Immunochemical characterization of L-isoaspartyl-protein carboxyl methyltransferase from mammalian tissues

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Polyclonal antibodies were raised against a synthetic peptide corresponding to a sequence of 14 amino acid residues found near the C-terminus of L-isoaspartyl (D-aspartyl)-protein carboxyl methyltransferase (PCMT). The affinity-purified antibodies were used to detect the methyltransferase by Western-blot analysis in cytosolic and membrane fractions from several mammalian tissues. A protein of 27 kDa was detected in the cytosol of most tissues; co-incubation with the peptide used for immunization abolished the detection. The identity of the 27 kDa protein as a PCMT was demonstrated by renaturation of PCMT activity from SDS/polyacrylamide gels. The methyltransferase from brain cytosol was immunoprecipitated by the anti-PCMT antibodies and Protein A–agarose, indicating that the native protein was recognized by the antibodies. PCMT was also immunodetected in crude membranes from brain, testes and heart, and in purified membranes from kidney cortex. The expression of the methyltransferase was higher in bovine and human brain than in rat tissues. The bovine enzyme had a greater electrophoretic mobility, suggesting small structural differences. The membrane-bound methyltransferase could be extracted with detergents above their critical micellar concentration, but not with salt, alkaline or urea solutions, suggesting that the binding of the enzyme to membranes is hydrophobic by nature. Anti-PCMT antibodies provide an interesting tool for studies regarding the expression of these enzymes in both soluble and membrane fractions of various cell types.

INTRODUCTION

Carboxyl methylation reactions can occur in cells at four different amino acid residues in proteins. These reactions are catalysed by distinct protein carboxyl methyltransferases (PCMT) that transfer a methyl group from *S*-adenosyl-L-methionine (AdoMet) to carboxyl groups of various acceptor proteins [1–3]. The first class of PCMT is found exclusively in chemotactic bacteria, where membrane receptors are reversibly methylated at specific glutamic acid residues [4]. Class II, L-isoaspartyl (D-aspartyl)-PCMT is a widely distributed enzyme that methylates abnormal aspartic acid residues in proteins [1]. C-terminal isoprenylcysteine PCMT (class III) catalyses the methyl esterification of C-terminal cysteine residues previously modified by a farnesyl or geranylgeranyl group [5–7]. Recently, a fourth class of PCMT that methylates the C-terminal leucine residue of a 36 kDa polypeptide has been identified in brain cytosol [8]. This substrate was later identified as the catalytic subunit of protein phosphatase 2A [9, 10].

Class II PCMTs methylate proteins and peptides that contain L-isoaspartyl [11–13] and D-aspartyl [14, 15] residues produced by spontaneous deamidation of asparagine residues or by racemization [16]. Although the physiological role of class II PCMT has not been conclusively defined, it has been suggested that this enzyme is involved in the repair of damaged proteins by facilitating the conversion of these altered residues into normal residues in proteins [17]. Class II PCMT is primarily a cytoplasmic enzyme that has been purified from the cytosols of many sources, including erythrocytes [18], brain [19], *Escherichia coli* [20] and wheat-germ [21]. However, in contrast with other classes of carboxymethyltransferases which are found exclusively in cytosol (class I) or in membranes (class III), class II PCMT are distributed in both compartments, at least in bovine brain [22,23], rat kidney [24,25] and chicken erythrocytes [26].

PCMT amino acid sequences have been determined by amino acid sequencing [27,28] and deduced from the nucleotide sequences of corresponding cDNAs [29,30]. Isoforms of PCMT have been identified in most mammalian tissues [31] and purified from bovine brain [19] and from human and bovine erythrocytes [18]. The molecular mass calculated from the amino acid sequences is 24.5 kDa, in good agreement with the apparent molecular sizes of purified PCMTs determined by SDS/PAGE, which are in the 25–27 kDa range [31]. However, larger sizes of 30 and 34 kDa have also been reported for the enzyme from *Torpedo ocellata* electric organ [32] and *Xenopus laevis* oocytes [33] respectively.

