Developmental changes in the activity of phosphatidylethanolamine N-methyltransferases in rat brain

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The activity of phosphatidylethanolamine N-methyltransferase (PeMT), an enzymic system that catalyses the synthesis of phosphatidylcholine (PtdCho) via sequential methylation of phosphatidylethanolamine (PtdEtn) using S-adenosylmethionine (AdoMet) as a methyl donor, was examined in brain homogenates from rats of various ages. The data thus obtained were consistent with the existence of two distinct enzyme activities within this enzyme system, i.e. one catalysing the methylation of PtdEtn to form phosphatidyl-N-monomethylthanolamine (PtdMeEtn), and the other catalysing the methylation of PtdMeEtn and phosphatidyl-NN-dimethylethanolamine (PtdMe₂Etn) (to form PtdMe₂Etn and PtdCho, respectively).

PeMT (PtdEtn-methylating) activity per g of brain was 4-fold higher in neonatal than in adult brains. The enzyme activity in adult brains exhibited Michaelis– Menten kinetics for AdoMet, and its affinity for AdoMet was high (apparent \( K_m \) 1.6 \( \mu \)M). In neonatal brain the relationships between AdoMet concentrations and PtdMeEtn formation were more complex: a sigmoidal component (with a Hill coefficient of 2.7), requiring 90 \( \mu \)M-AdoMet for half-saturation predominated over the high-affinity component (similar to that of the adult brain). PeMT (PtdMe₂Etn-methylating) activity per g of brain increased 2-fold between the 5th and the 20th postnatal days and remained constant thereafter; it was higher than that of PeMT (PtdEtn-methylating) activity at all ages studied, and its affinity for AdoMet was low (apparent \( K_m \) 99 \( \mu \)M). No sexual dimorphism in brain PeMT activity was observed at any age. We conclude that PeMT (PtdEtn-methylating) catalyses the rate-limiting step in PtdCho synthesis in rat brain, and that PtdCho formation via this pathway may be greatest during the neonatal period.

INTRODUCTION

Phosphatidylethanolamine N-methyltransferase (PeMT) activity catalyses the synthesis of phosphatidylcholine (PtdCho) by the sequential methylation of phosphatidylethanolamine (PtdEtn), using S-adenosylmethionine (AdoMet) as a methyl donor. This activity may represent one (Audubert & Vance, 1983), or two enzymes, the first catalysing the methylation of PtdEtn (Hirata & Axelrod, 1978; McGivney et al., 1981) and the second catalysing the methylation of phosphatidyl-N-monomethylthanolamine (PtdMeEtn) and phosphatidyl-NN-dimethylethanolamine (PtdMe₂Etn) (Rehbinder & Greenberg, 1965; Hirata & Axelrod, 1978; Rama Sastry et al., 1981; Percy et al., 1982). The PeMT pathway constitutes the only mechanism for generating new choline molecules in mammalian tissues (Bremer & Greenberg, 1961). The PtdCho formed by this pathway in brain constitutes a metabolic pool that turns over rapidly, liberating free choline (Blusztajn & Wurtman, 1981), some of which may be available as a precursor of neurotransmitter, acetylcholine. PeMT may also serve to create and maintain the asymmetry of distribution of PtdEtn and PtdCho across biomembranes (Hirata & Axelrod, 1978; Higgins, 1981) and may modulate the transmission of certain biological signals across plasma membranes in a variety of tissues (Hirata & Axelrod, 1980), including brain (Leprohon et al., 1983). However a modulatory role for PeMT remains controversial (Randon et al., 1981; Hotchkiss et al., 1981; Schanche et al., 1982; Moore et al., 1984). In brain, PeMT is primarily localized within nerve endings (Blusztajn et al., 1979; Crews et al., 1980). The developmental pattern of brain PeMT activity might therefore be correlated with synaptogenesis, a process that, in the rat, occurs largely postnatally (Aghajanian & Bloom, 1965). We now report that the PeMT activity in homogenates of rat brain changes during postnatal development. We also describe a novel form of PtdMeEtn-forming PeMT; the kinetic properties of its interactions with cofactor (AdoMet) differ from those previously observed in any tissue. This type of PtdMeEtn-forming PeMT activity is present in brains of neonatal animals only.

