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PROTEIN CARBOXYL-*O*-METHYLTRANSFERASE ACTIVITY IN CULTURED C-1300 NEUROBLASTOMA CELLS*

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Abstract—Protein carboxylmethyltransferase (PCM) has been identified in a variety of tissues derived from neural crest anlage, including *in vivo* C-1300 murine neuroblastoma (MNB). These observations have stimulated interest in further defining the role of PCM as a potential modulator of neoplastic cell behavior. The subcellular distribution and kinetic behavior of PCM have been characterized in a tissue culture line derived from the C-1300 murine neuroblastoma (clone NB41A3). The specific and total activities of PCM in the presence and absence of exogenous substrate were determined in subcellular fractions of MNB cells prepared by differential centrifugation. In the presence of exogenous substrate (+ gelatin), 40% of the total PCM activity was present in the 100,000 *g* supernatant fraction and 41% in the 800 *g* particulate fraction, whereas the higher specific activity of PCM was present in the 100,000 *g* supernatant fraction. Enzyme activity measured in the absence of gelatin, which reflects the concentration of endogenous methyl acceptor proteins in a cell fraction, was negligible. This activity represented less than 1.6 and 0.4% of the total PCM activity present in the 800 *g* particulate and 100,000 *g* soluble fractions respectively. Cytosolic PCM had an apparent K_m of 13.9×10^{-6} M for AdoMet and a V_{max} of 33 pmoles per min per mg protein. Cytoplasmic PCM was inhibited competitively by *S*-adenosylhomocysteine ($K_i = 3.0\ \mu M$) and its analogues, sinefungin ($K_i = 1.7\ \mu M$) and A-9145C ($K_i = 0.2\ \mu M$). These data demonstrate that the specific activity of PCM was greatest in the soluble component of subcellular fractions prepared from cultured MNB cells. This distribution pattern of PCM is similar to that observed in the C-1300 MNB tumor grown *in situ* and in non-malignant neural tissues. In contrast to the latter tissues, cultured MNB cells exhibited low PCM activity when assayed in the absence of exogenous substrate.

Protein carboxylmethyltransferase (EC 2.1.1.24; PCM§) reacts with a variety of proteins and polypeptides by transferring a methyl group from *S*-adenosyl-L-methionine (AdoMet) to form protein carboxyl methylesters [1–3]. This reaction has been implicated in the enzymatic mechanisms that regulate chemotaxis [4–8], exocytotic secretion [9–11], cellular growth and differentiation [12]. PCM is widely distributed in tissues of neural crest origin, including the adrenal medulla [9], brain [1, 10, 13], and pituitary gland [11, 14].

Previous investigations in our laboratory have demonstrated the presence of PCM in C-1300 murine neuroblastoma (MNB) tumors grown in the A/J mouse [15, 16]. PCM obtained from this neural crest tumor exhibits subcellular distribution, kinetic behavior, and responses to enzyme inhibitors similar to those observed in other neural tissues [1, 9, 14]. The present study was undertaken to determine whether the enzymatic characteristics of PCM extracted from subcellular fractions of MNB cells grown in tissue culture resemble those described for MNB tumor grown *in situ*.

METHODS

Chemicals. *S*-Adenosyl-L-[^{3}H-methyl]methionine (15 Ci/mmole) was purchased from the Amersham Corp. Arlington Heights, IL. Unlabeled AdoMet, AdoHcy, and gelatin (swine skin, Type 1) were obtained from the Sigma Chemical Co., St. Louis, MO. The antifungal antibiotics sinefungin and its analogue, A-9145C (Lilly) (Fig. 1), were provided by Dr. Ray Fuller, Lilly Research Laboratories, Indianapolis, IN. All other reagents were obtained from commercial sources.

Tissue source. C-1300 murine neuroblastoma (clone NB41A3; Atcc No. CCL-147) was obtained from the American Type Culture Collection Laboratories, Rockville, MD. The cells were stored at −180° suspended in Dulbecco's modified Eagles' medium containing 5% dimethylsulfoxide and 15%

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§ Abbreviations: PCM, *S*-adenosyl-L-methionine: protein carboxylmethyltransferase, EC 2.1.1.24; AdoMet, *S*-adenosyl-L-methionine; and AdoHcy, *S*-adenosylhomocysteine.

