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Rat and human mammary tissue can synthesize choline moiety via the methylation of phosphatidylethanolamine

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The normal mammal requires large amounts of choline for maintenance and growth of tissue mass. Since milk, the only food for neonates, has many-fold higher free choline concentration than does maternal plasma, it is possible that mammary gland can synthesize choline molecules. The only known mammalian pathway for the synthesis *de novo* of choline molecules is catalysed by phosphatidylethanolamine *N*-methyltransferase (PeMT), which synthesizes phosphatidylcholine (PtdCho) via sequential methylation of phosphatidylethanolamine (PtdEtn) using *S*-adenosylmethionine (AdoMet) as a methyl donor. We identified PeMT activity in rat mammary tissue, and differences in affinities for substrate, as well as in activities as a function of pH, suggest that at least two distinct enzyme activities are involved [i.e. one catalysing the methylation of PtdEtn to form phosphatidyl-*N*-methylethanolamine (PtdMeEtn) and the other catalysing the methylation of PtdMeEtn and phosphatidyl-*NN*-dimethylethanolamine ($PtdMe_2Etn$) to form $PtdMe_2Etn$ and PtdCho, respectively]. The relationships between AdoMet concentrations and PtdCho formation from endogenous PtdEtn in rat mammary homogenate were complex: a sigmoidal component (with a Hill coefficient of 2.2), requiring 55 μM-AdoMet for half saturation ($V_{max.}$ = 9 pmol/h per mg of protein), and a high affinity component ($K_{apparent}$ = 8.7 μM and $V_{max.}$ = 3.8 pmol/h per mg of protein) were identified. When exogenous $PtdMe_2Etn$ was added as substrate, PtdCho formation exhibited Michaelis–Menten kinetics for AdoMet, and its affinity for AdoMet was high ($K_{apparent}$ = 9 μM, $V_{max.}$ = 85 pmol/h per mg of protein). In the presence of endogenous substrates, the rates of PeMT-catalysed PtdCho formation within homogenates of rat mammary tissue were similar in tissue from lactating and non-lactating animals. When exogenous $PtdMe_2Etn$ was added to homogenates of rat mammary tissue, tissue from lactating rats made twice as much PtdCho as did tissue from non-lactating rats. Isolated mammary epithelial cells also exhibited PeMT activity; the rate of formation of PtdCho was much greater in intact versus broken cells. We also identified PeMT activity in homogenates of mammary tissue from non-lactating humans. The rate of PtdCho formation was of similar magnitude to that seen in rat tissue. This evidence supports the hypothesis that some of the choline found in milk could have been synthesized *de novo* in the mammary gland.

INTRODUCTION

The neonatal mammal requires large amounts of choline for maintenance and growth of tissue mass. Choline is a precursor of phosphatidylcholine (PtdCho; lecithin) which is the major phospholipid in mammalian membrane [1,2]. Choline is also required for the synthesis of acetylcholine, and is an important methyl donor [3]. Choline deficiency in rats has been associated with fatty infiltration of the liver, renal dysfunction, and increased sensitivity to carcinogens [3]. A significant portion of required choline is derived from the diet [3,4], and milk is the main component of the diet for the neonate. Milk has many-fold higher free choline concentration than does maternal plasma [4]. There are two ways that choline can be accumulated into milk: concentrative uptake of choline and biosynthesis of choline molecules by mammary gland. We have previously described choline transport by mammary epithelial cells [5]. We now describe synthesis *de novo*, by rat and human mammary gland, of choline molecules via a reaction catalysed by phosphatidylethanolamine-*N*-methyltransferase (PeMT) which synthesizes phosphatidylcholine (PtdCho) by sequentially methylating phosphatidylethanolamine (PtdEtn) using *S*-adenosylmethionine (AdoMet) as a methyl donor.

PeMT activity has been previously characterized in the liver, brain, lung, erythrocyte, polymorphonuclear leukocyte and adrenal medulla [6–13], but has never been identified in mammary tissue. It may represent one [12,13] or two enzymes, the first methylating PtdEtn and the second methylating phosphatidyl-*N*-methylethanolamine (PtdMeEtn) and $PtdMe_2Etn$ [10]. This is the only known mammalian pathway for the synthesis *de novo* of choline molecules [8].

Abbreviations used: PeMT, phosphatidylethanolamine *N*-methyltransferase; PtdCho, phosphatidylcholine (lecithin); PtdEtn, phosphatidylethanolamine; AdoMet, *S*-adenosylmethionine; PtdMeEtn, phosphatidyl-*N*-methylethanolamine; $PtdMe_2Etn$, phosphatidyl-*NN*-dimethylethanolamine; PNA, peanut agglutinin.

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MATERIALS AND METHODS

Sources of tissue

Lactating female Sprague–Dawley rats (Charles River Breeding Laboratories, Wilmington, MA, U.S.A.) were housed in polyethylene tubs with their litters (nine or ten pups). Non-lactating females were housed individually. They were offered food (Rodent Lab Chow, Farmers Exchange, Framingham, MA, U.S.A.) and water *ad libitum*. Rats were decapitated, and mammary tissue was collected (on postpartum day 15 for lactating rats). Mammary epithelial cells were isolated from the fresh lactating tissue as described below. Some of the tissue was stored at −95 °C until used.