In the present study we examined the expression of L-isoaspartyl (D-aspartyl) methyltransferase in various mammalian tissues with antibodies directed against the C-terminus of the enzyme. PCMT was not only detected in all the cytosols tested, but also in membranes from brain, testis and kidney. The expression of PCMT was higher in bovine and human than in rat tissues. The extraction behaviour of membrane-bound PCMT suggests a hydrophobic association with the lipid bilayer, since it was extracted by detergents but not significantly by salts and urea.

MATERIALS AND METHODS

Materials

Male Sprague–Dawley rats (150–300 g body weight) and New Zealand White rabbits (2 kg body weight) were obtained from Charles River (St.-Constant, Qué., Canada). All products required for peptide synthesis were from Applied Biosystems

Abbreviations used: PCMT, protein carboxymethyltransferase; AdoMet, *S*-adenosyl-L-methionine; KLH, keyhole-limpet haemocyanin; TBS, Tris-buffered saline; BBM, brush-border membrane; BLM, basolateral membrane; IM, intracellular membranes; CMC, critical micellar concentration.

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(Mississauga, Ont., Canada). Freund's adjuvants and keyhole-limpet haemocyanin (KLH) were purchased from Pierce (Rockford, IL, U.S.A.). HiTrap Protein A and CNBr-activated Sepharose were from Pharmacia–LKB (Dorval, Qué., Canada), *S*-Adenosyl-L-[*methyl*-^{3}H]methionine ([^{3}H]AdoMet; 60–85 Ci/mmol), from du Pont–New England Nuclear (Boston, MA, U.S.A.), and reagents for electrophoresis from Bio-Rad (Mississauga, Ont., Canada). All other reagents were purchased from Sigma (St. Louis, MO, U.S.A.).

Peptide synthesis

The peptide (C)VIYVPLTDKEKQWS, corresponding to the amino acid residues 210–223 from sequences found in mammalian PCMTs (see references in Table 1), was synthesized by Fmoc (9-fluorenylmethyloxycarbonyl) solid-phase chemistry with an Applied Biosystems 431A peptide synthesizer (Mississauga, Ont., Canada). The crude peptide was purified on a semi-preparative (1.0 cm × 25 cm) reverse-phase C_{18} HPLC column (Vydac), and the purity was confirmed by analytical HPLC and fast-atom-bombardment MS. A cysteine residue was added at the N-terminus to allow coupling of the peptide through a disulphide bond to KLH as described previously [34].

Immunization

Rabbits were injected subcutaneously at four sites on the back with 200 μg of peptide–KLH in complete Freund's adjuvant. Booster injections were administered 2 and 3 weeks after initial immunization with the same quantity of antigen in incomplete Freund's adjuvant. Reactivity of the antiserum against peptide and KLH was monitored by an ELISA as described previously [34]. The antibodies were purified successively by affinity chromatography on HiTrap Protein A and on a column of peptide coupled to CNBr-activated Sepharose according to the instructions of the manufacturer.

Western-blot analysis

SDS-PAGE was performed as described by Laemmli [35] with a MINI-PROTEAN II apparatus (Bio-Rad). Briefly, 10–20 μg of protein were heated at 100 °C in Laemmli's sample buffer for 3 min before loading on to 0.75 mm-thick 12.5 % polyacrylamide gels. Proteins were electroblotted on to 0.45 μm-pore-diameter poly(vinylidene difluoride) membranes (Immobilon-P; Millipore) in transfer buffer (192 mM glycine/25 mM Tris/20 % methanol) at 100 V for 2 h [36]. The blots were blocked overnight at 4 °C in Tris-buffered saline (TBS; 20 mM Tris/137 mM NaCl, pH 7.5) containing 0.1 % (v/v) Tween 20 and 5 % non-fat dry milk (Carnation). Each blot was incubated with a 1:200 dilution of affinity-purified antibodies (about 1 μg/ml final concn.) in TBS containing 1 % Tween 20 and 5 % non-fat dry milk for 2 h at room temperature, followed by a 1 h incubation with a 1:1000–1:2000 dilution of donkey anti-rabbit IgG conjugated to horseradish peroxidase (Amersham, Oakville, Ont., Canada) in the same incubation medium. Immunoreactive bands were revealed with enhanced chemiluminescence (ECL) Western-blotting kit (Amersham). Blots were exposed to Fuji films for about 10–30 min and scanned with an LKB Ultroscan XL laser densitometer. Molecular-mass determination was performed using the following standards: β-galactosidase (116.2 kDa), phosphorylase *b* (97.4 kDa), BSA (66.2 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa), soybean trypsin inhibitor (21.5 kDa) and lysozyme (14.4 kDa).