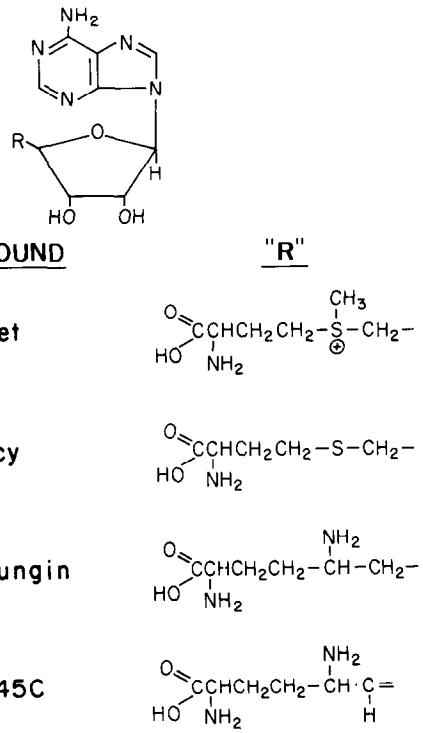

Fig. 1. Chemical structures of AdoMet, AdoHcy, sinefungin and A-9145C.

fetal calf serum. The cells were originally cloned from a wild strain of C-1300 neuroblastoma that had appeared spontaneously in the male A/J mouse, as described by Dunham and Stewart [7] and Augusti-Tocco and Sato [18].

Culture methods. The cells were grown in Dulbecco's modified Eagle's medium containing 25 mM glucose, 4 mM L-glutamine, and no pyruvate. This medium was supplemented further with 15% horse serum (Grand Island Biologicals Co. (GIBCO), Grand Island, NY), 2.5% fetal calf serum (Flow Laboratories, McLean, VA), 10,000 units/ml penicillin and 100 μg/ml streptomycin in an atmosphere of 7.5 CO_2/92.5% air at 37°. The cells were grown as monolayers in 75 cm^2 Falcon (Cockeysville, MD) tissue culture flasks containing 20 ml media flask. Cultures were initiated by the addition of 2.5×10^6 cells to each flask. The medium was changed every other day. After attaining confluency (7 days), adherent cells were removed by the addition of 5 ml of 0.05% trypsin–0.6 mM EDTA (GIBCO) to each flask followed by incubation for 10–15 min at 37°. Each flask yielded approximately 0.5×10^8 cells with an average viability of 90% as assessed by trypan-blue dye exclusion.

Preparation of cellular homogenates. After harvesting, the cells were centrifuged at 250 *g* for 5 min and the supernatant fraction was decanted. The cell pellet was resuspended in 5 ml of isotonic phosphate buffered saline (pH 7.4). This washing procedure was repeated twice. The final cell pellet was resuspended in 5 ml of a homogenization medium containing 75 mM sodium acetate (pH 6), 10 mM $MgCl_2$ and 0.1 mM EDTA. Homogenization was performed at 3000 rpm for 2 min at 4° in a Teflon–glass homogenizer (Kontes, Vineland, NJ). The homogenate was then sonicated for three sequential 10-sec intervals at 4° in a Branson cell disrupter at a setting of 60% intensity. Following the latter disruption, no intact cells were detected by phase contrast microscopy.

Differential centrifugation. The 100,000 *g* soluble and resuspended particulate fractions of the MNB homogenate, prepared as described above, were used in most experiments. The subcellular distribution pattern of PCM in these homogenates was determined by using differential centrifugation procedures [9]. A sonicated cellular homogenate prepared as described above, was centrifuged at 800 *g* for 10 min to prepare a crude nuclear and cell debris fraction. The resulting supernatant fraction was recentrifuged at 20,000 *g* for 20 min to obtain a crude mitochondrial fraction. The mitochondrial-free supernatant fraction was centrifuged at 100,000 *g* for 60 min to obtain a post-microsomal supernatant fraction and microsomal pellet. All particulate fractions were resuspended in homogenization buffer to a final protein concentration of approximately 1 mg/ml. Protein determinations were performed by the method of Lowry *et al.* [19].