Non-lactating human breast tissue was obtained from a surgical biopsy from a subject with a left breast lesion identified as mild fibrocystic disease. A portion of the biopsy, which was considered to be normal mammary gland, was excised and stored at −95 °C until used.

Preparation of mammary homogenates

Tissue homogenate (1%) was prepared in 0.32 M-sucrose by sonification (model W-225R, Heat Systems Ultrasonics, Plainview, NY, USA).

Isolation of epithelial cells

Mammary epithelial cells were isolated and enriched using the method of Richards *et al.* [14]. Mammary glands were freed of fascia and minced into 0.4 mm pieces on a chilled work surface. These were suspended in isolation buffer [Krebs–Henseleit bicarbonate buffer, pH 7.4, containing 4% (w/v) bovine serum albumin (Sigma, St. Louis, MO, U.S.A.) and 10 mM-glucose (Sigma Chemicals)]. Collagenase (10 mg/g of tissue; type III; Worthington Biochemicals, Freehold, NJ, U.S.A.) was added. Tissue was incubated for 1.5 h at 37 °C, with gentle mixing every 30 min. The mixture was centrifuged at 500 *g* for 10 min at 4 °C, and the supernatant was aspirated (removing adipocytes). The pellet was resuspended in isolation buffer and filtered through a nylon mesh (400 μm pore) to remove undigested tissue. The cells which passed through the screen were washed three times with the buffer and were resuspended so that there were 1.5×10^7 cells/ml in isolation buffer. Deoxyribonuclease (50 μg/ml; type I; Sigma Chemicals) was added to prevent cell clumping. This tissue digest was further enriched for epithelial cells by isopycnic banding on a density gradient. The density gradient was prepared in a 50 ml polycarbonate tube containing 10.8 ml of Percoll (Pharmacia, Piscataway, NJ, U.S.A.), 2.8 ml of 9% (w/v) NaCl, and 14.4 ml of water. A sigmoid gradient was formed after centrifugation in a fixed angle rotor at 20000 *g* for 60 min at 4 °C. The cells were layered onto the gradient, and centrifuged in a swinging bucket rotor at 800 *g* for 15 min at 4 °C. Epithelial cells banded at a density (1.05–1.07 g/ml) below most stromal cells and cell debris, and above nuclei and erythrocytes. The epithelial band was collected and diluted in 7 vol. of isolation buffer, then centrifuged at 100 *g* for 5 min at 4 °C, and the pellet was used.

Cell survival was assessed using Trypan Blue exclusion. In all experiments viability exceeded 80–95%. Our mammary cell preparation accumulated 3-*O*-methyl[^{3}H]-glucose (60 Ci/mmol; New England Nuclear, Boston, MA, U.S.A.) at rates that were similar to those reported by other investigators [15]. We used immunohistochemical methods to evaluate the purity of epithelial cells [16,17]. Cells were delivered onto microscope slides using cytocentrifugation, and were fixed in acetone. These were incubated with peanut lectin (PNA; Vector Laboratories, Burlingame, CA, U.S.A.) which specifically binds to galactose disaccharide marker on the surface of the mammary epithelial cell. The cell–PNA complex was then incubated with rabbit anti-PNA antibody (DAKO Corporation, Santa Barbara, CA, U.S.A). Horseradish peroxidase–rabbit anti-(horseradish peroxidase) immune complexes (PAP; DAKO Corporation) were added, and bound to the pig anti-(rabbit Ig) antibody (DAKO Corporation). Slides were washed, and H_2O_2 was added as a substrate for the peroxidase. 3′,3-Diaminobenzidine tetrahydrochloride (Aldrich Chemicals, Milwaukee, WI, U.S.A.) was used as a chromagen. Cells were counterstained with haematoxylin. Of our isolated cells, 99% stained positively as epithelial cells. When appropriate controls were used (no PNA, or PNA with galactose added to prevent binding), cells did not stain. The cells had characteristic features of mammary epithelial cells when examined with electron microscopy (results not shown).

PeMT assay

PeMT activity was assayed using a modification of the method of Hirata and colleagues [7,10]. The assay medium contained 25 μl of a solution containing 50 mM-Tris/HCl buffer (pH 7.5), 15 mM-$MgCl_2$, and 0.2 mM EDTA, to which was added 20 μl of tissue homogenate (20–50 μg of protein). The reaction was started by the addition of 5 μl of [*methyl*-^{3}H]AdoMet (0.5 μCi; 14.7 Ci/mmol; New England Nuclear). In some of the experiments, the media also contained 100 μg of dipalmitoyl-PtdMe$_2$Etn (Sigma Chemicals). The reaction was continued for 30 min at 37 °C in a Dubnoff shaker, and was stopped by the addition of 3 ml of chloroform/methanol/HCl (100:50:1, by vol.). These extracts were washed twice with 2 ml 0.1 M-KCl in 50% methanol. Individual ^{3}H-labelled phospholipids were purified by t.l.c. as described below. A heated preparation of enzyme (30 min at 100 °C) was used as a blank. At least three samples were assayed for each data point.

PeMT activity in intact cells was determined using methionine as a methyl donor. The assay medium contained 25 μl of Krebs–Henseleit bicarbonate buffer [pH 7.4, containing 4% (w/v) bovine serum albumin and 10 mM-glucose], to which was added 20 μl of isolated epithelial cell suspension (about 50 μg of protein). The reaction was started by adding 5 μl of [*methyl*-^{3}H]-methionine (0.5 μCi; 15 Ci/mmol; New England Nuclear). Individual ^{3}H-labelled phospholipids were purified by t.l.c., as described below.