Cytosol and crude membrane preparations

Sprague–Dawley male rats were anaesthetized with CO_2, killed by decapitation, and the tissues were washed with an ice-cold 0.15 M NaCl solution. Fresh bovine brains and kidneys were obtained from a local abattoir and human brains were obtained *post mortem* (with the informed consent of the next-of-kin). Tissues were minced in 250 mM sucrose/5 mM Hepes/Tris, pH 7.5, and homogenized with a Polytron homogenizer (Brinkman Instruments). The crude homogenates were centrifuged at 3000 *g* for 10 min at 4 °C to remove unbroken cells and nuclei. The resultant supernatants were centrifuged at 100000 *g* for 1 h to obtain the soluble and crude membrane fractions. Protein concentration was determined using the Coomassie Blue-binding assay [37] with BSA as the standard.

Rat kidney membrane preparations

Brush-border membranes (BBM) were purified from rat kidney cortex by an Mg^{2+}-precipitation method [38]. Basolateral membranes (BLM) were purified from rat kidney cortex with a self-orienting-Percoll-density-gradient-centrifugation method [39] with minor modifications [40]. Nuclei and mitochondria were prepared as described previously [41]. Intracellular membranes (IM) were prepared as follows: plasma membranes (BBM and BLM) were sedimented by centrifugation at 40000 *g* for 20 min, and the resulting supernatant was centrifuged at 100000 *g* for 60 min to sediment the IM. Isolated membranes were resuspended in 50 mM mannitol/5 mM Hepes/Tris, pH 7.5, to a concentration of 10–15 mg of protein/ml and stored in liquid nitrogen. The purity of the BBM, BLM and IM preparations was determined by measuring alkaline phosphatase [42], Na^+–K^+-ATPase [43], and NADPH:cytochrome *c* reductase [44] activities respectively. BBM showed a 10–12-fold enrichment in alkaline phosphatase activity over the cortex homogenate. BLM were enriched 15–20-fold in Na^+–K^+-ATPase activity, and IM were enriched 3–4-fold in NADPH:cytochrome c reductase activity.

PCMT assay

Samples were incubated at 37 °C in 100 mM Hepes/Tris buffer, pH 7.0, with 10 μM [^{3}H]AdoMet and 500 μg of ovalbumin in a final volume of 30 μl. After 20 min the reaction was stopped by the addition of an equal volume of 1 % (w/v) SDS/0.2 M NaOH. An aliquot of 40 μl was quickly spotted on to a piece of pleated filter paper (1.5 cm × 8.5 cm) that was inserted in the neck of a 20 ml vial containing 10 ml of FORMULA 963 scintillation fluor (NEN). The vial was capped and left at room temperature to allow the diffusion of the volatile methanol resulting from the hydrolysis of the methyl esters [45]. After 2 h, the paper was removed and the radioactivity was measured by liquid-scintillation spectrophotometry. Samples incubated without ovalbumin were run concurrently to establish the background level.

