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Characterization of the binding of *S*-adenosyl-L-methionine to plasma membranes of HL-60 promyelocytic leukemia cells

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S-Adenosyl-L-methionine (AdoMet) has been found to bind specifically to the plasma membrane of promyelocytic leukemia cells, HL-60. The K_d for AdoMet is $4.2 \cdot 10^{-6}$ M and the B_{max} is $4.0 \cdot 10^{-12}$ mol / 10^7 HL-60 cells. The binding is not related to the adenosine receptor since neither adenosine, ADP, nor ATP affect the ligand-receptor reaction. When HL-60 cells were incubated with physiological concentrations of [*methyl*-^{3}H]AdoMet (20 μM) at 36 °C, AdoMet did not equilibrate with the intracellular pool, nor were any [^{3}H]methyl groups incorporated into nucleic acids or proteins. In contrast, significant amounts of [^{3}H]methyl groups were incorporated into membrane phospholipids. When cells were incubated with 20 μM [*methyl*-^{3}H]AdoMet, [^{3}H]methyl groups were transferred to phosphatidylethanolamine, -monomethylethanolamine, and -dimethylethanolamine yielding phosphatidylcholine. However, the rate of methyl transfer with AdoMet was only 22% of that observed when cells were incubated with a comparable amount of [*methyl*-^{3}H]methionine. Both the binding of AdoMet and the methylation of phospholipids were inhibited by exogenous *S*-adenosyl-L-homocysteine. Therefore, the binding may be linked to a phospholipid methyltransferase.

Introduction

Over the past few years, *S*-adenosyl-L-methionine (AdoMet) has been utilized in the treatment of certain neurological disorders as an antidepressant [1–3] as well as in the treatment of osteoarthritis [4–6]. This compound functions as the universal methyl donor in the cell and also serves as a substrate in the biosynthesis of polyamines. AdoMet is synthesized from methionine and adenosine triphosphate (ATP) via the methionine adenosyltransferase (EC 2.5.1.6). Orally administrated AdoMet apparently crosses the intestinal wall, increasing the plasma concentration of AdoMet [1,3,5]. AdoMet has been reported to display anti-inflammatory and analgesic activities. The pharmacological effects can not be mimicked by methionine, a relatively toxic precursor of AdoMet. Little AdoMet is found in the plasma, and cells are generally found to be impermeable to this compound [1,7–10]. Hence, there is considerable uncertainty as to how this compound functions pharmacologically.

AdoMet may exert its effect by altering the transport of specific compounds across the cell membrane. Pacheco et al. [9] has found that exogenous AdoMet enhances histamine release. AdoMet also enhances proteoglycan synthesis and secretion by human chondrocytes in culture [11].

In view of the increasing use of AdoMet clinically, and its high polarity which impedes penetration through cellular membranes, we investigated the binding of AdoMet and its effects on methylation reactions in promyelocytic leukemia cells (HL-60). In this investigation, we report that exogenous AdoMet will bind specifically to receptors in plasma membranes of HL-60 cells, resulting in methylation of membrane phospholipids.

Materials and Methods

Pulse labeling of cultured cells. HL-60 cells were cultured for 5 days in RPMI-1640 medium containing 5% fetal calf serum. The cells were harvested by centrifuga-

Abbreviations: AdoMet, *S*-adenosyl-L-methionine; AdoHcy, *S*-adenosyl-L-homocysteine; ADP, adenosine diphosphate; ATP, adenosine triphosphate; Met, L-methionine; PME; phosphatidylmonomethylethanolamine, PDE, phosphatidyldimeethylethanolamine; Pc, phosphatidylcholine.

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tion and washed twice with phosphate-buffered saline. The cells were suspended in RPMI-1640 medium lacking methionine (20 ml aliquots) at a concentration of $2 \cdot 10^7$ cells/ml. After the cells were incubated at 35°C for 10 min, 10 μM L-[*methyl*-^{3}H]methionine (10 Ci/mmol) was added. At varying times, the reaction was terminated with chilling to 0°C. After centrifugation at 3000 × *g* for 4 min, the medium was removed and discarded. The cells were washed two times with cold phosphate buffered saline and suspended in 0.25 M sucrose at a concentration of $4 \cdot 10^7$ cells/ml.