Of the PeMT activity in rat mammary homogenates, 95% was inhibited by addition of 200 μM-*S*-adenosylhomocysteine (98% pure; Bioresearch Corporation, Milan, Italy), a known inhibitor of AdoMet-dependent reactions (results not shown).

Identification of reaction products

The products were identified using the method of Blusztajn *et al.* [7]. The chloroform phase was dried using a vacuum centrifuge (SpeedVac, Savant Industries, Farmingdale, NY, U.S.A.). The pellet was resuspended and was applied to a silica gel t.l.c. plate (Si250-PA; J. T. Baker Chemicals, Phillipsburg, NJ, U.S.A.) along

with internal standards of PtdMeEtn, $PtdMe_2Etn$, and PtdCho (Avanti Polar Lipids, Birmingham, AL, U.S.A.). These were developed using acetone, dried, and then developed using chloroform/propionic acid/1-propanol/water (2:2:3:1, by vol.). Bands containing phospholipids were visualized by exposure to iodine vapour, and scraped into scintillation vials. Phospholipids were eluted with 0.5 ml of methanol, and then 5 ml of scintillation fluid (ScintiVerse; Fisher Chemicals, Medford, MA, U.S.A.) was added and the samples were counted using liquid-scintillation spectrophotometry.

To establish that the radioactivity found in the newly formed PtdCho actually was present as [^{3}H]choline, we used a modification of the method of Liscovitch *et al.* [18]. The chloroform phase was chromatographed and eluted as described above, evaporated to dryness in a vacuum centrifuge, resuspended in 100 μl of 6 M-HCl in methanol, and heated in a stoppered tube at 100 °C for 1 h to hydrolyse the PtdCho, forming free choline. This choline was then isolated using the h.p.l.c. technique described below. Some of this choline was then treated with choline oxidase (12 units; from *Alcaligenes* spp.; Sigma Chemicals) in 0.2 M-sodium phosphate buffer (pH 7.8) at 30 °C for 1 h, thereby converting the choline to betaine. The heated mixtures purportedly containing choline liberated from PtdCho, or betaine made from this choline, were evaporated to dryness, and were applied to a silica h.p.l.c. column (Pecosphere-3CSi 3 μm; 4.6 mm × 83 mm; Perkin–Elmer, Norwalk, CT, U.S.A.). Hydrolysed authentic [*methyl*-^{14}C]PtdCho was used as a control, and was treated in the same way. Metabolites were eluted with a linear gradient, from 0 to 100%, of acetonitrile/ethanol/acetic acid/1 M-ammonium acetate/10 mM-sodium phosphate/water (800:68:2:3:10:127, by vol., changing to 400:68:44:88:10:400, by vol.) at a flow rate of 1.5 ml/min. Fractions were collected and counted using liquid-scintillation spectrophotometry.

AdoMet assay

The endogenous AdoMet concentration in mammary tissue was determined using a modification of the reversed-phase h.p.l.c. technique described by Shivapurkar [19]. Protein in mammary homogenates was precipitated with trichloroacetic acid, and the supernatant was washed three times with peroxide-free diethyl ether, and dried using vacuum centrifugation (SpeedVac, Savant Industries). After resuspension, an aliquot was applied to a reverse-phase h.p.l.c. column (Supelguard LC-18 guard column; 5 μm; 4.6 mm × 20 mm; Supelco Inc., Bellefonte, PA, U.S.A.) in series with a Microsorb C-18 column (5 μm; 4.6 mm × 250 mm; Rainin Instruments, Woburn, MA, U.S.A.). Column temperature was maintained at 55 °C using a heater (Eldex Laboratories, Menlo Park, CA, U.S.A.). AdoMet was eluted with a 5 mM-heptanesulphonic acid in 20% (v/v) methanol, adjusted to pH 3.5 with acetic acid; flow rate was 2 ml/min (model 2350 pump; ISCO, Lincoln, NE, U.S.A.). Absorbance was monitored at 254 nm (model LC-85B on-line spectrophotometric detector; Perkin–Elmer). An internal standard of *S*-adenosylethionine was used to calculate recoveries. Concentrations of AdoMet were calculated using an external standard curve.

Methionine assay

The endogenous methionine concentration in mammary epithelial cells was determined using the methods of Bidlingmeyer *et al.* [20] and Scholze [21]. The phenylisothiocyanate derivative of methionine was formed and applied to a reverse-phase h.p.l.c. column (Zorbax C-8: 5 μm; 4.6 mm × 250 mm; Dupont, Wilmington, DE, U.S.A.) using the h.p.l.c. system described above. Methionine was eluted using a nonlinear binary gradient of 10 mM-potassium phosphate with 10% acetonitrile (pH 6.5) changing to 100% acetonitrile at a flow rate of 2 ml/min. Absorbance was monitored at 254 nm.

Protein assay

Protein was measured using the colorimetric assay of Bradford [22].

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 and $PtdMe_2Etn$) and the product (PtdCho) of the pathway [12]. This treatment assumes that endogenous concentrations of the intermediates (PtdMeEtn and $PtdMe_2Etn$) are insignificant. We have chosen to report the amount of [^{3}H]methyl transferred rather than estimates of actual synthetic rates. Data were analysed using Student's *t*-test, and one way analysis of variance and Tukey or Dunnet's test [23]. Analyses of kinetic data were performed 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

PeMT activity was observed in mammary tissue from lactating and non-lactating rats, in isolated epithelial cells from lactating rats, and in mammary tissue from non-lactating human (Figs. 1, 2, 6, and 7).