RESULTS

Polyclonal antibodies directed against a synthetic peptide corresponding to 14 amino acid residues found in the C-terminal portion of mammalian L-isoaspartyl PCMTs were raised in rabbit. The last three C-terminal amino acid residues encoded by the PCMT gene (Table 1) were not included in the synthetic peptide, since they are not found in both isoenzyme species arising from alternative splicing of a unique PCMT gene [46,47]. The antibodies from rabbit serum were affinity-purified successively by chromatography on Protein A–agarose and peptide–agarose columns, the first column providing total immuno-

Table 1 C-terminal sequences of PCMTs and the sequence of the peptide used as an antigen for the preparation of anti-PCMT antibodies

PCMT species	Sequence
Isoenzyme I, bovine brain [27], rat brain [29], human erthrocyte [28] and mouse testis [30]	210 V I Y V P L T D K E K Q W S R W K 226
Isoenzyme II, bovine brain [46] and human brain [54]	210 V I Y V P L T D K E K Q W S R D E L 227
Wheat-germ [21]	215 V R Y V P L T S R S A Q L Q D S 230
Escherichia coli [20]	196 V R F V P L V K G E L A 207
Peptide	(C) V I Y V P L T D K E K Q W S

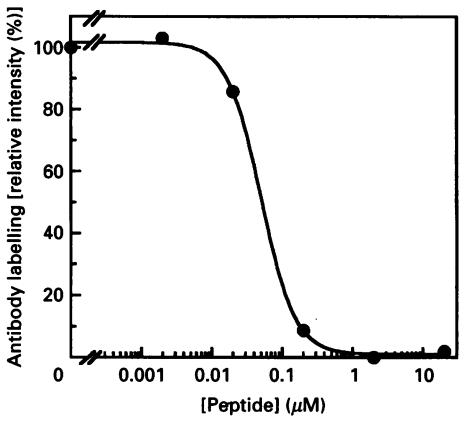

Figure 1 Immunodetection of PCMT in rat brain cytosol

Cytosolic proteins (10 μg/lane) from rat brain were separated by electrophoresis on SDS/12.5%-polyacrylamide gels and transferred to poly(vinylidene difluoride) membranes. The blots were incubated with affinity-purified anti-PCMT antibodies (1:200) in the presence of 0–20 μM of the synthetic peptide used for immunization. The blots were then incubated with donkey anti-rabbit IgG horseradish peroxidase-conjugated antibodies (1:2000) and the labelled antigens were revealed with an enhanced chemiluminescence (ECL) detection kit. The intensity of the 27 kDa (●) labelled protein band was measured by densitometric scanning of the film.

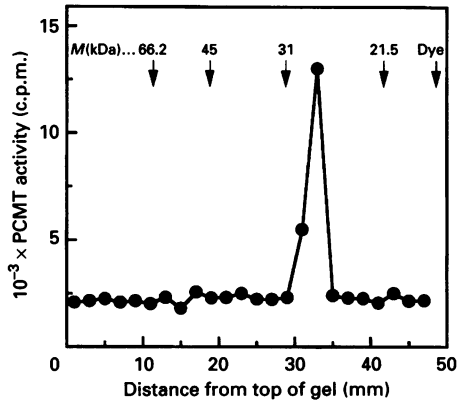

Figure 2 Determination of the molecular mass (*M*) of PCMT by renaturation from SDS/polyacrylamide gel

Cytosolic proteins (10 μg/lane) from rat brain were separated by electrophoresis on an SDS/12.5%-polyacrylamide gel, without prior heating of the sample. After electrophoresis, the gel was sliced (2 mm/slice) and the gel pieces were incubated overnight at 20 °C in 250 μl of 1% Triton X-100/50 mM Tris/HCl (pH 8.0)/15 mM β-mercaptoethanol. PCMT activity was determined by incubating 20 μl aliquots for 1 h at 37 °C in the presence of [^{3}H]AdoMet and ovalbumin.