Preparation of cell membranes. The cell suspension was equilibrated with nitrogen in a Parr cell disruption bomb at 180 lb/in^2 for 10 min and released dropwise. The resultant cell lysate was centrifuged at 1000 × *g* for 5 min. The pellet (crude nuclei) was suspended in 0.25 M sucrose and subjected to centrifugation at 1000 × *g* for 5 min. The supernatant fluids were combined and $MgCl_2$ added to a concentration of 2.5 mM. The supernate was gently layered on a discontinuous Percoll gradient [12]. Stock Percoll (100%) was diluted to 5, 25 and 35% Percoll solution in 0.25 M sucrose: 2.5 mM $MgCl_2$: 50 mM Tricene (pH 8.8) and 10 ml each of these solutions were layered into a 50 ml clear plastic centrifuge tube using a graduated syringe. The gradients were centrifuged at 27000 × *g* for 30 min. The membranes at the top of the 35% Percoll were collected by suction using a syringe. This fraction was diluted with phopshate buffered saline: 1.0 mM $MgCl_2$ and centrifuged at 100000 × *g* for 30 min. The pellet was suspended in phosphate-buffered saline containing 1.0 mM $MgCl_2$ and stored at −70°C. The membranes were essentially devoid of the enzymes, succinate dehydrogenase (mitochondrial), hexosaminidase (lysosomal) and lactate dehydrogenase (cytosolic), but contained measurable amounts of NADPH–cytochrome-*c* reductase, an endoplasmic reticulum marker. The cytochrome-*c* reductase activity in the membrane fraction was 2.4 nmol cytochrome *c* reduced per min per mg protein compared to a value of 14.4 for the microsomal fraction.

Preparation of microsomal fraction. An aliquot of the post-nuclear supernate was subjected to centrifugation at 27000 × *g* for 30 min to sediment crude membranes and mitochondria. Prior to centrifugation, $MgCl_2$ was added to a concentration of 1.0 mM. The supernate was centrifuged at 100000 × *g* for 1 h to sediment the microsomes. The microsomes were washed once with 0.25 M sucrose: 1.0 mM $MgCl_2$ and stored at −70°C.

Preparation of DNA. DNA was purified from chromatin by a modification of the procedure of Kalousek and Morris [13]. The resultant DNA was hydrolyzed in 1.0 M perchloric acid at 70°C for 25 min. DNA was quantitated by the method of Burton [14] and radioactivity was determined.

Extraction of phospholipids. Phospholipids were extracted from the membrane and microsomal fractions by the method of Folch et al. [15]. After the microsomes were homogenized with Folch reagent, the extract was centrifuged and filtered. Biphasic separation was achieved by adding 0.05% $CaCl_2$. To remove residual proteins, the chloroform extract was mixed with 0.75 ml saline: 0.75 ml methanol and centrifuged. This step was repeated and the organic layer was taken to dryness under a stream of nitrogen. The lipids were dissolved in 300 μl of hexane/isopropanol/methanol/water (37 : 53 : 4 : 6, v/v). 25-μl aliquots of the extract were removed for the determination of total [^{3}H]methyl groups incorporated into lipids. All fractions were stored under nitrogen at −80°C pior to analysis.

Fractionation of phospholipids. The method used to separate phosphatidylcholine and phosphatidylethanolamine is a modification of the methods described by Hanson et al. [16], Patton et al. [17] and Briand et al. [18]. The phospholipids were fractionated on an Ultrasil-amine-bonded-column employing a Gilson model 911 high pressure liquid chromatography system, and an inline Flo-One/Beta (RIC) radioactive detector.