PeMT activity in homogenates of rat mammary tissue

Putative PtdCho, formed via the PeMT pathway in rat mammary homogenates, could be hydrolysed to form a radiolabelled product which co-chromatographed with choline in our h.p.l.c. separation. This choline peak could be collected, treated with choline oxidase, and converted to a product which co-chromatographed with betaine in our h.p.l.c. separation (results not shown).

There were no significant differences in PeMT activity (PtdCho forming) between fresh and frozen tissue (Fig. 1). Tissue from lactating rat had approx. 2-fold more PeMT activity (PtdCho forming) than did tissue from non-lactating rats ($P < 0.05$ different by *t*-test; Fig. 1). We found that the AdoMet concentration in fresh intact tissue was 30 nmol/g wet weight. In homogenates of mammary tissue from lactating rats AdoMet concentration was 0.73 μM after the addition of radioactivity. At these low concentrations of AdoMet, the rate of incorporation of methyl groups into PtdCho was 1.4 pmol/h per mg of protein (±0.6; S.E.M.). The rates of PtdEtn and $PtdMe_2Etn$ methylation were 0.6 (±0.2) and 1.1 (±0.4) pmol/h per mg of protein, respectively (Fig. 2). Addition of 100 μg of $PtdMe_2Etn$ as substrate greatly increased the rate of formation of PtdCho [to 10.2 pmol/h per mg of protein (±0.6); $P < 0.05$ different from

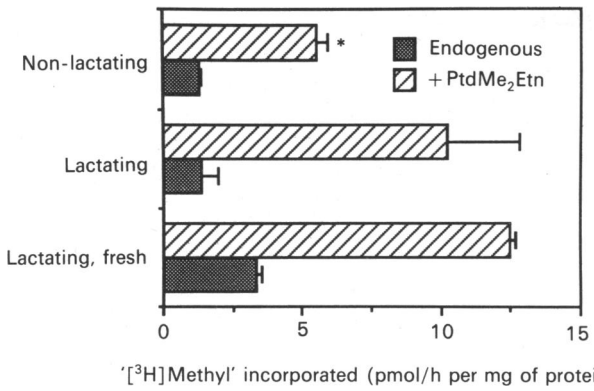

Fig. 1. Phosphatidylcholine synthesis within homogenates of rat mammary gland

Rats were killed by decapitation and mammary tissues were collected and used immediately (Lactating, fresh) or frozen at −95 °C until used ('Non-lactating', 'Lactating'). Homogenates of mammary gland were incubated in the presence of 0.73 μM-[*methyl*-^{3}H]AdoMet. In some samples, 100 μg of dipalmitoyl-phosphatidyl-*NN*-dimethylethanolamine was added to tissue homogenates ('+PtdMe$_2$Etn'). Phospholipids were extracted and purified by t.l.c. as described in the Materials and methods section. Data are expressed as pmol of '[^{3}H]methyl' incorporated into PtdCho/h per mg of protein (±S.E.M.). At least three samples were assayed for each data point. $*P < 0.05$ different from 'Lactating' by *t*-test.

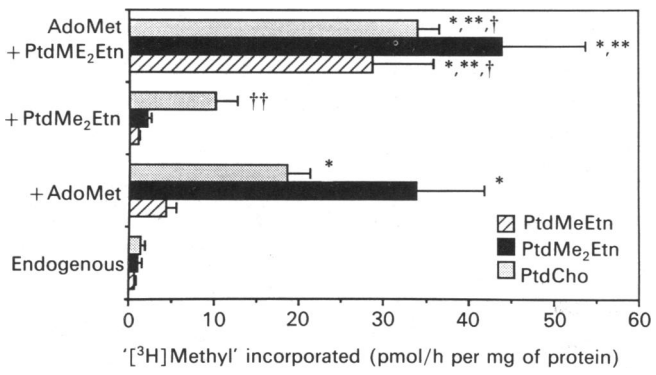

Fig. 2. PeMT activity in homogenates of mammary gland from lactating rats

Mammary tissue was collected from rats which had been lactating for 15 days. Tissue was frozen at −95 °C until used. The mammary homogenates were incubated in the presence of 0.73 μM-[*methyl*-^{3}H]AdoMet ('Endogenous'), 200 μM-[*methyl*-^{3}H]AdoMet ('+AdoMet'), 100 μg of dipalmitoyl-PtdMe$_2$Etn ('+PtdMe$_2$Etn'), or 200 μM-[*methyl*-^{3}H]AdoMet and 100 μg of dipalmitoyl-PtdMe$_2$Etn ('PtdMe$_2$Etn+AdoMet'). Phospholipids were extracted and purified by t.l.c. as described in the Materials and methods section. Data are expressed as pmol of '[^{3}H]-methyl' incorporated into products/h per mg of protein (±S.E.M.). At least three samples were assayed for each data point. $*P < 0.01$ different from 'Endogenous', $**P < 0.01$ different from '+PtdMe$_2$Etn', $†P < 0.01$ different from '+AdoMet' and $††P < 0.05$ different from 'Endogenous' and from PtdMe$_2$Etn+AdoMet', by one-way analysis of variance.