PCM assay. PCM activity was assayed by a modification of methods previously described [9, 14, 20]. Each incubation mixture contained 75 mM sodium acetate buffer (pH 6.0), 10 mM $MgCl_2$, 0.1 mM EDTA, and 20 μl of water or gelatin (the exogenous protein substrate) at a final saturating concentration of 10 mg/ml. [^{3}H]AdoMet (4×10^{-6} M; 0.5 μCi) and variable amounts of subcellular fraction (10–100 μg protein) were then added to achieve a final incubation volume of 100 μl. The mixture was incubated at 37° for 30 min, and the reaction was terminated by the addition of 1 ml of 10% trichloracetic acid followed by the addition of 100 μl of 1% bovine serum albumin as a protein coprecipitant. After centrifugation for 15 min at 20,000 *g*, the precipitated protein methylesters were hydrolyzed by the addition of 300 μl of 1 M borate buffer (pH 11.0) containing 2.6% methanol (v/v). The tritiated methanol was then extracted into 3 ml of toluene–isoamyl alcohol (3:2), added directly to the hydrolyzate. After centrifugation for 10 min at 1500 *g*, paired 1-ml portions of the organic phase were transferred to scintillation vials. The radioactivity in the first vial was determined by liquid scintillation spectrometry immediately after the addition of 10 ml of Biofluor (New England Nuclear Corp., Boston, MA). The residual radioactivity in the other vial was determined after evaporation of the organic solvent (80° for 2 hr). The difference in radioactivity before and after evaporation stoichiometrically relates to the quantity of protein methylesters formed [8]. In all experiments incubation blanks prepared without protein substrate (gelatin or membranes) or enzyme were carried through the entire assay procedure. The activity of the blank averaged 0.23 ± 0.02 pmole [^{3}H]methanol/30 min (mean ± S.E.M., N = 7 experiments) and was always subtracted from the activity obtained in the presence of substrate and enzyme. All data points displayed in either the table

Table 1. Subcellular distribution of protein carboxylmethyltransferase in cultured C-1300 murine neuroblastoma*

Homogenate fraction	Specific PCM activity		Total PCM activity			
	− Gel (units/mg protein)	+ Gel (units/mg protein)	− Gel (units/10^8 cells)	% Total	+ Gel (units/10^8 cells)	% Total
Crude homogenate	1.4 ± 0.7	117 ± 15	35 ± 19	100	2666 ± 695	100
800 *g* Particulate	1.3 ± 0.4	76 ± 16	18 ± 6	51	1093 ± 268	41
20,000 *g* Particulate	1.4 ± 0.4	35 ± 11	1.4 ± 0.9	3.9	19 ± 2.3	0.7
100,000 *g* Particulate	0.9 ± 0.4	52 ± 12	1.5 ± 1.1	4.2	17 ± 1.7	0.6
100,000 *g* Supernatant	0.9 ± 0.5	198 ± 31	4.7 ± 2.5	13.6	1068 ± 456	40

* Subcellular fractions were prepared from cellular homogenates (6×10^8 cells/experiment), and PCM activity was assayed as described in Methods. The values shown represent the mean ± S.E.M. for triplicate determinations in three separate experiments. PCM activity was determined in the presence and absence of a saturating concentration of gelatin (10 mg/ml). One unit of PCM activity represents 1 pmole of [^{3}H]methanol formed/30 min at 37°.

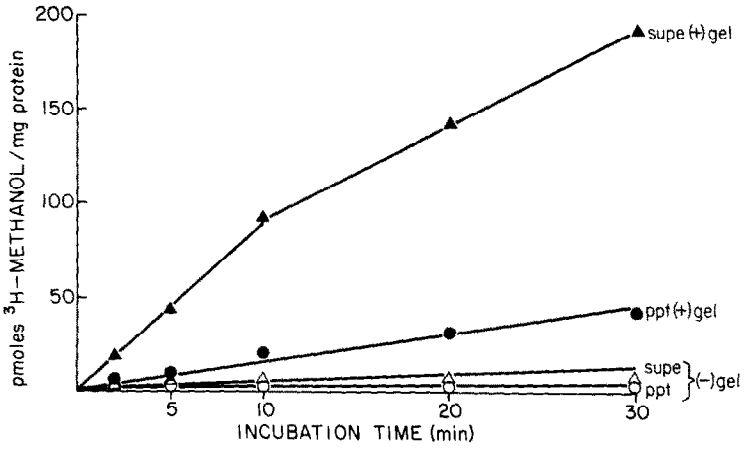

Fig. 2. Time course of PCM in cultured murine neuroblastoma cells. PCM activity was measured after 0, 2.5, 5, 10, 20 and 30 min of incubation using the 100,000 *g* supernatant (supe) and particulate (ppt) fractions. (+) Gel represents a saturating concentration of gelatin (10 mg/ml); (−) gel refers to the absence of gelatin. Each point represents the average of triplicate determinations.