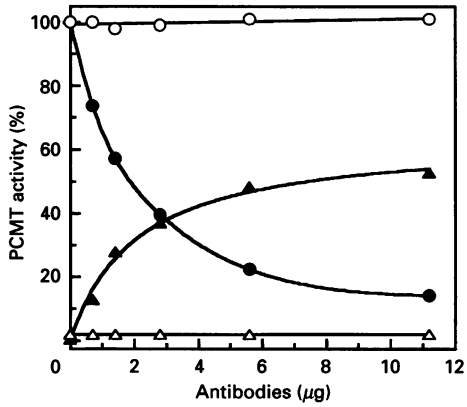

Figure 3 Immunoprecipitation of PCMT activity

Rat brain cytosol (1 mg of protein/ml) was incubated at 4 °C for 2 h in the presence of immune (filled symbols) or non-immune (open symbols) antibodies. Protein A–agarose beads (10 μl, prewashed in TBS) were added to the sample and incubated overnight. The beads were pelleted by centrifugation at 3000 *g*, 2 min and washed four times with PBS. PCMT activity was measured in the supernatants (●,○) and in the beads pellets (▲, △) as described in the Materials and methods section.

globulins and the second only the peptide-specific antibodies. Immunoblot analysis showed that one predominant cytosolic protein of 27 kDa from brain cytosol was strongly labelled in most tissues (Figure 4 below). Co-incubation of the peptide with the antibodies resulted in a dose-dependent decrease in the labelling of the 27 kDa protein (Figure 1), half-displacement occurring at a peptide concentration of 50 nM. Cloned PCMT genes predict a polypeptide size of 24.5 kDa, and most purified PCMTs were reported to have an apparent size of 25–27 kDa on SDS/polyacrylamide gels.

We have previously shown that PCMT can be partially renatured from SDS/polyacrylamide gels [24]. Using this technique with rat brain cytosol, only one peak of activity was detected, corresponding to a protein of 27 kDa (Figure 2). In the absence of β-mercaptoethanol in the SDS/PAGE sample buffer, the migration of the 27 kDa band was unaffected (results not shown), indicating that the C-terminal binding site is recognized under both reducing and non-reducing conditions.

The ability of these antibodies to immunoprecipitate the methyltransferase activity was then studied (Figure 3). After incubation of cytosol with immune antibodies and Protein A–agarose beads, PCMT activity decreased in the supernatant and, conversely, increased in the washed Protein A–agarose

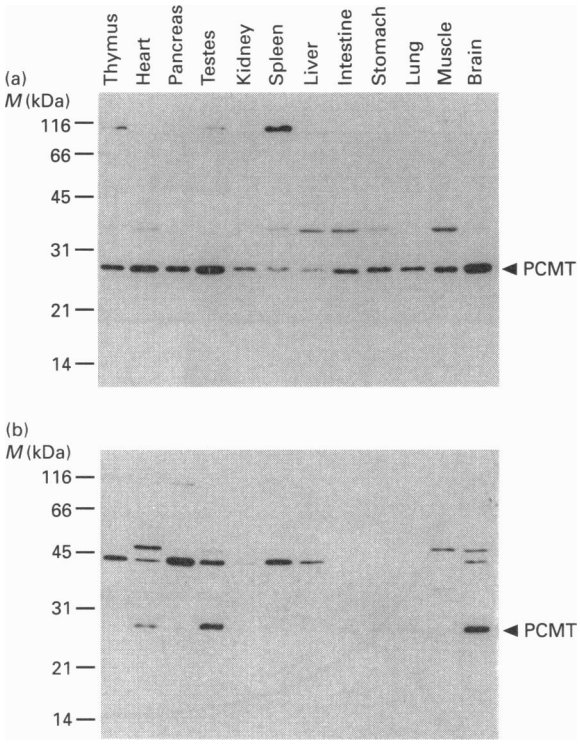

Figure 4 Distribution of PCMT in cytosols and membranes from rat tissues

PCMT was detected by Western-blot analysis as described in Figure 1 in cytosol (**a**) and membranes (**b**) (10 μg of protein) from various rat tissues. Gels and blots from (**a**) and (**b**) were processed together, allowing comparison of the levels of expression in both fractions. Abbreviation: *M*, molecular mass.