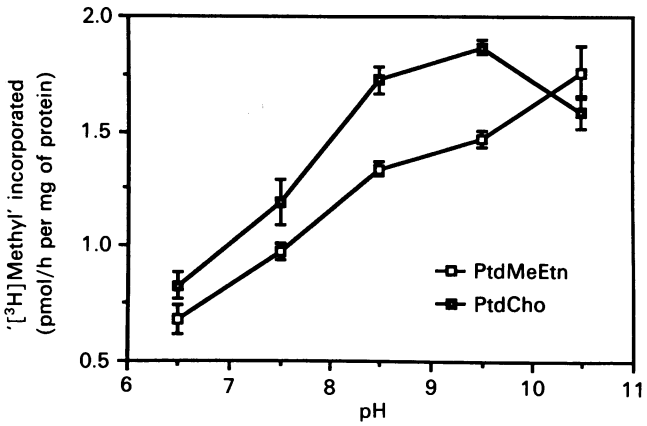

Fig. 3. PeMT (PtdEtn methylating) and PeMT (PtdMe$_2$Etn methylating) differ in pH-dependence

Homogenates of lactating rat mammary gland were incubated as described in Fig. 2. Sodium acetate buffer (50 mM) was used to generate medium pH 6.5, Tris/HCl (50 mM) was used for pH 7.5–8.5, and NaOH/glycine (50 mM) for pH 9.5–10.5. AdoMet concentration was 2.33 μM. Data are expressed as pmol of '[^{3}H]methyl' incorporated into product/h per mg of protein (±S.E.M.). At least three samples were assayed for each data point.

endogenous substrates by one-way ANOVA (Fig. 2). In the presence of 200 μM-AdoMet all products accumulated more rapidly, though the greatest increase was in PtdMe$_2$Etn formation [to 34 (±8) pmol/h per mg of protein; $P < 0.01$ different from endogenous substrates by one-way ANOVA (Fig. 2)]. The addition of both 200 μm-AdoMet and 100 μg of PtdMe$_2$Etn further increased the rates of formation of PtdMeEtn and PtdCho ($P < 0.01$ different from endogenous substrates, added AdoMet or added PtdMe$_2$Etn by one-way ANOVA; Fig. 2).

In homogenates of lactating rat mammary gland, methylation of PtdMe$_2$Etn exhibited a pH optimum of 9.5, while the rate of methylation of PtdEtn increased linearly between pH 6.5 and 10.5 (Fig. 3). The relationships between AdoMet concentrations and PtdCho formation from endogenous PtdEtn in rat mammary homogenate were complex: a sigmoidal component, requiring 55 μM-AdoMet for half saturation ($V_{max.}$ = 9 pmol/h per mg of protein), and a high-affinity component ($K_{apparent}$ = 8.7 μM and $V_{max.}$ = 3.8 pmol/h per mg of protein) were identified (Fig. 4). The Hill plot of the data was non-linear and it was resolved into two components, one exhibiting a Hill coefficient of 0.85 (over the range 1.73–31 μM-AdoMet) and another exhibiting a Hill coefficient of 2.19 (over the range 41–300 μM-AdoMet). When exogenous PtdMe$_2$Etn was added as substrate, PtdCho formation exhibited Michaelis–Menten kinetics for AdoMet, and its affinity for AdoMet was high ($K_{apparent}$ = 9 μM, $V_{max.}$ = 85 pmol/h per mg of protein; Fig. 5). We observed no significant decrease of the enzyme activity when magnesium was omitted from the incubation medium (results not shown).

PeMT activity in rat mammary epithelial cells

Homogenates of rat epithelial cells, at low concentrations of AdoMet (0.6 μM), incorporated methyl groups

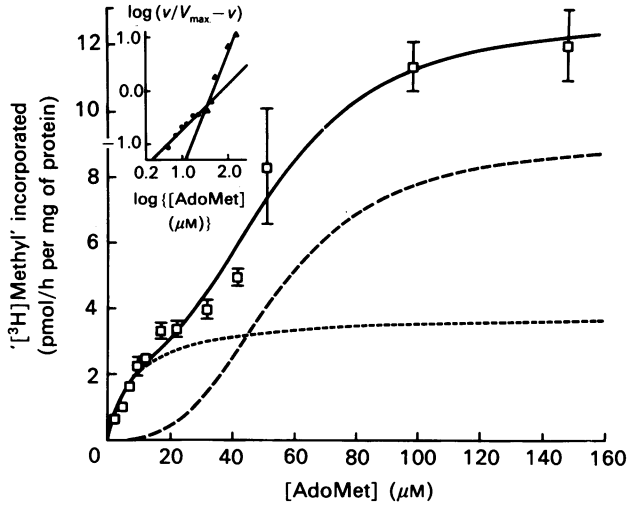

Fig. 4. Kinetic properties of rat mammary PeMT activity (endogenous phospholipid substrate)

Homogenates prepared from frozen lactating rat mammary tissue were incubated with various concentrations of [*methyl*-^{3}H]AdoMet; [^{3}H]PtdCho was extracted and purified by t.l.c. as described in the Materials and methods section. The kinetic parameters were calculated using the best fit to a hypothetical curve which was a combination of a rectangular hyperbola and a logistic curve. The graph shows the experimental points (±S.E.M.) and the best fit curve (solid line) as well as its two components (broken lines), the logistic:

$$\text{velocity} = (9.0[\text{AdoMet}]^3)/([\text{AdoMet}]^3 + 55.4^3)$$

and the hyperbolic:

$$\text{velocity} = (3.8[\text{AdoMet}])/(8.7 + [\text{AdoMet}])$$

The Hill plot (inset) was constructed from the data and the two lines that are the results of linear regressions using [AdoMet] between 1.73 and 31 μM (Hill coefficient 0.9) and between 41 and 300 μM (Hill coefficient 2.2). Data are expressed as pmol of '[^{3}H]methyl' incorporated into product/h per mg of protein (±S.E.M.). At least three samples were assayed for each data point.