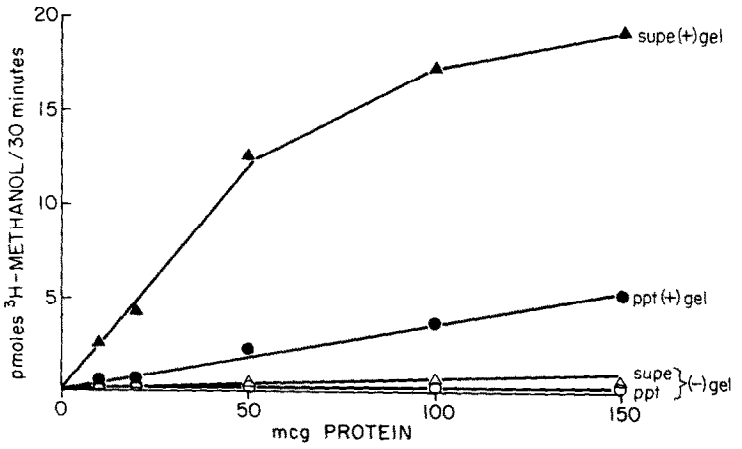

Fig. 3. Effect of protein concentration on PCM activity in cultured murine neuroblastoma cells. PCM activity in the 100,000 *g* supernatant (supe) and particulate (ppt) fractions was determined in the presence (+ gel) and absence (− gel) of gelatin. Each experimental point represents the average of triplicate determinations.

or figures represent the average of triplicate determinations with an inter-sample variation of less than 5%.

RESULTS

Distribution of PCM in subcellular fractions of cultured MNB. Initial experiments were performed to determine the activity of PCM in various subcellular fractions prepared from MNB cells. In the presence of gelatin, 40% of the total PCM activity was localized in the 100,000 *g* supernatant fraction, whereas 41% was detected in the 800 *g* particulate fraction (Table 1). When enzyme activity was determined in the absence of exogenous substrate, 51% was present in the 800 *g* particulate fraction. Approximately 73–83% of the total PCM activity present in the cellular homogenate was recovered after subcellular fractionation.

The time–activity relationship for PCM was determined in the supernatant and particulate fractions obtained after centrifugation at 100,000 *g* (Fig. 2). In the presence of gelatin, PCM activity in both fractions increased linearly for nearly 30 min, whereas in the absence of gelatin very little methylester formation was detected in either fraction. The ratio of PCM activity in the presence versus the absence of gelatin $\frac{\text{PCM}(+)\text{ gel}}{\text{PCM}(-)\text{ gel}}$ was 220 in the supernatant fraction and 58 in the particulate fraction.

The specific activity of PCM in the 100,000 *g* supernatant fraction was 5-fold greater than that observed in the 100,000 *g* particulate fraction when assayed in the presence of gelatin (Fig. 3). A sharp distinction in this pattern was observed when the specific activities of these fractions were estimated in the absence of gelatin. Under these conditions, no enzymatic activity was detected in either fraction (Fig. 3).

Kinetics of PCM in the presence and absence of enzyme inhibitors. The kinetic characteristics of PCM were analyzed in 100,000 *g* supernatant fractions to which were added various concentrations of [^{3}H]AdoMet. PCM exhibited Michaelis–Menten kinetics with an apparent K_m of 13.9×10^{-6} M and a V_{max} of 33 pmoles [^{3}H]methanol per mg protein per min.

The responses of PCM to inhibition by the enzyme inhibitors AdoHcy and its analogues, sinefungin and A-9145C (structures shown in Fig. 1), were determined using the 100,000 *g* supernatant fraction. All compounds were potent inhibitors of PCM activity as illustrated in the Dixon plots [21] of these data (Fig. 4). The inhibition of PCM by AdoHcy and sinefungin appeared to be strictly competitive, whereas A-9145C inhibited PCM in a mixed competitive–noncompetitive manner (Fig. 4C). The inhibitory constant (mean $K_i \pm$ S.D.) for each compound was as follows (increasing order of potency): AdoHcy ($3.01 \pm 0.44 \times 10^{-6}$ M); sinefungin ($1.67 \pm 0.22 \times 10^{-6}$ M); and A-9145C ($2.14 \pm 0.72 \times 10^{-7}$ M).

