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Inhibition of Phospholipid Methyltransferase(s) from Rat Liver Plasma Membranes by Analogues of *S*-Adenosylhomocysteine

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SUMMARY

The kinetics of the enzymatic incorporation of [^{3}H]methyl groups from *S*-adenosyl-L-[*methyl*-^{3}H]methionine into phospholipids of isolated plasma membranes from rat liver were determined. Physiological pH (7.4) and the presence of Mg^{2+} (1 mM) favored the incorporation of the first methyl group into phosphatidylethanolamine to form phosphatidyl-*N*-monomethylethanolamine, whereas pH 10.0 favored the incorporation of two methyl groups into phosphatidyl-*N*-monomethylethanolamine to form phosphatidylcholine. The apparent K_m values for *S*-adenosylmethionine were 4.1 μM (pH 7.4) and 92 μM (pH 10.0). The inhibition of phospholipid methylation by *S*-adenosylhomocysteine and some *S*-adenosylhomocysteine analogues known to be effective in other biological systems was determined. Linear competitive inhibition was observed with *S*-adenosylhomocysteine and some analogues, and among these compounds *S*-tubercidinylhomocysteine was particularly effective. The inhibitor constants at pH 7.4 (in the presence of Mg^{2+}) were 0.5 μM (*S*-adenosylhomocysteine), 0.3 μM (*S*-tubercidinylhomocysteine), 1.5 μM (*S*-3-deazaadenosylhomocysteine), 15 μM (*S*-adenosyl-D-homocysteine), and 20 μM (sinefungin). 5′-Deoxy-5′-*S*-isobutyl-thioadenosine and the sinefungin metabolite A9145C did not inhibit phospholipid methylation. Similar results were obtained at pH 10.0, except that the inhibition constants were about 10-fold higher. The results are discussed in relationship to the biological properties of these analogues of *S*-adenosylhomocysteine.

INTRODUCTION

Enzymatic methylation of phosphatidylethanolamine to form phosphatidylcholine was first demonstrated by Bremer and Greenberg (1) in rat liver. The stepwise methylation of phosphatidylethanolamine seems to involve two methyltransferases, termed methyltransferase I and methyltransferase II, which are localized on the cytoplasmic and outer surfaces of the membrane, respectively. Methyltransferase I catalyzes methylation of phosphatidylethanolamine to form phosphatidyl-*N*-monomethylethanolamine, whereas methyltransferase II catalyzes the incorporation of two methyl groups into phosphatidyl-*N*-monomethylethanolamine to form phosphatidylcholine via phosphatidyl-*N*,*N*-dimethylethanolamine. These enzymes can be distinguished with respect to pH optimum, magnesium requirement, and affinity for AdoMet[1] (2). The existence of two phospholipid methyltransferases has been questioned by some workers (3, 4).

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[1] The abbreviations used are: AdoMet, *S*-adenosyl-L-methionine; AdoHcy, *S*-adenosyl-L-homocysteine; D-AdoHcy, *S*-adenosyl-D-homocysteine; SIBA, 5′-deoxy-5′-*S*-isobutyl thioadenosine; c^{7}AdoHcy, *S*-tubercidinyl-L-homocysteine; c^{3}AdoHcy, *S*-3-deazaadenosyl-L-homocysteine.

Hirata and Axelrod (2) have suggested that phospholipid methylation plays an important role in various biological processes related to membrane function and structure. These processes include *beta*-adrenergic receptor coupling to adenylate cyclase (5), benzodiazepine receptor occupation (6), Ca^{2+} uptake and histamine release from mast cells (7), lymphocyte mitogenesis (8), and leukocyte chemotaxis (9). It has been proposed that enzymatic methylation of phospholipids is linked to transduction of receptor-mediated signals through the membranes of a variety of cells (2).

Methylation of phospholipids and AdoMet-dependent methylation of other cellular components are associated with the formation of AdoHcy from AdoMet. AdoHcy is a potent inhibitor of a number of transmethylases when AdoMet is the methyl donor, and the tissue level of AdoHcy may play a critical role in the regulation of cellular methylation (10). Analogues of AdoHcy, which are potent inhibitors of certain transmethylases, have been synthesized and some of these compounds have oncostatic and antiviral properties as well as antimalarial and antifungal effects (11, 12). These data suggest that AdoHcy analogues may be useful chemotherapeutic agents in future.

The central role attributed to phospholipid methyltransferase(s) in various cellular processes (2) warrants investigation into the effects of AdoHcy analogues on the

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enzyme(s). The present paper reports on the inhibition of phospholipid methyltransferase(s) from rat liver plasma membranes by AdoHcy analogues. The biological properties of most compounds tested have been studied in some detail in other laboratories.