MATERIALS AND METHODS

Animals

Sprague-Dawley rats (Charles River Breeding Laboratories, Wilmington, MA, U.S.A.) were housed singly in an ambient temperature of 24°C, exposed to light

Abbreviations used: PeMT, phosphatidylethanolamine N-methyltransferase; AdoMet, S-adenosylmethionine; PtdEtn, phosphatidylethanolamine; PtdCho, phosphatidylcholine (lecithin); PtdMeEtn, phosphatidyl-N-monomethylethanolamine; PtdMe₂Etn, phosphatidyl-NN-dimethylethanolamine.

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Vol. 232
(300 W/cm²; VitaLite, Duro-Test Corp., North Bergen, NJ, U.S.A.) for 12 h per day, and allowed free access to food (Charles River Rat–Mouse–Hamster Maintenance Formula; 23% protein, 4.5% lipid, 72% carbohydrate, 0.183% free choline) and water. Infant rats were allowed free access to their mother and to rat chow. At each postnatal age (2, 5, 9, 12, 20, 30, 42, and 61 days), infants were selected from separate litters (reducing litter size uniformly) and decapitated; their brains were immediately frozen, and stored at −80 °C until assay. [In preliminary experiments we determined that homogenates prepared from frozen brains retained at least 95% of the PeMT activity present in fresh tissue (results not shown).]

PeMT assay

PeMT activity was assayed by a modification of the method described by Hirata and colleagues (Hirata & Axelrod, 1978; Hirata et al., 1978). The assay medium consisted of 50 μl of a solution containing 50 mM-Tris/HCl buffer (pH 7.5), 15 mM-MgCl₂ and 0.2 mM-EDTA, to which was added 20 μl of the tissue preparation (10% homogenate of brain in 0.32 M-sucrose). The enzymatic reaction was initiated by addition of 5 μl of [methyl-³H]AdoMet, (13 Ci/mmol; New England Nuclear, Boston, MA, U.S.A.), which had been diluted with unlabelled AdoMet (a gift from the Bio research Co., Milan, Italy) to achieve the desired concentration in the incubation mixture (see legends to Figures and Table). Since PeMT activity may represent one or two enzymes (see the Introduction) the enzyme(s) were assayed either in the presence of endogenous phospholipid substrate only (to approximate the physiological situation), or in the presence of saturating concentrations of the phospholipid substrates (0.1 mg of PtdEtn (Sigma, St. Louis, MO, U.S.A) or PtdMeEtn (Grand Island Biologicals Co., Grand Island, NY, U.S.A.) sonicated in the assay medium. The putative second enzyme catalyses the methylation of both PtdMeEtn and PtdMeEtn. To simplify the data analyses PtdMeEtn was used.

The reaction was carried out for 30 min at 37 °C in a Dubnoff metabolic shaker, and was stopped by addition of 3 ml of a chloroform/methanol/HCl mixture (100:50:1, by vol.). After the reaction was stopped the solution was washed twice with 0.1 M-KCl in 50% methanol; an aliquot of the chloroform phase was then concentrated and applied to a silica gel t.l.c. plate (LKB 5; Whatman Corp., Clifton, NJ, U.S.A.), along with internal standards for PtdEtn, PtdMeEtn, PtdMsEtn and PtdCho. This was developed using chloroform/propanic acid/propan-1-ol/water (2:2:3:1, by vol.). Segments were visualized with I₂ vapour and scraped into scintillation vials; phospholipids were eluted with 0.5 ml of methanol, and the radioactivity was determined after addition of 15 ml of ScintiVerse (Fisher Scientific Co., New York, NY, U.S.A.) using a Beckman LS-7500 scintillation counter.

Protein was determined by the method of Bradford (1976).