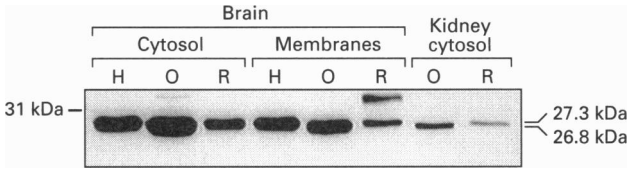

Figure 5 Immunodetection of PCMT in different species

PCMT was detected in brain cytosol and crude membranes from human (H), ox (O), and rat (R), and in kidney cytosol from ox (O) and rat (R).

beads. Control rabbit IgGs had no effect. This result indicates that the antibodies are able to react with native PCMT in addition to the denatured form detected on blots. Recovery of PCMT activity in the immunoprecipitate, up to 52%, suggests that the binding of the antibodies did not interfere extensively with enzyme activity.

The tissue distribution of PCMT was investigated by Western-blot analysis. PCMT was detected in the cytosol of all tissues tested (Figure 4a), brain, testes and heart showing the highest levels. In crude membranes prepared from tissue homogenates, PCMT was immunodetected at a high level in brain and testes (Figure 4b); it was also detected in all other tissues after longer film exposures (results not shown). The antibodies react with other proteins (about 43-48 kDa) in membranes from some tissues but since these proteins are not detected in cytosols, where most of PCMT activity is found, we conclude that they are not related to PCMT. The tissue distribution of PCMT estimated here by immunoblot analysis is in good agreement with those previously obtained by measuring enzyme activity [48] and by Northern-blot analysis [30,49].

Comparison of the PCMT levels detected in brains from different species showed 1.6- and 2.2-fold higher expression of the enzyme in human and ox respectively compared with rat (Figure 5). In brain membranes, PCMT levels were 4-fold higher in ox and human than in rat. The bovine enzymes from brain and kidney appeared to be slightly smaller than those from human and rat tissues. This small but significant difference in polypeptide size was estimated to 0.5 kDa. A similar difference in migration between PCMT from bovine brain and murine testes has been previously reported [50].

Epithelial cells from kidney cortex are characterized by the differentiation of the plasma membrane into two distinct domains: a luminal brush-border membrane (BBM) and an antiluminal basolateral membrane (BLM). These cells represent a useful model for studies on the subcellular distribution and targetting of membrane-bound proteins. We have recently purified and characterized a membrane-bound L-isoaspartyl PCMT from kidney cortex [24]. This enzyme was found to be kinetically and structurally similar to the cytosolic form. Western-blot analysis confirmed that PCMT is present in BBM and BLM, in addition to the cytosol (Figure 6). The enzyme was also detected in a crude mitochondrial fraction and in nuclei. A substantial amount of PCMT activity associated with the mitochondrial fraction of rat brain has been previously reported [51]. Surprisingly, a protein with a slightly higher molecular mass was also detected in BBM, BLM, and mitochondria, but not in cytosol, IM and nuclei from rat kidney (Figure 6). This extra band appears to be due to non-specific binding, since (i) only the 27 kDa protein displays enzymic activity after renaturation from polyacrylamide gels [24,25] and (ii) this additional band is not present in cytosol and membranes from other tissues (see Figure 4).

Figure 7 shows the expression of PCMT in cytosol and membrane fractions from brains of rats of various ages. Large increases of PCMT occur between 15 and 77 days after birth in both cytosolic and membrane fractions. Afterward, PCMT expression was 55% higher in brain membranes from old rats (390 days) than in those from young rats (77 days), but remained

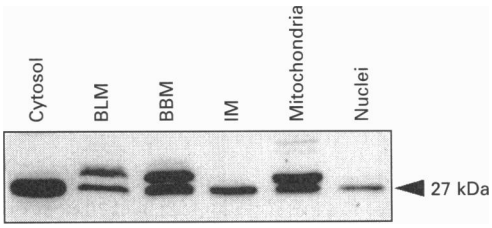

Figure 6 Immunodetection of PCMT in subcellular fractions of rat kidney cortex

PCMT was detected in different fractions of kidney cortex as described in the Materials and methods section, except that 20 μg of protein of each fraction was loaded on the gel. The subcellular fractions used were: cytosol, BLM, BBM, IM, mitochondria and nuclei.