MATERIALS AND METHODS

Chemicals. AdoHcy, D-AdoHcy, adenine, methylthioadenosine, L-α-phosphatidylethanolamine, L-α-phosphatidyl-*N*,*N*-dimethylethanolamine dipalmitoyl, L-α-phosphatidylcholine, and DL-homocysteine were purchased from Sigma Chemical Company (St. Louis, Mo.) and L-α-phosphatidyl-*N*-monomethylethanolamine dipalmitoyl from Calbiochem-Behring Corporation (La Jolla, Calif.). 3-Deazaadenosine was kindly supplied by Dr. J. Montgomery, Southern Research Institute (Birmingham, Ala.). The following reagents were gifts from the producers: sinefungin and A9145C (Lilly Laboratories, Indianapolis, Ind.), SIBA (Dr. E. Lederer, Centre National de la Recherche Scientifique, Gif-sur-Yvette, France), and c^7AdoHcy (Dr. J. Coward, Rensselaer Polytechnic Institute, Troy, N. Y.). AdoMet was obtained from Koch-Light Laboratories Ltd. (Colnbrooks Bucks., England), and *S*-adenosyl-L[*methyl*-^{3}H]methionine (74 Ci/mmole) was obtained from the Radiochemical Centre (Amersham, England).

c^3AdoHcy was synthesized enzymatically from DL-homocysteine and 3-deazaadenosine using homogenous *S*-adenosylhomocysteine hydrolase from mouse liver (13) as source of enzyme. The compound was purified by high-pressure liquid chromatography on a Nuncleosil 50 5-μm silica column eluted isocratically with 68% acetonitrile in 1.7 mM potassium phosphate buffer (pH 6.0) at a flow rate of 2 ml/min. c^3AdoHcy eluted from the column was pooled, lyophilized, resuspended in distilled water (0.5 ml), and applied to a Sephadex G-10 column (0.5 × 15 cm) equilibrated with distilled water; it was eluted with water. The fractions containing UV-absorbing material were pooled and lyophilized, and c^3AdoHcy was resuspended in water to a final concentration of 0.8 mM.

Preparation of rat liver plasma membranes. Rat liver plasma membranes were prepared according to a procedure which involves differential centrifugation in a hypotonic medium, flotation of the membranes in a discontinuous sucrose gradient, and, finally, washing of the membranes (14).

Assay for phospholipid methyltransferase. The assay for phospholipid methyltransferase is based on the determination of [^{3}H]methyl groups incorporated into phospholipids of the plasma membranes in the presence of [^{3}H]AdoMet. The incubation mixture contained [^{3}H]AdoMet, membranes, potential inhibitors, and buffer. The incubations were routinely carried out at pH 7.4 (25 mM Tris-glycine buffer) in the presence of 1 mM $MgCl_2$ or at pH 10.0 (50 mM KOH-glycine buffer) and were started by the addition of membranes. The temperature was 37° and the incubation times were 2, 5, 10, and 20 min. Three procedures were evaluated for the determination of total radioactive phospholipids.

1. Aliquots (100 μl) from the incubation mixture were mixed with 1 ml of ice-cold 20% sodium trichloroacetate (pH 7.0). After 10 min at 0°, the precipitated lipids were collected by centrifugation at 27,000 × *g* for 10 min. The pellets obtained were dissolved in 3 ml of chloroform-methanol (2:1, v/v), and the chloroform phase was washed twice with 2 ml of 0.1 M KCl in 50% methanol, as described by Crews *et al.* (15). Samples (1 ml) of the chloroform phase were transferred to vials, evaporated to dryness at 80°, and 5 ml of Scint-Hei 4 (Ingeniør Heidenreich, Oslo, Norway) were added.

2. This procedure was carried out as described under Procedure 1 up to the centrifugation step. The precipitated lipids were collected on glass fiber filters (Whatman type GF/A) using a Millipore sampling manifold. The filters were washed discontinuously three times with 2 ml of 20% sodium trichloroacetate (pH 7.0) and then transferred to 10-ml capacity tubes containing 3 ml of chloroform-methanol; the phospholipids were extracted from the filters by vigorous shaking for at least 15 min. The chloroform phase was washed twice, and the amounts of radioactive phospholipids were determined as described above.

3. This procedure was carried out as described under Procedure 2 except that the sodium trichloroacetate was replaced by ice-cold 1.5 N HCl. The phospholipids were extracted from the glass fiber filters in 10-ml capacity tubes containing 2 ml of 1-butanol. The 1-butanol was washed twice with 2 ml of 1.5 N HCl, and 1 ml of the 1-butanol phase was transferred to vials and evaporated to dryness at 80°; the radioactivity was determined by liquid scintillation counting.