Phospholipids were eluted from the column with a gradient composed of buffer A (Hexane/isopropanol (41 : 59, v/v)) and buffer B (methanol/water (40 : 60, v/v). The system is equilibrated with 7% buffer B, 93% buffer A prior to the run at a flow rate of 2 ml/min. The concentration of buffer B is held at 7% for the first 5 min of the run, then increased linearly to 15% over the next 22 min, then held at 15% for the next 5 min. Ultraviolet detection was performed at 205 nm. The ^{3}H and ^{14}C counts were corrected for both efficiency and crossover in both channels. The results are presented as dpm/μg protein.

Determination of carboxyl methylated proteins. The extent of in vivo carboxyl methylation of proteins was determined by the amount of [^{3}H]methanol released upon alkaline hydrolysis [19]. After the proteins were incubated with [*methyl*-^{3}H]AdoMet, they were hydrolyzed in 0.05 M NaOH for 1 h at 37°C. The [^{3}H]methanol released was extracted with a mixture of toluene/isoamyl alcohol 3 : 2, v/v). After vigorous mixing, the organic phase was separated from the aqueous phase by centrifugation. One ml aliquots of the organic phase were placed in two scintillation vials; one of these was evaporated to dryness. The difference in radioactivity between the evaporated and non-evaporated samples was used to quantitate the amount of alkaline labile methyl groups. After removal of the lipids, the background counts were less than 10% of the total counts present in the organic phase.

Assay for binding of AdoMet. The binding of AdoMet to the plasma membrane was determined at pH 7.4 using an oil centrifugation technique [20]. The oil is a mixture of dinoylphthalate/dibutylphthalate (2 : 3, v/v). Intact cells or the membrane fraction were sus-

pended in phosphate-buffered saline at a concentration 0.1–2.0 mg protein/ml. Aliquots of the cell suspension (5.0 ml each) were incubated with [^{3}H]AdoMet to determine total binding. To determine nonspecific binding, the cells were incubated with 40 μM AdoMet for 30 min at 4°C prior to the addition of [^{3}H]AdoMet. The nonspecific binding counts were subtracted from total binding counts to obtain the specific binding values [21].

After incubating these mixtures for 30 min at 4°C, 0.5 ml of the cell suspension was added to tubes containing varying concentrations of [^{3}H]AdoMet (0.001–0.1 μM). After incubating at 0°C for 20 min, 0.25 ml samples from the incubation mixture were layered upon oil and centrifuged at 12 000 × g for 45 s in 1.5 ml Eppendorf tubes using an Eppendorf centrifuge.

The oil and aqueous phases were removed by pipette and the bottom of the tube containing packed cells or membranes was cut off. The pellet was dissolved in 1 ml of Protosol, neutralized with glacial acetic acid, and counted by liquid scintillation spectrometry. The dissociation constants were determined by Scatchard analysis [22,23].

Determination of S-adenosylmethionine concentration by high-performance liquid chromatography (HPLC). HL-60 cells ($2 \cdot 10^7$ cells/ml) were grown on RPMI-1640 medium containing either L-[*methyl*-^{3}H]methionine or *S*-adenosyl-L-[*methyl*-^{3}H]methionine (148 nCi/nmol) for various times. Cells were washed three times with cold buffered saline and the AdoMet extracted with 5% sulfosalicylic acid. AdoMet was fractionated by HPLC on Vydac resin as described previously [24].

Results

Specific binding of [^{3}H]AdoMet to the plasma membrane of HL-60 cells occurs linearly up to approx. $0.8 \cdot 10^7$ cells per assay (about 1 mg of cell protein, Fig. 1A). Typically, about $0.6 \cdot 10^7$ cells were used in further experiments. The specificity of [^{3}H]AdoMet binding was demonstrated by inhibition with AdoMet and *S*-adenosylhomocysteine, but not adenosine, adenosine diphosphate (ADP) or adenosine triphosphate (ATP) (Table I). Binding of [^{3}H]AdoMet to the plasma membrane reached equilibrium within 5 min (Fig. 1B), and reached saturation at 4.5 μM (Fig. 2).

