Characterization of Protein Carboxyl-O-methyltransferase in the Spontaneous in Vivo Murine C-1300 Neuroblastoma

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ABSTRACT

Protein carboxyl-O-methyltransferase (PCM) activity was determined in subcellular fractions prepared from C-1300 neuroblastoma tumors following transplantation and growth in male A/J mice. Fractions were obtained by differential centrifugation, and PCM activity was determined in all fractions in the presence (+gel) and absence (-gel) of an exogenous substrate, gelatin. Sixty % of the PCM activity in the absence of exogenous substrate (-gel) was contained in the crude 800 x g particulate fraction, whereas 80% of the PCM activity in the presence of gelatin (+gel) was present in the postmicrosomal (100,000 x g) supernatant. The latter fraction also contained the highest specific activity of PCM. A K_m of 3.2 x 10^{-6} M and a V_max of 5.3 pmol per mg protein per min were obtained for PCM activity (+gel) in the high-speed supernatant.

Cytoplasmic PCM was highly sensitive to competitive inhibition by S-adenosylhomocysteine and the S-adenosyl-homocysteine analog sinefungin except that it has a double bond at the 4',5'-position (see Chart 4). The role of PCM as a potential modulator of neuronal function is suggested by observations that stimulation of catecholamine secretion from the adrenal medulla promotes a concurrent increase in carboxymethylation of membrane protein(s) (7). Strittmatter et al. (27) also demonstrated an increase in PCM and MAP activity following in vitro or in vivo exposure of the parotid gland to 1-isoproterenol. An elevation in PCM activity, in the absence of endogenous substrates only, has also been observed in differentiating human monocytes and pulmonary alveolar macrophages in tissue culture, suggesting a potential role of PCM in cellular differentiation (29).

Since its discovery in 1940 as a spontaneous tumor occurring in the male A/J mouse, considerable interest has been expressed in the murine C-1300 neuroblastoma as an experimental model of neural behavior (11). Several studies have demonstrated that certain neuroblastoma cell clones extend neurites in tissue culture (28), suggesting that expression of this "neural" behavior is regulated by changes in cyclic nucleotides (23), cyclic nucleotide binding proteins (15, 24), or other undefined biochemical events. Because of the hypotheses that PCM may modulate cellular growth and certain neuronal functions, this enzyme system in the in vivo murine neuroblastoma tumor model has been partially characterized, especially with regard to its inhibition by specific methyltransferase inhibitors.

MATERIALS AND METHODS

Chemicals. S-Adenosyl-L-[methyl-3H]methionine (15 Ci/mmoll was purchased from Amersham Corp., Arlington Heights, Ill. Unlabeled AdoMet, AdoHcy, and gelatin (swine skin, type 1) were obtained from Sigma Chemical Co., St. Louis, Mo. The antifungal antibiotic sinefungin and its analog A-9145C (Lilly) were kindly provided by Dr. Ray Fuller, Lilly Research Laboratories, Indianapolis, Ind. Sinefungin differs from AdoHcy only in having the sulfur replaced by an amino-substituted methylene linkage; however, sinefungin is neither an inhibitor of nor a substrate for AdoHcy hydrolase (4). A-9145C is structurally similar to sinefungin except that it has a double bond at the 4',5'-position (see Chart 4). All other reagents were obtained from Sigma Chemical Co.

Animals. Male A/J mice (20 to 25 g body weight) were obtained from The Jackson Laboratory, Bar Harbor, Maine. Murine C-1300 neuroblastoma (strain T.B.J.-734), was kindly supplied by the E. G. and G. Mason Research Institute, Worcester, Mass. The tumor was maintained via serial transplants in male A/J mice following a.c. implantation of either solid tumor (- x 1-mm segments) or 10^6 viable, dispersed tumor cells. All mice were killed 20 to 21 days following implantation, and the tumor tissue was excised. Additional information on the maintenance and characterization of this tumor line is contained in a paper published previously (22).

Tumor Homogenates. The tumor was dissected from surrounding connective tissue, trimmed of necrotic tumor, rinsed in 0.9% sodium chloride solution, and weighed. A 5% (v/w) suspension in either 25 mm sodium acetate (pH 6.0) or 0.3 M sucrose (pH 7.4) was prepared using a Kontes glass/glass homogenizer. Homogenization was performed at 300 rpm for 2 min at 4°C. With the use of these conditions for

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cellular breakage, less than 5% of the tumor cells remain intact and refractile, as assessed by phase-contrast microscopy. Furthermore, less than 0.5% of the cells retain the capacity to exclude trypan blue following homogenization.