Calculations

It has been suggested that the rates of formation of N-methylated derivatives of PtdEtn are best calculated by using equations which include contributions of labelled methyl groups from each of the intermediates (PtdMeEtn, PtdMsEtn) and the product (PtdCho) of the pathway (Audubert & Vance, 1983). This treatment of data is appropriate for studies of liver PeMT, as no significant amounts of labelled intermediates accumulate, and it can be assumed that all of the labelled PtdCho contains three newly transferred methyl groups (Audubert & Vance, 1983). In brain this is not the case; all three methylated derivatives of PtdEtn accumulate to a similar extent, and the amounts of endogenous intermediates might be significantly higher than the amounts of labelled methyl groups transferred by PeMT. Thus the endogenous PtdMeEtn or PtdMsEtn are likely substrates for methylation by PeMT in brain preparations. [For example, the amount of PtdMsEtn in brain has been found to constitute 0.3% of the PtdCho pool (Tacconi & Wurtman, 1985) whereas the enrichment of the synapto- somal PtdCho pool with labelled methyl groups derived from PeMT during a 30 min incubation was at best 0.0002% (Blusztaż & Wurtman, 1981)]. Therefore the amount of [³H]methyl transferred is reported here, rather than the actual synthetic rates which are impossible to assess not knowing the contributions of endogenous PtdMeEtn and PtdMsEtn to the PeMT substrate pool.

The enzymatic activities are expressed both per mg of protein and per g of fresh tissue, since in the developing brain there is a marked increase in the content of protein (associated with cellular growth) per unit weight (Benjamins & McKhann, 1976).

Statistical analyses were performed using one-way analysis of variance and the Tukey test. Analyses of kinetic data were performed by the method of Wilkinson (1961) or by curve fitting using the RS/1 software package (Bolt Beranek & Newman Inc., Cambridge, MA, U.S.A.) on a VAX/VMS computer (Digital Equipment Corp., Maynard, MA, U.S.A.).

RESULTS

Developmental changes in PtdMeEtn accumulation

The PeMT that converts brain PtdEtn to PtdMeEtn apparently was saturated with endogenous PtdEtn throughout development, since adding PtdEtn to the reaction mixture never enhanced product formation (results not shown). Highest enzyme activity was observed in rats 2 days of age (770 pmol/h per g wet wt. or 16 pmol/h per mg of protein) after which it declined (Fig. 1). The relationships between the velocity of PtdMeEtn formation by this neonatal enzyme and the concentration of AdoMet suggested the action of two distinct enzymes, with different affinities for AdoMet (Fig. 2). The Hill plot of the data was nonlinear and could be resolved into two components, one exhibiting a Hill coefficient of 1.01 ± 0.03 (S.E.M.) (over the range of 0.8–13 μM-AdoMet) and another exhibiting a Hill coefficient of 2.66 ± 0.24 (S.E.M.) (over the range 43–200 μM-AdoMet) (Fig. 2, inset). In order to fit an equation to the observed kinetic data (Fig. 2) it was necessary to postulate the existence of two PtdEtn-methylating enzymes, one exhibiting Michaelis–Menten-type kinetics and a high affinity for AdoMet, and another with sigmoidal kinetics and with a low affinity for AdoMet. [Attempts to fit a single Michaelis–Menten equation, a linear combination of two such equations or a single sigmoidal (logistic) equation to these data resulted in higher residual terms]. The apparent Km for AdoMet of the high-affinity component was 10.1 ± 7.2 μM (S.E.M.) [maximal velocity 3.3 ± 1.0 (S.E.M.) pmol/h per mg
Fig. 1. Developmental changes in activity of PeMT (PtdEtn-methylating) in rat brain

Brain homogenates were incubated in the presence of 3.3 μM- (●) or 200 μM- (○) [methyl-3H]AdoMet, as indicated. [3H]PtdMeEtn was extracted and purified by t.l.c. as described in the Materials and methods section. Data are expressed as pmol of [3H]methyl incorporated into PtdMeEtn/h per g wet wt. of tissue (±S.E.M.) in (a) and as pmol/h per mg of protein (±S.E.M.) in (b). Statistical analyses utilized one-way analyses of variance followed by the Tukey test. (a) In the presence of 200 μM-[methyl-3H]AdoMet, enzyme activity on days 2 and 5 was higher than at any other age (P < 0.01). At 3.3 μM-[methyl-3H]AdoMet, activity on day 12 was higher than activities noted on days 2, 9, 30, and 42 (P < 0.05). (b) In the presence of 200 μM-[methyl-3H]AdoMet, the enzyme activity on day 2 was higher than on days 5–61 (P < 0.01).