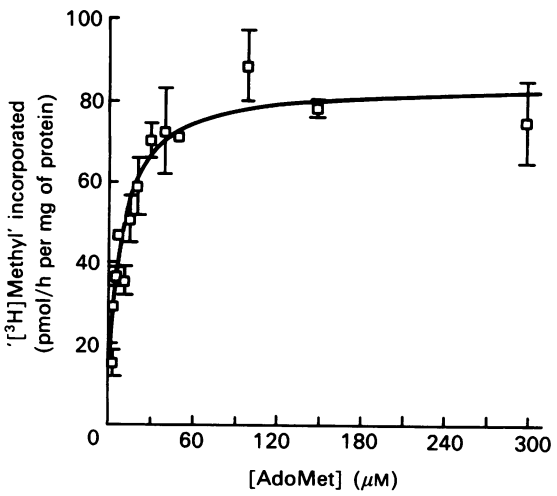

Fig. 5. Kinetic properties of rat mammary PeMT ($PtdMe_2Etn$ methylating)

Homogenates from frozen lactating rat mammary tissue were incubated with various concentration of [*methyl*-^{3}H]AdoMet and 100 μg of $PtdMe_2Etn$. [^{3}H]PtdCho was extracted and purified by t.l.c. as described in the Materials and methods section. The graph shows the experimental points (±S.E.M.) and the best fit to a hypothetical curve which was a rectangular hyperbola:

$$\text{velocity} = (84.7[\text{AdoMet}])/(8.6 + [\text{AdoMet}])$$

Data are expressed as pmol of '[^{3}H]methyl' incorporated into product/h per mg of protein (±S.E.M.). At least three samples were assayed for each data point.

into PtdCho at the rate of 1.6 pmol/h per mg of protein (±0.4; Fig. 6). Addition of 100 μg of $PtdMe_2Etn$ as substrate increased the rate of formation of PtdCho [to 6.4 pmol/h per mg of protein (±1.2); Fig. 6]. In the presence of 200 μM-AdoMet all products accumulated more rapidly, though the greatest increase was in $PtdMe_2Etn$ formation (to 221±13 pmol/h per mg of protein; $P < 0.01$ different from endogenous substrates by one-way ANOVA; Fig. 6). The addition of both 200 μM-AdoMet and 100 μg of $PtdMe_2Etn$ further increased the rates of formation of all products ($P < 0.01$ different from endogenous substrates, added AdoMet or added $PtdMe_2Etn$ by one-way ANOVA; Fig. 6).

Intact rat mammary epithelial cell mixtures contained 4 μM-methionine after the addition of radioactivity. Much more methyl incorporation (from methionine) into PtdCho occurred in this preparation (54 pmol/h per mg of protein; Fig. 6) than occurred in homogenates (from AdoMet). Addition of 200 μM-methionine increased the rate of formation of all products, especially that of PtdCho (to 7527 pmol/h per mg of protein; $P < 0.01$ different by one-way ANOVA; Fig. 6). The addition of 100 μg of $PtdMe_2Etn$ had no effect on product formation (Fig. 6).

PeMT activity in homogenates of human mammary tissue

Homogenates of human mammary tissue, at low concentrations of AdoMet (0.7 μM), incorporated methyl groups into PtdCho at the rate of 0.5 pmol/h per mg of protein (±0.1; Fig. 7). Addition of 100 μg of $PtdMe_2Etn$ as substrate increased the rate of formation of PtdCho [to 11 pmol/h per mg of protein (±0.7); Fig. 7]. In the presence of 200 μM-AdoMet all products accumulated more rapidly, and the greatest increase was in $PtdMe_2Etn$ methylation [to 42 (±6) pmol/h per mg of protein; $P < 0.01$ different from endogenous substrates by one-way ANOVA; Fig. 7]. The addition of both 200 μM-AdoMet and 100 μg of $PtdMe_2Etn$ further increased the rates of formation of PtdCho ($P < 0.01$ different from endogenous substrates, added AdoMet or added $PtdMe_2Etn$ by one-way ANOVA; Fig. 7).