A Scatchard plot of [^{3}H]AdoMet binding to HL-60 cells is shown in Fig. 3. The K_d is 4.2 ($\pm$0.2) $\cdot 10^{-6}$ M and the B_{max} is 4.0 ($\pm$0.3) $\cdot 10^{-12}$ mol/10^7 HL-60 cells. Therefore, there are approx. $2.4 \cdot 10^5$ AdoMet binding sites/cell. The linear Scatchard plot indicates that there is no heterogeneity among the AdoMet binding sites measured in these experiments.

There was no evidence that the receptors function in the transport of AdoMet across the cell membrane.

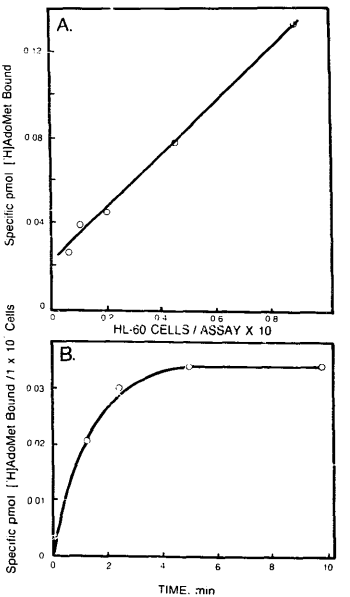

Fig. 1. Specific binding of [^{3}H]AdoMet to HL-60 cells. The standard deviation for the points were less than 6%. (A) Specific binding of [^{3}H]AdoMet with HL-60 cells as a function of cell number. HL-60 cells were incubated in 50 nM [^{3}H]AdoMet (15 Ci/mmol) at 4°C for 20 min. (B) Binding of [^{3}H]AdoMet to HL-60 cells as a function of time.

When HL-60 cells were incubated with 20 μM [^{3}H]AdoMet at 36°C, the specific radioactivity of the endogenous AdoMet was less than 1% of the exogenous [^{3}H]AdoMet (Fig. 4 and Table II). In contrast, when the HL-60 cells were incubated with [^{3}H]Met, the specific radioactivity of the endogenous AdoMet increased to 80% of the exogenous methionine after 2 h (Fig. 4). Further evidence that AdoMet failed to cross the cell membrane under these conditions was obtained by measuring the incorporation of [^{3}H]methyl groups from AdoMet into various cell fractions. No radioactive methyl groups were detected in nucleic acids or proteins when the concentration of AdoMet was maintained at 20 μM or less (Table II and Fig. 5).

TABLE I

Binding of AdoMet to HL-60 cell fractions

Cell fractions were suspended in 1.2 μM [*methyl*-^{3}H]AdoMet (15 Ci/mmol) and incubated at 4°C for 20 min. AdoHcy, adenosine, ADP or ATP were added at the same time. Proteins were precipitated with Cl_3CCOOH (10%) on glass fiber filters and washed several times with 10% Cl_3CCOOH.

Fraction	Additions	Bound AdoMet (dpm/mg)
Cell lysate	none	26800
Cell lysate	50 μM AdoHcy	11350
Cell lysate	40 μM AdoMet	13600
Soluble	none	9920
Membrane	none	41000
Membrane	50 μM AdoMet	18450
Membrane	50 μM AdoHcy	16200
Membrane	100 μM adenosine	40200
Membrane	100 μM ADP	42100
Membrane	100 μM ATP	41300

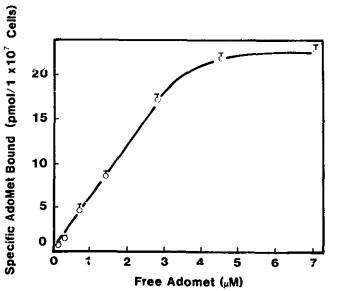

Fig. 2. Specific binding of [^{3}H]AdoMet to HL-60 cells as a function of increasing concentration of free AdoMet. Cells were incubated for 30 min at 4°C; nonspecific binding was determined with 40 μM AdoMet and subtracted from the total counts yielding the specific [^{3}H]AdoMet bound. Each point represents the mean ± S.E. of at least three separate assays.