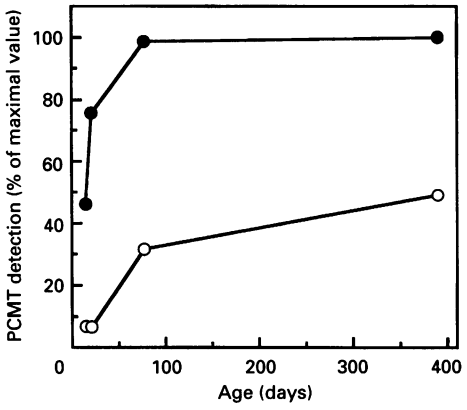

Figure 7 Age-dependency of PCMT expression in rat brain cytosol and membranes

Cytosol (●) and crude membranes (○) were prepared from brains of male rats aged 15, 21, 77 and 390 days, and 10 μg of proteins were used to detect PCMT by Western-blot analysis as described in the Materials and methods section.

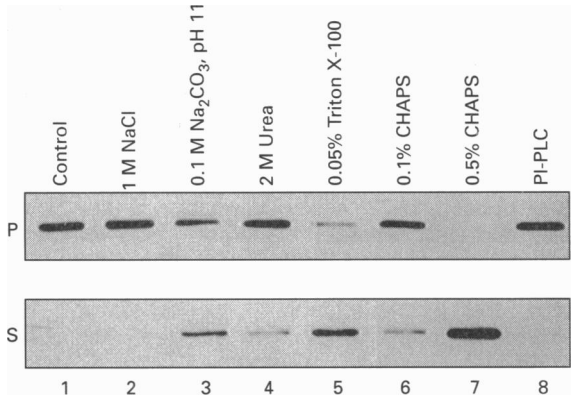

Figure 8 Extraction of PCMT from rat brain membranes

Membranes (1 mg of protein/ml) were incubated at 20 °C for 30 min in the absence (1) or presence of 1 M NaCl (2), 0.1 M Na_2CO_3, pH 11 (3), 2 M urea (4), 0.05% Triton X-100 (5), 0.1% CHAPS (6), 0.5% CHAPS (7) or 1 unit of phosphatidylinositol-specific phospholipase C (PI-PLC) (8); 20 mM Tris/HCl, pH 7.5, was present in all incubation media except for (3). The mixtures were centrifuged in a Beckman Airfuge at 124.2 kPa (18 lbf/in^2) for 15 min, and the resulting supernatants (S) and pellets (P) were used for Western-blot analysis as described in the Materials and methods section.

unchanged in the cytosol. PCMT activity in rat brain cytosol has been previously reported to increase considerably during the development [51]. The ratio of cytosolic/membrane-bound PCMT is decreased with age: 12:1, 3:1 and 2:1 after 21, 77 and 390 days respectively.

The membrane association of brain PCMT was examined by testing whether the enzyme was solubilized by compounds known to extract peripheral or integral proteins (Figure 8). PCMT was not extracted by NaCl (1 M) and urea (2 M) solutions, whereas an alkaline buffer (pH 11) was partially effective. PCMT was solubilized by the non-ionic detergent Triton X-100 at 5-fold its critical micellar concentration (CMC). CHAPS also solubilized PCMT at a concentration equal to its CMC (0.5%), but not below its CMC (0.1%). Phosphatidylinositol-specific phospholipase C, known to release proteins anchored in the membranes with a glycophosphatidylinositol moiety, was ineffective on membrane-associated PCMT.