DISCUSSION

We have identified, for the first time within mammary tissue of humans and rats, an enzyme (or enzymes) activity that catalyses the synthesis *de novo* of choline molecules via the sequential methylation of PtdEtn. We carefully established that we were, indeed, characterizing PtdCho formation via the methylation of PtdEtn. Radiolabel was incorporated into the choline moiety of phosphatidylcholine (Fig. 1). *S*-Adenosylhomocysteine was a potent inhibitor of the activity. Addition of

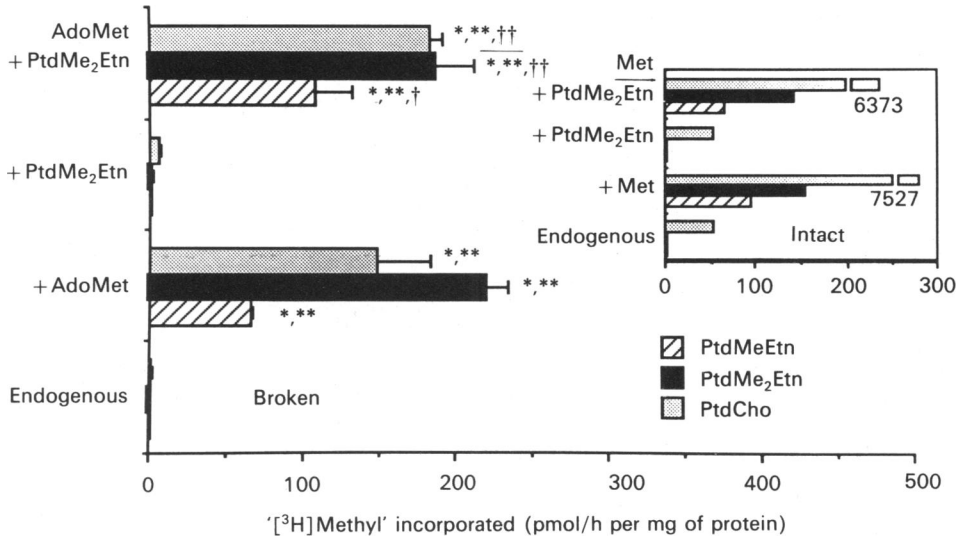

Fig. 6. PeMT activity in isolated mammary epithelial cells from lactating rats

Epithelial cells were isolated from lactating rat mammary tissue as described in the Materials and methods section. Homogenates ('Broken') were incubated in the presence of 0.63 μM-[*methyl*-^{3}H]AdoMet ('Endogenous'), 200 μM-[*methyl*-^{3}H]AdoMet (' + AdoMet'), 100 μg of dipalmitoyl-PtdMe$_2$Etn (' + PtdMe$_2$Etn'), or 200 μM-[*methyl*-^{3}H]AdoMet and 100 μg of dipalmitoyl-PtdMe$_2$Etn ('AdoMet + PtdMe$_2$Etn'). Intact cells (inset) were incubated in the presence of 4 μM-[*methyl*-^{3}H]methionine ('Endogenous'), 200 μM-[*methyl*-^{3}H]methionine (' + Met'), 100 μg of dipalmitoyl-PtdMe$_2$Etn (' + PtdMe$_2$Etn) or 200 μM-[*methyl*-^{3}H]methionine and 100 μg of dipalmitoyl-PtdMe$_2$Etn ('Met + PtdMe$_2$Etn'). Phospholipids were extracted and purified by t.l.c. as described in the Materials and methods section. Data are expressed as pmol of '[^{3}H]methyl' incorporated into product/h per mg of protein (±S.E.M.). At least three samples were assayed for each data point. $*P < 0.01$ different from 'Endogenous', $**P < 0.01$ different from ' + PtdMe$_2$Etn', $\dagger P < 0.01$ different from ' + AdoMet' and $\dagger\dagger P < 0.05$ different from ' + AdoMet' by one-way analysis of variance.

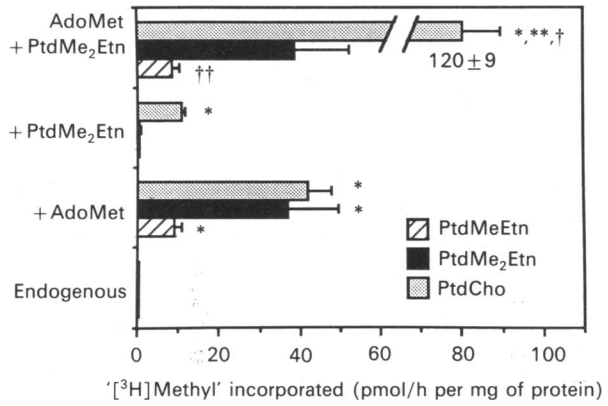

Fig. 7. PeMT activity in homogenates on non-lactating human mammary tissue

Human mammary tissue was collected and frozen at −95 °C until used. Homogenates were incubated in the presence of 0.73 μM-[*methyl*-^{3}H]AdoMet ('Endogenous'), 200 μM-[*methyl*-^{3}H]AdoMet (' + AdoMet'), 100 μg of dipalmitoyl-PtdMe$_2$Etn (' + PtdMe$_2$Etn), or 200 μM-[*methyl*-^{3}H]AdoMet and 100 μg of dipalmitoyl-PtdMe$_2$Etn ('AdoMet + PtdMe$_2$Etn'). Phospholipids were extracted and purified by t.l.c. as described in the Materials and methods section. Data are expressed as pmol of '[^{3}H]methyl' incorporated into product/h per mg of protein (±S.E.M.). At least three samples were assayed for each data point. $*P < 0.01$ different from 'Endogenous', $**P < 0.01$ different from ' + PtdMe$_2$Etn', $\dagger P < 0.01$ different from ' + AdoMet', and $\dagger\dagger P < 0.05$ different from 'Endogenous', ' + PtdMe$_2$Etn' and ' + AdoMet' by one-way analysis of variance.