Fig. 2. Kinetic properties of neonatal PeMT (PtdEtn-methylating) activity

Homogenates prepared from brains of 2-day-old rats were incubated with various concentrations of [methyl-3H]AdoMet; [3H]PtdMeEtn was extracted and purified by t.l.c. as described in the Materials and methods section. The data are triplicate determinations of representative experiments repeated three times. The kinetic parameters were calculated using the best fit to a hypothetical line being a linear combination of a rectangular hyperbola and a logistic curve. This model produced the best results (P < 0.001) as compared with a single rectangular hyperbola, single logistic, or a linear combination of two rectangular hyperbolas. The graph shows the experimental points and the best fit as well as the two components; the logistic:

\[ \text{velocity} = \frac{(8.4[\text{AdoMet}]^3)}{([\text{AdoMet}]^3 + 90.1^3)} \]

and the hyperbola:

\[ \text{velocity} = \frac{(3.3[\text{AdoMet}])}{(10.1 + [\text{AdoMet}])} \]

The inset shows the Hill plot constructed from the data and the two lines that are the results of linear regressions using the concentrations of AdoMet between 0.2 and 20 μM (dotted line, Hill coefficient 1.01) and between 43 and 200 μM (broken line, Hill coefficient 2.7) as the independent variable.
but was saturated when PtdMe₂Etn was added at a concentration of 1 mg/ml. When homogenates were incubated with 200 μM-AdoMet and 1 mg of PtdMe₂Etn/ml, the incorporation of [³H]methyl groups into PtdCho, expressed per wet weight of tissue, was slowest in 2- and 5-day-old rats (2 nmol/h per g); it doubled by 20 days of age (to 4.3 nmol/h per g; \( P < 0.01 \)) and then declined gradually (to 3.3 nmol/h per g by day 61; \( P < 0.01 \); Fig. 3a). Homogenates incubated with lower concentrations of AdoMet (3.3 μM) showed similar developmental changes in [³H]PtdCho accumulation, though the rate of PtdCho formation was slower (Fig. 3a).

When homogenates were incubated with saturating concentrations of AdoMet (200 μM) and with PtdMe₂Etn, the specific activity of PeMT (PtdMe₂Etn-methylating) was high at 2 days of age (42 pmol/h per mg of protein) but decreased by 5 days of age. At all AdoMet concentrations tested in the presence of added PtdMe₂Etn, the specific activity of PeMT (PtdMe₂Etn-methylating) was highest at 12 days of age (Fig. 3b). However when no exogenous PtdMe₂Etn was added, PtdCho formation was greatest near birth and declined thereafter (Fig. 4).

The PtdMe₂Etn-methylating enzyme followed Michaelis–Menten kinetics for AdoMet at all ages examined regardless of the presence or absence of PtdMe₂Etn. The \( K_m \) for AdoMet was approx. 100 μM and did not vary during development (results not shown).

**Sex-related differences in PeMT activity**

At all ages studied, PeMT activities were the same in brain homogenates from male and female rats (Table 1).
Table 1. Formation of PtdCho in brain homogenates from male or female rats

<table>
<thead>
<tr>
<th>Sex</th>
<th>Age (days)</th>
<th>9</th>
<th>12</th>
<th>30</th>
<th>42</th>
<th>61</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male</td>
<td></td>
<td>35.53±2.51 (3)</td>
<td>42.13±5.34 (3)</td>
<td>29.82±1.22 (7)</td>
<td>23.80±1.63 (6)</td>
<td>20.88±1.70 (5)</td>
</tr>
<tr>
<td>Female</td>
<td></td>
<td>32.77±0.71 (4)</td>
<td>43.93±5.78 (4)</td>
<td>29.27±2.58 (3)</td>
<td>24.48±2.75 (4)</td>
<td>25.19±4.83 (5)</td>
</tr>
</tbody>
</table>