When the concentration of AdoMet was increased to 180 μM, radiolabeled AdoMet leaked across the cell membrane. After 3 h at 36°C the specific radioactivity of the endogenous AdoMet reached about 19% of that of the exogenous AdoMet (Fig. 4). Under these conditions, all the cell fractions assayed contained radiolabeled methyl groups (Table II). On the other hand, at 20 μM or less [^{3}H]AdoMet, the phospholipids were the only compounds which contained significant amounts of [^{3}H]methyl groups (Fig. 5). Even at 1 μM [^{3}H]AdoMet, there was significant incorporation of [^{3}H]methyl groups into membrane phospholipids (Table II). When the HL-60 cells were incubated with 10 μM [^{3}H]methionine, all cellular fractions of the cells incorporated [^{3}H]methyl groups (Fig. 5).

After incubation of HL-60 cells with 10 μM AdoMet at 36°C analysis of all the cell fractions revealed that 80–90% of the radiolabeled phospholipids were recoverable in the plasma membrane fraction. The value obtained for total phospholipid was about 100 000 dpm/mg for the membrane fraction compared to less than 8000 dpm/mg for the microsomal fraction. Thus, there was essentially no transport of radiolabeled phospholipid from the plasma membrane into the cell.

When HL-60 cells, had been incubated with [^{3}H]AdoMet, methyl groups were transferred to phosphatidylethanolamine, resulting in the formation of radiolabeled phosphatidylcholine (Fig. 6B). However, the rate was only about 22% of that found when cells were incubated with a comparable amount of L-[*methyl*-^{3}H]methionine (Fig. 6A). There also appeared to be a

TABLE II

Utilization of extracellular S-adenosylmethionine by HL-60 cells

HL-60 cells ($1 \cdot 10^8$ per ml) were incubated in RPMI-1640 medium without L-methionine. After 10 min, *S*-adenosyl-L-[*methyl*-^{3}H]methionine ($5.2 \cdot 10^5$ dpm/nmol) was added and the cells were incubated for 1 h at the designated temperature.

S-Adenosyl-L-methionine				Temp. (°C)	Methylation (pmol/1 per 10^7 cells)		
Extracellular	intracellular				phospholipids	protein carboxyl	DNA
	nmol/10^7 cells	dpm/nmol	% added AdoMet				
1 μM	0.35	1900	0.4	4	0.01	0	0
1 μM	0.32	2200	0.4	36	0.60	0	0
20 μM	0.39	4400	<1.0	4	0.03	0.02	0
20 μM	0.31	5000	<1.0	36	1.20	0.07	0.01
180 μM	0.43	30400	5.8	4	0.20	0.14	0.02
180 μM	0.45	62000	11.9	36	9.60	0.70	5.10

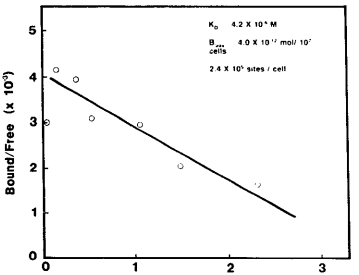

Fig. 3. Representative Scatchard plot of [^{3}H]AdoMet binding to HL-60 cells at 4 °C. Each point represents at least two replicates. The standard deviation for the points was less than 6%.

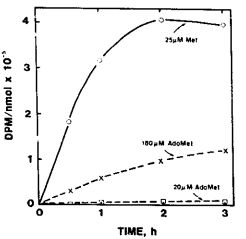

Fig. 4. Specific radioactivity of intracellular AdoMet (ordinate) after culturing HL-60 cells in the presence of L-[*methyl*-^{3}H]methionine (25 μM) or [*methyl*-^{3}H]AdoMet (20 μM or 180 μM). The specific activity for both radioactive compounds was $5.2 \cdot 10^5$ dpm/nmol.

disproportionate amount of phosphatidylmonoethanolamine in membranes from cells incubated with [*methyl*-^{3}H]AdoMet compared to those cultured with [*methyl*-^{3}H]methionine. This suggests that the rate-limiting step for phospholipid methylation in HL-60 cells observed with exogenously supplied AdoMet, which does not enter the cell, is the methylation of phosphatidylmonomethylethanolamine, and is different from that observed by exogenously supplied methionine, which does enter the cell. This implies (a) the existence of asymmetry of the methyltransferase enzymes in the HL-60 membranes and (b) that methyltransferase(s) are indeed present in the outer leaflet of the lipid bilayer.