PtdMe$_2$Etn as a lipid substrate accelerated the rate of incorporation of radiolabel into PtdCho, as did addition of AdoMet or methionine (Figs. 1–7). We were able to demonstrate that PeMT activity was present in purified preparations of mammary epithelial cells, the site of milk production. Bhattacharya & Vonderhaar [24] have previously reported incorporation of radiolabel derived from [^{3}H]AdoMet into lipids of mouse mammary membranes; however, they made no attempt to identify the lipid labelled. We have frequently observed accumulation of radiolabel in unidentified lipid which migrates near the solvent front of our t.l.c. system. For this reason, measurement of radioactivity in lipids is not sufficient if quantification of PeMT is desired.

In mammary tissue, we are describing enzyme activities with different affinities for AdoMet ($K_{apparent} = 55$ μM and $K_{apparent} = 9$ μM). The relationship between the velocity of production of product by this enzyme(s) in mammary tissue and the concentration of AdoMet, and differences in activities as a function of pH, suggest the action of two distinct enzymes or active sites (Figs. 3, 4 and 5). It has been suggested that the conversion of PtdEtn to PtdCho is carried out by two enzyme activities in brain and red blood cells [7,10]. In liver, however, it is unclear whether there are one or two enzyme activities. Ridgeway & Vance [13] found that, within rat liver, a single membrane-bound enzyme catalysed all three methylating steps for the conversion of PtdEtn to PtdCho. However, other investigators have found that rat liver PeMT can exist in two forms, as a monomer and as a dimer [25]. Only the monomer has a high affinity for AdoMet [25]. In rat liver endoplasmic reticulum the enzyme catalysing the first methylation is located only on

the inner surface of the membrane, while the second and third methylations are catalysed by activities on both the inner and outer surfaces [26]. Perhaps Ridgeway and Vance [13] have characterized the inner surface enzyme but not the outer surface enzyme. The most compelling evidence that the conversion of PtdEtn to PtdCho is carried out by two enzyme activities in some systems comes from the study of PeMT in the yeast mutant *Saccharomyces cerevisiae opi*3-3 [27]. This mutant is defective in the $PtdMe_2Etn$ methylating step, and possibly in the methylation of PtdMeEtn. It accumulates PtdMeEtn and, to a lesser extent, $PtdMe_2Etn$, and it does not accumulate PtdCho via the methylation pathway. This mutant does not have any difficulty in making PtdCho from exogenous choline.

The first methylation may be rate-limiting (as it is in brain [7]), and therefore, we probably are describing the kinetic properties of this first enzyme in Fig. 4. The kinetic properties for this methylation were different than those previously described for most other tissues [6–12], but were remarkably similar to those previously described for a special PeMT (PtdEtn methylating) transiently present in neonatal rat brain [7]. Affinity for AdoMet was low, and its [AdoMet]-versus-velocity relationship could best be described by a sigmoidal curve. The Hill coefficient of this enzymic activity was 2.19 (calculated from a Hill plot) or 3.0 (calculated from a best fit of the [AdoMet]-versus-velocity data). It is impossible to state whether this indicates more than one form of PtdEtn methylating enzyme, with distinct binding sites, or a co-operative effect. PeMT ($PtdMe_2Etn$ methylating) had high affinity for AdoMet, and exhibited pure Michaelis–Menten type kinetics (Fig. 5). PeMT ($PtdMe_2Etn$ methylating) activity was greater in lactating than in non-lactating tissue, suggesting that this activity may be influenced by the hormonal status of the mammary gland.

When intact epithelial cells were studied, using [^{3}H]-methionine as the methyl donor, we observed much higher PtdCho forming activity than in broken cells. In homogenates, required substrates are diluted 10-fold. When tissue was broken by sonication, the structural relationships between phospholipid and AdoMet substrates and enzyme may have been perturbed, and the enzyme may have been physically damaged. It is interesting that PeMT ($PtdMe_2Etn$ methylating) was the most sensitive to cell disruption (Fig. 6). This suggests that this methylation may have been catalysed by a distinct enzyme activity, rather than by the same activity which catalyses the methylation of PtdMeEtn (Fig. 6). In intact cells exogenous $PtdMe_2Etn$ did not affect the rate of PtdCho formation (Fig. 6), suggesting that this phospholipid could not enter the cells to reach the PeMT activity.

We have identified a new capacity within mammary tissue for generating PtdCho molecules. PtdCho can be cleaved to form choline in reactions catalysed by several phospholipases [28]. Could this be a significant source of the choline excreted in milk? The mammary gland has only one other source for the choline in milk — uptake from maternal blood. Under physiological conditions, 500 pmol of choline/h per mg of protein are taken up by mammary gland [5]. We observed that intact mammary cells make, *de novo*, 50–7000 pmol of PtdCho/h per mg of protein (Fig. 6). Thus a significant portion of the choline in milk could be derived from the methylation pathway. This may be one mechanism whereby milk choline levels are maintained at concentrations far in excess of those present in maternal blood. The high choline content of milk is, in part, responsible for the very high choline concentrations in neonatal animal's blood [29,30]. The choline concentration of human milk briefly increases from 150 μM to 650 μM shortly post partum [4]. Maternal plasma choline concentration (approx. 10 μM) does not change during this period (our unpublished results). It would be interesting to determine whether changes in mammary PeMT activity are responsible for these transient changes in milk choline concentration. Perhaps PeMT is influenced by hormones such as oxytocin, prolactin, oestrogen or progesterone.

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