DISCUSSION

The activity of PeMT changes in a complex fashion during the postnatal development of rat brain. Synthesis of PtdCho is highest in neonatal animals (2 days of age) because of the presence of relatively large amounts of a special form of PeMT that catalyses the first (and probably rate-limiting) reaction in the process, the conversion of PtdEtn to PtdMeEtn. This form of PeMT has a low affinity for AdoMet [requiring 90 μM-AdoMet to reach half-maximal velocity (Figs. 1 and 2)] and cannot be detected in brains of rats older than 5 days of age. Later in the animals’ lives PtdMeEtn is synthesized by a PeMT that has a high affinity for AdoMet (apparent \( K_m \) 1.6 μM) and whose activity reaches its maximum by 12–20 days of age (Fig. 1). This activity (\( V_{max} \)), however, is lower than that of the neonatal form of PeMT that catalyses the conversion of PtdEtn to PtdMeEtn. The activity of PeMT that catalyses the conversion of PtdMeEtn to PtdCho is highest in the 12–20-day-old brain and has a tendency to decrease thereafter. Its affinity for AdoMet is fairly constant [apparent \( K_m \) 100 μM, similar to the previously reported value of 110 μM (Crews et al., 1980)].

Conversion of PtdEtn to PtdMeEtn

The brains of neonatal rats contain a novel methyltransferase that catalyses the formation of PtdMeEtn. The activity of this enzyme is kinetically different from that of previously described forms of PeMT (PtdEtn-methylating) (Hirata & Axelrod, 1978; Hirata et al., 1978; Leprohon et al., 1983; Rama Sastry et al., 1981; Percy et al., 1982). Its affinity for AdoMet is low, its [AdoMet]-versus-velocity relationship is best characterized by a sigmoidal curve (Fig. 2) and it can be detected only in animals less than 5 days old. The Hill coefficient of this enzyme is 2.7±0.2 (S.E.M.) (as obtained from the Hill plot, Fig. 2) or 3.7±0.8 (S.E.M.) (as obtained from the best fit of the [AdoMet]-versus-velocity data, Fig. 2) which do not differ statistically from each other. From the available data it is not possible to establish whether the sigmoidal character of the line relating AdoMet concentrations to velocity reflects more than one form of methyltransferase, with distinct binding sites for AdoMet, or a co-operative effect. In order to answer that question it might be necessary to purify the enzyme or to use various analogues of AdoMet that might act as selective ligands for the hypothetical AdoMet binding sites. The activity of this form of PeMT (PtdEtn-methylating) accounts for most of PtdMeEtn and, consequently, PtdCho synthesis by the methylating pathway in the brains of neonates [since the rate of PtdMeEtn formation appears to be rate-limiting (see below)] (Figs. 1 and 4). It is likely that this enzyme is also present in fetal brains. The other form of PeMT (PtdEtn-methylating) has a high affinity for AdoMet (apparent \( K_m \) of 10.1 μM in the 2-day-old and apparent \( K_m \) of 1.6 μM in the 61-day-old rat) and is therefore similar to the enzyme studied previously in brain (Crews et al., 1980; Percy et al., 1982) and other tissues (Hirata & Axelrod, 1978; Hirata et al., 1978; Rama Sastry et al., 1981).

The activity of PeMT in synaptosomes prepared from brains of 7- and 14-day-old rats and from adult animals was studied by Hitzemann (1982). PeMT (PtdEtn-methylating) had slightly lower affinity for AdoMet at 7 days of age (apparent \( K_m \) 8 μM) than in the adult (apparent \( K_m \) 5.3 μM). The disappearance of the low-affinity component of PeMT (PtdEtn-methylating) soon after birth (Fig. 1) might explain why the differences in \( K_m \) values obtained in that study were much less striking than the ones reported here, i.e. the brains of 7-day-old rats might contain only small amounts of the low-affinity form of PeMT (Fig. 1). The subcellular origin of the preparation termed ‘synaptosomal’ in that study is also uncertain, since it has been shown that, although one can prepare from neonatal animals (by means of centrifugations in discontinuous sucrose density gradients), a fraction whose density is equivalent to that of synaptosomes from the adult animals, these preparations are not at all similar to synaptosomes prepared from adult brains (as assessed by electron microscopy and marker enzyme assays) (Jones & Revell, 1970; Gontzas et al., 1971; Kanerva et al., 1977); their subcellular origin is unclear (Jones & Revell, 1970; Gontzas et al., 1971; Kanerva et al., 1977).