**Differential Centrifugation.** The majority of experiments were performed on soluble and particulate tumor fractions obtained after centrifugation of the 5% homogenate in sodium acetate, pH 6.0, at 20,000 x g for 20 min. In other experiments, subcellular fractions of the neuroblastoma were prepared by differential centrifugation (7). A 5% homogenate in 0.3 M sucrose (pH 7.4) was used to prepare a low-speed particulate fraction (Table 1, Fraction 2) by centrifugation at 800 x g for 10 min. The supernatant obtained after the 800 x g centrifugation was then centrifuged at 20,000 x g for 20 min to obtain a crude mitochondrial fraction (Table 1, Fraction 3). This supernatant was recentrifuged at 100,000 x g for 60 min to obtain a postmicrosomal supernatant and microsomal pellet (Table 1, Fractions 4 and 5). Particulate preparations were gently resuspended in 0.3 M sucrose, pH 7.4, to achieve a protein concentration of approximately 1 mg/ml. Proteins were measured by the method of Lowry et al. (16).

**PCM.** PCM activity was assayed with methods previously described (5, 7, 19). Protein methyl esters formed enzymically through the transfer of the methyl group from \( ^3H \)AdoMet to carboxyl groups of proteins were precipitated with TCA. Following hydrolysis of the precipitated protein methyl ester in alkaline buffer, the released \( ^3H \)methanol was extracted into an organic solvent and quantitated. The incubation mixture contained 7.5 \( \mu \)mol of sodium acetate buffer (pH 6.5), 20 \( \mu \)l of water or gelatin at a final saturating concentration of 10 mg/ml, 0.40 nmol of \( ^3H \)AdoMet (2.7 Ci/mmol), adequate 0.1 N NaOH to neutralize the \( H_2SO_4 \) in the \( ^3H \)AdoMet solution, and variable amounts of tumor fraction (10 to 150 \( \mu \)g protein) in a final incubation volume of 100 \( \mu \)l. Incubations were carried out at 37\(^\circ\)C for 20 min, and the reaction was terminated with 1 ml of ice-cold 10% TCA followed by the addition of 100 \( \mu \)l of 1% bovine serum albumin as a coprecipitant. The acidified incubation mixture was centrifuged for 10 min at 20,000 x g and then hydrolyzed by the addition of 300 \( \mu \)l of 1 M borate buffer, pH 11, containing 2.6% (v/v) unlabeled methanol as a carrier. The hydrolysates were extracted by the addition of 3 ml of toluene:isoamyl alcohol (3:2) and agitated. After centrifugation for 10 min at 200 x g, 1-ml aliquots of the organic phase were transferred into 2 scintillation vials, A and B. The radioactivity was determined directly after the addition of 10 ml of Biofluor (New England Nuclear, Boston, Mass.) to Vial A. The organic extract in Vial B was evaporated to dryness, and the residual tritium was measured after the addition of Biofluor. The difference in radioactivity between Vials A and B represents the volatile methanol formed by the hydrolysis of the protein methyl esters (28). In all experiments, blank incubation tubes containing neither protein substrate nor enzyme were carried through the entire assay procedure. These values averaged 7.6 ± 0.71 pmol \( ^3H \)methanol/min (mean ± S.E.; \( N = 7 \) experiments) and were always subtracted from activity obtained in the presence of substrate and enzyme.