Evaluation of the procedures used for the determination of total radioactive phospholipids. The same results were obtained when the amount of radioactive phospholipids was determined by the three procedures described in the preceding section. In our hands, the method described under Procedure 1 gave high and variable background. This seriously interfered with the determination of ^{3}H-labeled phosholipids when a small fraction of the total radioactivity was incorporated into phospholipids.[2] Procedures 2 and 3, which involved washing of the precipitated membranes retained on glass fiber filters, were characterized by blank values not significantly above the background of the scintillation counter. Extraction of the radioactive phospholipids with 1-butanol was preferred for two reasons. Some extraction tubes leaked chloroform. Second, the aqueous medium used to wash the chloroform or 1-butanol phase contained relatively high amounts of radioactive material which obscured the results when carried over into the scintillation vials. This problem was easily avoided when the lipophilic phase was layered upon the aqueous phase.

Separation of phospholipids by thin-layer chromatography. To separate and quantitate the various [^{3}H]methyl-labeled phospholipids of the membranes, the lipids were extracted into a chloroform phase (according to Procedure 1), the chloroform was dried under a stream of N_2, and the lipids were dissolved in 100 μl of chloroform containing standard phospholipids (about 1 mg/ml). The extract was applied to silica plates (Silica Gel 60 plates, E. Merck, Darmstadt, Germany) using an Analytical

[2] The fraction of [^{3}H]methyl groups of [^{3}H]AdoMet incorporated into phospholipids was 0.02–0.14%.

Instrument Specialties thin-layer chromatography multispotter. The plates were developed in propionic acid/1-propanol/chloroform/water (2:2:1:1, v/v/v/v) (16). The spots were visualized by exposure of the plates to iodine vapor, and the spots corresponding to the methylated phospholipids were scraped off and eluted with 1 ml of chloroform; radioactivity was determined by liquid scintillation counting.

Determination of chemical decomposition of AdoMet. AdoMet (100 μM) was incubated in the assay buffer used for the determination of phospholipid methyltransferase activity at pH 10.0. The temperature was 37°. At time intervals of 8 min, samples (20 μl) from the incubation mixture were subjected to high-pressure liquid chromatography on a Partisil 10 SCX column eluted isocratically with 0.4 M ammonium formate buffer (pH 4.0). The flow rate was 2 ml/min. The absorbance at 254 nm was recorded. The retention time for AdoMet and the retention times for some of its derivatives in this system were as follows: 5.97 min (AdoMet), 3.36 min (methylthioadenosine), 3.86 min (adenine), and 2.34 min (AdoHcy).

Determination of protein. Protein was determined according to the method of Lowry *et al.* (17), using bovine serum albumin as standard.

RESULTS

Time course for the incorporation of [^{3}H]methyl groups into phospholipids. The progress curves for the incorporation of [^{3}H]methyl groups from [^{3}H]AdoMet into total lipid fraction and into the separate phospholipids were determined at physiological pH (7.4) in the presence of Mg^{2+} (1 mM) and at pH 10.0 in the absence of Mg^{2+} (Fig. 1). At pH 7.4, the radioactivity was mainly identified as phosphatidyl-*N*-monomethylethanolamine, whereas pH 10.0 favored the incorporation of the second and third methyl groups giving the final product, phosphatidylcholine (Fig. 1). These data are largely in accordance with those reported for phospholipid methyltransferases from other tissues (15, 18, 19).

Kinetics of the enzymatic methylation of phospholipids at various pH values. The kinetic parameters of the enzymatic methylation of phospholipids in rat liver plasma membranes were determined at various pH values and in the absence and presence of magnesium. At pH 6.5 (50 mM sodium acetate buffer) in the presence of 1 mM $MgCl_2$, the double reciprocal plot for the methyltransferase activity versus the concentration of AdoMet was characterized by a main linear component corresponding to an apparent K_m of 3.2 μM for AdoMet and a high K_m component (data not shown). Similar results were obtained at pH 7.4 (in the presence of 1 mM $MgCl_2$). The double reciprocal plot at pH 7.4 was consistent with an apparent K_m of 4.1 μM for AdoMet, and the high K_m component of the graph was slightly more pronounced than at pH 6.5 (Fig. 2). The double reciprocal plot at pH 10.0 was linear, and the apparent K_m for AdoMet was 92 μM (Fig. 2). Thus, kinetically different forms of phospholipid methyltransferase can be demonstrated in rat liver membranes, as previously reported for this enzyme from other sources (15, 18, 19).

The possibility existed that the instability of AdoMet in alkaline solution (20) obscured the determination of enzyme kinetics at pH 10.0. The rate of decomposition of AdoMet in 50 mM KOH-glycine buffer (pH 10.0) at 37° was estimated to be 1.02 hr^{-1}, and the main split product was adenine. These data suggest that the slight reduction in the concentration of AdoMet which occurred during the determination of initial velocities of phospholipid methylation is not a source of erratic results.