Discussion

The binding of labeled [^{3}H]AdoMet to HL-60 cells at 0 °C was saturable, of low affinity, and the Scatchard

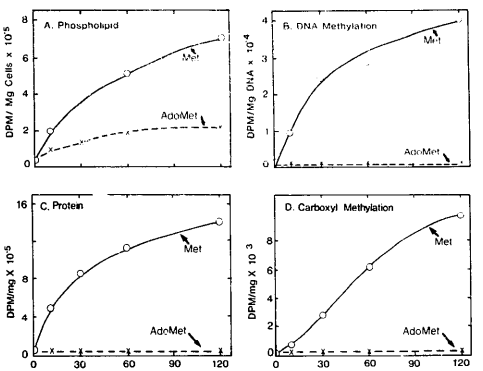

Fig. 5. Incorporation of [^{3}H]methyl groups from methionine or AdoMet into proteins, phospholipids, and DNA from HL-60 cells. Cells were cultured in RPMI-1640 media containing 10 μM L-[*methyl*-^{3}H]methionine ($5.2 \cdot 10^5$ dpm/nmol) or 10 μM *S*-adenosyl-L-[*methyl*-^{3}H]methionine ($5.2 \cdot 10^5$ dpm/nmol) at 36 °C. The reaction was terminated by chilling to 0 °C.

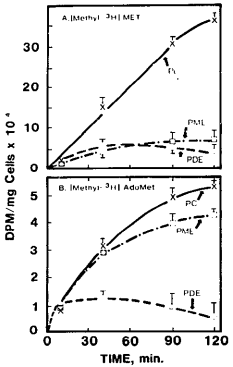

Fig. 6. Distribution of [^{3}H]methyl groups in phospholipids after HL-60 cells had been incubated with 10 μM [^{3}H]methyl labeled exogenous AdoMet or methionine. Cells were incubated as described in Fig. 5. Each point represents the mean±S.E. of at least two separate assay.

plot depicts a homogeneous population of receptors with respect to AdoMet binding. The K_d of $4.2 \cdot 10^{-6}$ M determined here compares with an overall apparent K_m of about $6.7 \cdot 10^{-6}$ M at pH 6.6 for rat liver microsomal AdoMet methyltransferase conversion of phosphatidylethanolamine (PE) to phosphatidylcholine [25]. A K_d value of $5.3 \cdot 10^{-7}$ M for the binding of AdoHcy to rat cortex membranes has been reported [26]. On HL-60 cells, the number of binding sites was about $2.4 \cdot 10^5$ per cell ($4.0 \cdot 10^{-12}$ mol/10^7 cells, 3.3 pmol/mg cells). For AdoHcy binding to rat liver, about 28 pmol/mg protein has been reported [26].

Physiological conditions are best studied in whole cells. With cells, however, there may be endocytosis of the tritiated ligand (AdoMet), or ligands may diffuse across the membrane and become trapped within the cell. Since essentially no [^{3}H]AdoMet could be detected in the intracellular pool at concentrations of extracellular AdoMet as high as 20 μM (Table II), trapping of this ligand seems very unlikely. In addition, [^{3}H]AdoMet binds specifically and with the highest activity to the membranes of HL-60 cells (Table I).