<table>
<thead>
<tr>
<th>Homogenate fraction</th>
<th>pmol ( ^3H )methanol/ mg protein/20 min</th>
<th>% of total activity</th>
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<tr>
<td></td>
<td>- Gelatin + Gelatin</td>
<td>- Gelatin + Gelatin</td>
</tr>
<tr>
<td>1. Crude homogenate</td>
<td>1.8 49.6</td>
<td>100 100</td>
</tr>
<tr>
<td>2. 800 x g particulate</td>
<td>1.7 22.0</td>
<td>30 30</td>
</tr>
<tr>
<td>3. 20,000 x g particulate</td>
<td>0.8 16.2</td>
<td>2 2</td>
</tr>
<tr>
<td>4. 100,000 x g particulate</td>
<td>0.8 12.0</td>
<td>2 2</td>
</tr>
<tr>
<td>5. 100,000 x g supernatant</td>
<td>1.4 81.0</td>
<td>32.5 32.5</td>
</tr>
<tr>
<td>Total</td>
<td>92.5% 113.6%</td>
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**RESULTS**

The time-activity relationship of PCM in the 20,000 x g tumor supernatant is shown in Chart 1. The enzymic reaction was linear for at least 30 min in the presence and absence of exogenous substrate. PCM activity obtained under either incubation condition was significantly different from control (blank) assays and from each other. The linearity of these data suggests that the influence of inhibitors and activators as well as the hydrolysis of protein methyl esters was minimal in this enzyme preparation.

The specific activities of PCM in the 20,000 x g soluble and particulate preparations are shown in Chart 2. PCM activity (±gel) in the 20,000 x g soluble fraction was significantly greater than the enzyme activity determined in the particulate fractionCrude.

![Chart 1](chart1.png)

**Chart 1.** Time course of PCM in C-1300 murine neuroblastoma. PCM activity was measured at various times in the 20,000 x g supernatant, as described in "Materials and Methods," in the presence (0) and absence (C) of a saturating concentration of gelatin (10 mg/ml). Points, means of values obtained in 2 separate experiments. ***, points significantly greater than control (−enzyme) or (−gel) gelatin activity (p < 0.01) by Student’s paired t analysis. * Significantly greater than control activity with (p < 0.01) by a similar analysis.

![Chart 2](chart2.png)

**Chart 2.** Effect of protein concentration on PCM activity in soluble and particulate preparations of C-1300 murine neuroblastoma. PCM activities in the 20,000 x g supernatant and pellet fractions were determined, as described in "Materials and Methods," in the presence (+) and absence (−) of gelatin. Points, means of values obtained in 2 separate experiments. ***, velocity significantly greater than control (−enzyme), pellet (+gel), supernatant (−gel), or pellet (−gel) with a p < 0.001, by Student’s unpaired t analysis.
fraction (+gel or -gel) or soluble fraction in the absence of added substrate (-gel). Although data from the 2 experiments shown in Chart 2 suggest that PCM activity in the particulate fraction (+gel) was greater than PCM activity in the supernatant (-gel) or particulate fraction (-gel), the differences were not statistically significant. In a large number of experiments (N = 5 to 13), the specific activities of PCM in the above 20,000 x g fractions were (pmol [3H]methanol per mg protein per min): supernatant (-gel), 0.25 ± 0.04 (S.D.); supernatant (+gel), 2.44 ± 0.36; particulate (-gel), 0.22 ± 0.08; and particulate (+gel), 0.29 ± 0.02. These distribution patterns for neuroblastoma PCM activity in the presence and absence of exogenous substrate were similar to those reported previously for other neural tissues (6, 7).

Further fractionation of the tumor tissue into subcellular fractions (Table 1) revealed that 80% of the PCM activity in the presence of exogenous substrate was located in the 100,000 x g supernatant (Fraction 5). The remainder of the activity was distributed throughout the other particulate fractions. Approximately 50% of the total PCM activity in the absence of exogenous substrate was present in the nuclear and cellular debris particulate fraction (800 x g). It is possible, however, that this value may be increased slightly due to slight contamination of the 800 x g particulate fraction with unbroken cells, as described in “Materials and Methods.” The activity measured under these conditions reflects the concentration of enzyme and protein substrate present in the tissue compartment.

PCM extracted from murine C-1300 neuroblastoma manifests classical Michaelis-Menten kinetics when incubated with increasing concentrations of the methyl donor, AdoMet (Chart 3A). It has an apparent Km of 3.2 x 10^-6 M, and Vmax of 5.3 pmol/min/mg protein that are comparable to other studies using PCM isolated from nonneoplastic tissues (6).