DISCUSSION

The distribution pattern, enzyme kinetics and effects of inhibitors on PCM activity have been elucidated in a C-1300 MNB tissue culture line. The characteristics of PCM in this *in vitro* neural crest tumor model differed from those observed in previous studies of the C-1300 MNB grown *in situ* [15] and from non-malignant tissues such as the adrenal and pituitary glands [9, 14]. Total PCM activity determined in the presence of gelatin was distributed equally between the 100,000 *g* supernatant and 800 *g* particulate fractions prepared from these cells (Table 1). In the absence of gelatin, however, very low enzyme activity was detected in all of the subcellular fractions (Table 1; Fig. 3). These observations sug-

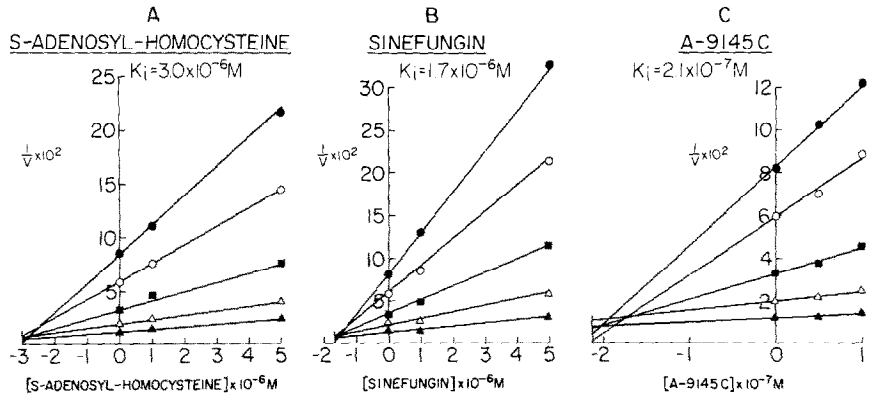

Fig. 4. Effect of methyltransferase inhibitors on PCM activity in cultured murine neuroblastoma cells. Each data point represents the average of triplicate determinations in a single experiment, displayed according to the method of Dixon [21]. Using these methods, a drawn line perpendicular to the point of intersection defines the apparent K_i on the abscissa. Individual lines represent the best fit as determined by linear regression analyses. The K_i values shown represent the mean of multiple (N = 6–9) line intersections, reflected upon the abscissa. The following symbols refer to different micromolar concentrations of AdoMet: (●——●) 0.67; (○——○) 1.0; (■——■) 2.0; (△——△) 4.0; and (▲——▲) 8.0. Key: (A) AdoHcy; (B) sinefungin; (C) A-9145C.

gest that the concentration of methyl-acceptor protein(s) in this cultured MNB cell line may be lower than that present in other tissues of neural crest origin [9, 11, 14] or MNB tumors grown in A/J mice [15]. This deficiency of methyl-acceptor proteins is analogous to that reported previously in erythrocyte cytosol [20]. Preliminary experiments in our laboratory have shown that AdoMet-dependent formation of alkaline-labile methyl esters is also extremely low in intact cultured MNB cells following exposure to [^{3}H]methionine*.

The enzyme kinetics of PCM derived from cultured MNB cells and the sensitivity of this enzyme to methyltransferase inhibitors also appear to differ from that previously described in other tissues [22]. The K_m for AdoMet under the conditions of this study was 14×10^{-6} M; this value exceeded that reported in other neural tissues [14, 15]. The competitive inhibitors AdoHcy, sinefungin and A-9145C were all effective antagonists of PCM activity over a wide concentration range (Fig. 4), a finding similar to observations in other systems [22, 23]. All compounds, however, were less effective inhibitors of PCM activity in the cultured MNB cells than in MNB tumors grown in A/J mice [15]. In the former, the K_i values of AdoHcy, sinefungin and A-9145C were 3.0, 1.7 and 0.21 μm, respectively, whereas in the latter they were 0.64, 0.47 and 0.046 μM, respectively.

The biologic role of the PCM system in the cultured murine line remains undefined although a regulatory role for PCM has been implicated in neurosecretion [9–11] and cellular differentiation [12]. Our goal in this investigation was to establish an *in vitro* model for the study of PCM using a cultured neural tumor cell system. The finding that PCM activity in the cultured cells was barely detectable in the absence of exogenous substrate contrasted with the presence of measurable activity when similar experiments were performed with the *in situ* MNB tumor model [15]. These observations suggest that the expression of this characteristic may be clonal specific or host-modulated.

* M. O'Leary, B. L. Mirkin and R. F. O'Dea, unpublished observations.

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