DISCUSSION

In the present study we used C-terminus-directed polyclonal antibodies to detect and characterize PCMT in mammalian tissues. The identity of the 27 kDa protein as a PCMT was strongly suggested by measurement of methyltransferase activity after renaturation from SDS/polyacrylamide gel. Furthermore, this apparent molecular mass is in good agreement with those previously reported for purified PCMTs and those calculated from their amino acid sequences.

The ubiquitous nature of PCMT in mammalian cells is well illustrated by the distribution of this enzyme in rat tissues as determined by Western-blot analysis. PCMT is expressed at higher levels in brain, testes and heart, moderately in pancreas, thymus, muscle, lung, stomach and intestine in comparison with kidney, spleen and liver. PCMT was also strongly detected in brain membranes, confirming previous identification of the enzyme activity in this tissue, and also moderately in testes. PCMT had been previously immunodetected in soluble extracts of mouse testes and in extracts from detergent-treated spermatogenic cells [50], but to our knowledge membrane-associated methyltransferase expression has not been previously reported in this tissue. In a recent study on PCMT gene expression in rat testis and brain, it has been suggested that the testicular enzyme may lack 31 amino acid residues found in the N-terminal portion of brain PCMT [49]. We detected a high level of the 27 kDa PCMT in this tissue, but no smaller form of the enzyme, which would be readily detectable with the C-terminus-directed anti-PCMT antibodies.

PCMT from bovine tissues migrated as a slightly-lower-molecular-mass protein in SDS/polyacrylamide gels than the enzyme from human and rat tissues. This difference in apparent size is rather surprising, because the amino acid sequence of PCMT from bovine brain [27] and that deduced from rat brain cDNA [29] show a similarity of 96% (nine residues different out of 226), and a calculated molecular-mass difference of only 60 Da. Moreover, human and rat PCMT migrate at exactly the same position in SDS/PAGE, even if their sequences [29,47] differ at ten amino acid residues (95.6% similarity). This may suggest that the bovine enzyme is post-translationally modified or that the few amino acid changes cause the protein to migrate at a higher apparent molecular mass.

Although PCMT activity is found predominantly in the cytosolic fraction of cells, there has been reports of membrane-associated PCMT activities from brain [22,23,52], erythrocytes [26] and kidney [24,25,53]. In rat kidney, immunoreactive PCMT is detected in different membrane fractions as well as in the cytosol. The primary structure of PCMT predicts a hydrophilic protein, but interestingly the sequence of the more acidic isoenzyme II (Table 1), which differs only at the C-terminus, ends with a sequence motif (-RDEL) known to act as a signal for retention in the endoplasmic reticulum. However, equal amounts of isoenzymes I and II were found in the microsomal fraction from rat brain [46]. Furthermore, more than 95% of the PCMT associated with the kidney BBM is the more basic isoenzyme I [24], whereas most of the PCMT activity associated with the kidney basolateral membrane is catalysed by isoenzyme II [25]. Thus the membrane localization of PCMTs does not appear to be isoform-specific.

The molecular basis of the membrane association of PCMT remains unclear. Although PCMT is found predominantly in the

cytosol of cells and although its primary structure predicts an hydrophilic protein, we believe that the presence of PCMT in membrane fractions is not due to contamination by the cytosolic enzyme and propose that the membrane-associated PCMT is an integral membrane protein, based on the following criteria: (1) washing the membranes with a hypo-osmolar solution, to release vesicle-entrapped enzymes, had no effect; (2) the amount of PCMT detected in membranes is much higher than that which would be predicted from simple intravesicular entrapment; in fact, PCMT levels are almost as high in the membranes as in the cytosols from brain and testes; (3) extraction of PCMT from the membranes could be achieved with detergents, but not with compounds known to extract peripheral and loosely bound proteins. Further work is needed to determine the exact molecular interaction of PCMT with either membrane lipids or proteins. The use of antibodies raised against PCMTs provides a useful tool to study the expression and subcellular distribution of this enzyme.

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