AdoHcy, a product of all AdoMet-dependent methyltransferase reactions and an effective competitive inhibitor of these enzymes was used to further define the kinetic behavior of murine neuroblastoma PCM (Chart 3A and B). An K_i of 0.64 x 10^-6 M was calculated from these studies. Recently, Fuller and Nagarajan (10), Borchardt et al. (2), and Robert-Gero et al. (25) reported that the AdoHcy analogs (see Chart 4), sinefungin and A-9145C, were also effective competitive inhibitors of PCM and to a lesser extent small-molecule methyltransferases. Sinefungin inhibited soluble neuroblastoma PCM with a calculated K_i of 0.47 x 10^-6 M, whereas a K_i of 4.6 x 10^-6 M was obtained when A-9145C was used as the antagonist (Table 2).

**DISCUSSION**

PCM is present in murine C-1300 neuroblastoma with a distribution pattern similar to that reported previously for other neuronal tissues [e.g. rat pituitary (6), rat hypothalamus (14), and bovine adrenal medulla (7)]. The majority of PCM activity present in the murine neuroblastoma appears to be localized to the cytosol fraction, whereas activity reflecting its endogenous methyl acceptor proteins is located primarily in the particulate fraction. This distribution pattern is consistent with the hypothesis that a major biological function of PCM is neutralization of negative charges of anionic proteins on membrane surfaces, facilitating thereby membrane-membrane interactions critical for stimulus-response coupling (7).

PCM in the murine neuroblastoma is very sensitive to competitive inhibition by AdoHcy and its analogs, sinefungin and A-9145C (1). The latter compound, in particular, has a high affinity for the AdoMet-active site on this enzyme with a calculated K_i of 4.6 x 10^-6 M. While it is possible that AdoHcy may undergo partial hydrolysis by hydrolases present in this enzyme preparation, thereby reducing the effective concentration of inhibitor present, this seems unlikely since the K_i for AdoHcy was quite similar to values reported previously for PCM from other sources (2, 3). Experiments are currently under way in our laboratory to examine whether AdoHcy or its analogs alter neuroblastoma growth in vivo or in vitro. As such, they may represent novel pharmacological probes to investigate neoplastic cell function and potentially useful antineoplastic agents.

**Table 2**

<table>
<thead>
<tr>
<th>Compound</th>
<th>K_i (μM)</th>
<th>± S.D. of 3 different concentrations of each inhibitor.</th>
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<tr>
<td>AdoHcy</td>
<td>0.64 ± 0.014</td>
<td></td>
</tr>
<tr>
<td>Sinefungin</td>
<td>0.47 ± 0.082</td>
<td></td>
</tr>
<tr>
<td>A9145-C (Lilly)</td>
<td>0.046 ± 0.002</td>
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**Chart 3.** Inhibition of C-1300 murine neuroblastoma PCM by AdoHcy. A. PCM activity in the soluble 20,000 x g fraction was determined at various concentrations of AdoMet in the presence and absence of AdoHcy. The apparent K_m and Vmax were determined by the method of Hofstee (12). B. Replot of the data shown in A according to the method of Dixon (8). The calculated K_i represents the point on the abscissa where the 3 lines intersect.

**Chart 4.** Chemical structures of AdoHcy analogs.

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The functional significance of PCM in the neuroblastoma cell remains unclear; however, its postulated relationship to neoplastic growth and differentiation constitutes an attractive possibility. The recent observation of Zuckerman et al. (29) that monocyte-to-macrophage differentiation in long-term culture was accompanied by a dramatic increase in endogenous PCM activity suggests that the availability of MAPs or their interaction with cytoplasmic PCM may be related to cell growth.

In addition to its possible role as a growth regulator, PCM may modulate other aspects of neural behavior. This hypothesis is supported by the observation that neural and endocrine tissues have the highest levels of PCM and MAPs (6). Furthermore, the stimulated release of secretory products from the adrenal and parotid glands have been shown to induce a concomitant increase in protein carboxymethylation (7, 27). Although the exact mechanisms underlying catecholamine production, storage, and release from neuroblastoma cells remain obscure, studies in our laboratory (22) have demonstrated significant quantities of biogenic amines in the in vivo uncloned tumor. Animals bearing these tumors also excrete large quantities of catecholamines into their urine. The role of protein carboxymethylation in the regulation of neuroblastoma growth and its neuronal behavior remains to be defined. Studies currently under way in our laboratory are exploring this relationship with both in vitro and in vivo neuroblastoma lines.

REFERENCES


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