When the HL-60 promyelocytic leukemia cells were cultured in the presence of L-[*methyl*-^{3}H]methionine, the specific radioactivity of the cellularly synthesized AdoMet increased gradually, reaching 80% of that of the exogenous methionine within 2 h (Fig. 4). In contrast HL-60 cells are impermeable to exogenous [^{3}H]AdoMet. If HL-60 cells were incubated at 36°C, methyl groups from [*methyl*-^{3}H]AdoMet were incorporated into phospholipids in the plasma membrane, but not into proteins or nucleic acids (Table II, Fig. 5). Both the binding of AdoMet and methylation of phospholipids were inhibited by AdoHcy.

Hirata and Axelrod [28] have examined rat erythrocyte ghosts for the presence of phospholipid methyltransferase enzyme(s). They concluded that there were two enzymes: methyltransferase I facing the cytoplasmic side of the membrane, and methyltransferase II (which methylates phosphatidylmonomethylethanolamine to phosphatidylcholine) facing the outside of the membrane. In contrast, Vance et al. [29] could find no evidence to support the hypothesis that there is more than one enzyme. Regardless of the number of enzymes involved, phospholipid methyltransferase(s) is sufficiently exposed to bind AdoMet, resulting in the synthesis of phosphatidylcholine (Fig. 6).

It is generally accepted that the levels of phosphatidylmonomethylethanolamine and phosphatidyldimethylethanolamine are lower than phosphatidylcholine and phosphatidylethanolamine [25]. This was readily observed when HL-60 cells were incubated with physiological levels of [*methyl*-^{3}H]methionine (Fig. 6A). Under such conditions, a significant amount of phosphatidyl[*methyl*-^{3}H]choline was synthesized in relation to its precursers. In contrast, incubation of HL-60 cells with extracellular [^{3}H]AdoMet resulted in roughly equivalent levels of phosphatidylcholine and phosphatidylmonomethylethanolamine (Fig. 6B). There is a rapid conversion of phosphatidylethanolamine to phosphatidylmonomethylethanolamine, as illustrated in Fig. 6B, but not in the conversion of phosphatidylmonomethylethanolamine to phosphatidyldimethylethanolamine. There is also a rapid conversion of phosphatidyldimethylethanolamine to phosphatidylcholine. Thus, rather than the methylation of phosphatidylethanolamine being the rate determining step when HL-60 cells are incubated with [*methyl*-^{3}H]methionine (Fig. 6A), the methylation of phosphatidylmonomethylethanolamine is the rate limiting step when cells are incubated with [^{3}H]AdoMet. These results provide further evidence for the existence of more than one phospholipid methyltransferase [28]. One of the transferases being limited to the outside of HL-60 cells, and that this enzyme differs from the phospholipid methyltransferases that is located on the inside of HL-60 membranes (Fig. 6). However, Scatchard analysis (Fig. 3) indicated homogeneous binding at one site.

Schanche et al. [27] found that AdoHcy would bind to numerous sites within the cell, particularly the plasma membrane and endoplasmic reticulum. They suggested that phospholipid methyltransferase may be the major sites for the binding of AdoHcy. This enzyme has a higher affinity for AdoHcy than AdoMet; however,

AdoMet should also bind to this enzyme. The finding that the membrane fraction from HL-60 cells binds four times as much AdoMet as soluble proteins would be consistent with this conclusion (Table I). However, phospholipid methyltransferases may not be the only site, since there are numerous other methyltransferases in the cell [23].

In conclusion, AdoMet binds to the cell surface of HL-60 cells with low affinity. AdoHcy but not adenosine, ADP, nor ATP could inhibit specific AdoMet binding. AdoMet is unable to cross the cell membrane of HL-60 cells at physiological concentrations, but will gradually leak across the membrane when added at inordinate concentrations. Culturing HL-60 cells in the presence of 1–20 μM AdoMet results in methylation of membrane phospholipids, but not components on the inside of the cell. A unique observation is the accumulation of [^{3}H]methyl groups from [^{3}H]AdoMet in phosphatidylmonomethylethanolamine, in addition to phosphatidylcholine. These results may indicate asymmetric location of methyltransferase(s) in the inner and outer leaflet of HL-60 cell membrane.

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