In the brains of newborn rats AdoMet concentrations are 40–50 nmol/g of tissue (Hoffman et al., 1979). These levels probably are sufficient to enable the neonatal form of PeMT (PtdEtn-methylating) to maintain high rates of PtdMeEtn synthesis, and it is in this concentration range that this enzyme is sensitive to changes in AdoMet levels. Once the PeMT (PtdEtn-methylating) is of the adult type (exhibiting high affinity for AdoMet) it probably is saturated with AdoMet [20 nmol/g of brain at 28 days of age (Hoffman et al., 1979)] and the rate of PtdCho formation would be expected to be slower and less affected by substrate [except in certain membrane domains where it may be modulated by various biological signals, e.g. dopamine (Leprohon et al., 1983) or in
cholinergic nerve endings where it might provide some of the choline for acetylcholine synthesis (Blusztajn & Wurtman, 1981).}

**Conversion of PtdMe₂Et to PtdCho**

The accumulation of PtdCho was markedly stimulated by the addition of PtdMe₂Et to the assay mixture at every age (Figs. 3 and 4), suggesting that the availability of PtdMe₂Et was rate-limiting in the formation of PtdCho. It is for this reason that the developmental pattern of PtdCho accumulation [when assayed in the absence of exogenous phospholipid substrates (Fig. 4)] mimics that of PeMT (PtdEtν-methylating) rather than PeMT (PtdMe₂Et-methylating) activity. The developmental pattern of PtdMe₂Et accumulation was virtually indistinguishable from that of PtdCho accumulation (results not shown), consistent with the notion that the methylations of PtdMe₂Et and PtdMe₆Et are catalysed by the same enzyme (Rehninger & Greenberg, 1965; Hirata & Axelrod, 1978; Rama Sastry, et al., 1981; Percy et al., 1982). When PeMT (PtdMe₂Et-methylating) activity was measured after the addition of saturating amounts of PtdMe₂Et, thereby eliminating the effect of developmental variations in PeMT (PtdEtν-methylating) activity, the incorporation of [3H]methyl into PtdCho expressed per wet weight of tissue was lowest in brains of neonates (Fig. 4). This may be partly due to the high water content of neonatal brain (84–88% versus 78% in the adult; Himwich, 1973). The specific activity of PeMT (PtdMe₂Et-methylating) is high in neonates, though it is highest in the brains from 12-day-old rats (Fig. 4). This reflects either an activation of existing PeMT molecules or an increase in the number of PeMT molecules relative to other brain proteins. At this age the brain grows rapidly (Himwich, 1973) and PtdCho is needed for membrane synthesis. There is a generalized increase in lipid-synthesizing activity during the second week of life in the rat brain, specifically choline incorporation into PtdCho by the CDP-choline pathway (Abdel-Latif & Smith, 1972). The saturation of fatty acids (Abberhard et al., 1969), cholesterol synthesis (Jones et al., 1975) and sulphate incorporation into sulphatides (McKinnan & Ho, 1967) are all highest during that period. Myelin synthesis is also maximal during this time (Welles & Dittmer, 1967); indeed it has been shown that cultured chick glia have higher activity of PeMT than cultured chick neurons (Dainous et al., 1982).

The effects of age on PeMT activity has been studied previously. Crews et al. (1981) observed that in synaptosomes the specific activity of PeMT (PtdEtν-methylating) increased 30% during aging (1–21 months), yet the activity of PeMT (PtdMe₂Et-methylating; measured in the presence of added PtdMe₂Et) remained constant. If PeMT (PtdEtν-methylating) activity is indeed limiting throughout life, then the synthesis of PtdCho via the methylation pathway may be rapid near birth, slowed down during adolescence and adulthood and high again during old age. Hoffman et al. (1979) studied developmental changes in PeMT activity of rat brain microsomes and found a fairly constant specific activity at all ages examined. The developmental changes in PeMT activity that we report may occur in a subcellular fraction other than microsomes (Blusztajn & Wurtman, 1981; Hirata et al., 1978; Hitzemann, 1982). Hoffman et al. (1979) carried out their assay in the presence of deoxycholate and at pH 8.6. It has been noted that the pH optima of rat brain PeMT may vary with the animal's age (Mozzi et al., 1980). These technical differences could explain the differences in the results obtained by Hoffman et al. (1979), and us. Our observations are consistent, however, with those of Chida & Arakawa (1971), who observed that PtdCho synthesis via the methylation pathway was highest in young rats in vivo.

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