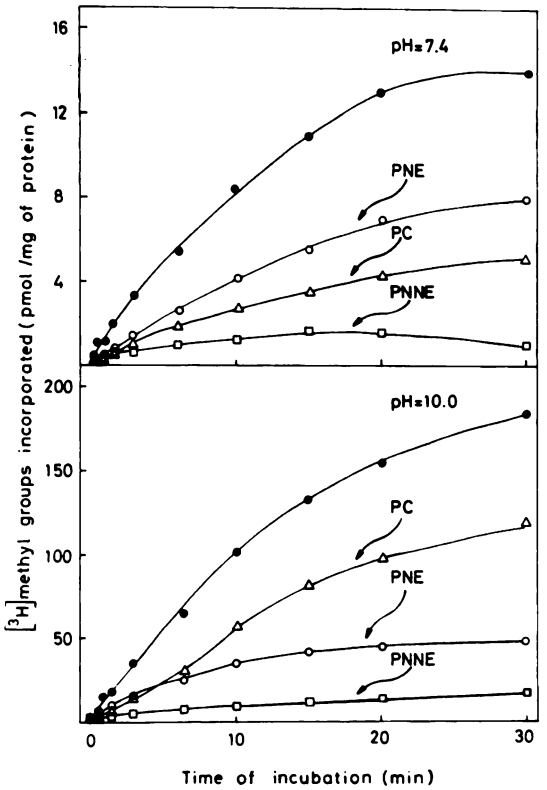


FIG. 1. *Progress curves for the methylation of separate phospholipids in rat liver membranes*

The incorporation of [^{3}H]methyl groups from [^{3}H]AdoMet into the total lipid fraction (●), phosphatidyl-*N*-monomethylethanolamine (*PNE*, ○), phosphatidyl-*N*,*N*-dimethylethanolamine (*PNNE*, □), and phosphatidylcholine (*PC*, △) in plasma membranes from rat liver (1.8 mg of protein per milliliter) was determined in the presence of 1 mM $MgCl_2$ at pH 7.4 (*upper panel*) and at pH 10.0 (*lower panel*). The concentrations of [^{3}H]AdoMet were 1.25 μM and 20 μM, respectively.

Inhibition of phospholipid methyltransferase(s) by AdoHcy and AdoHcy analogues. The effect of AdoHcy and some analogues of AdoHcy on phospholipid methylation in rat liver plasma membranes was determined at pH 7.4 in the presence of Mg^{2+} and at pH 10.0. Data in the preceding paragraphs suggest that these conditions distinguish between the two hypothetical enzyme participating in the stepwise methylation of phosphatidylethanolamine. The AdoHcy analogues tested and their structural formulae are shown in Table 1.

The initial velocities of the enzyme catalysis were determined under the conditions described above at two concentrations of AdoMet and increasing concentrations of inhibitor. The data obtained were plotted according to the method of Dixon (21), as shown for *S*-tubercidinylhomocysteine (c^{7}AdoHcy) in Fig. 3, and the inhibitor constants were determined directly from the graphs. The results are summarized in Table 2.

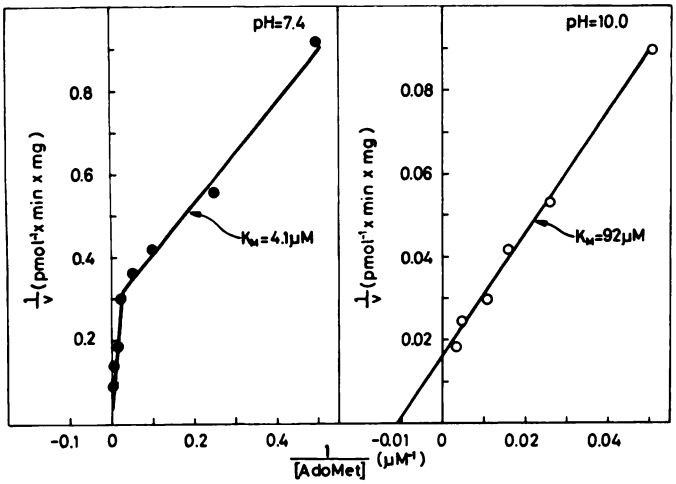


FIG. 2. *Double reciprocal plots of the enzymatic methylation of phospholipids in rat liver plasma membranes*

The initial velocities (v) of the incorporation of [^{3}H]methyl groups into phospholipids of rat liver plasma membranes (0.98 mg of protein per milliliter) were determined at various concentrations of [^{3}H] AdoMet in the presence of $MgCl_2$ at pH 7.4 (*left panel*) and at pH 10.0 (*right panel*).

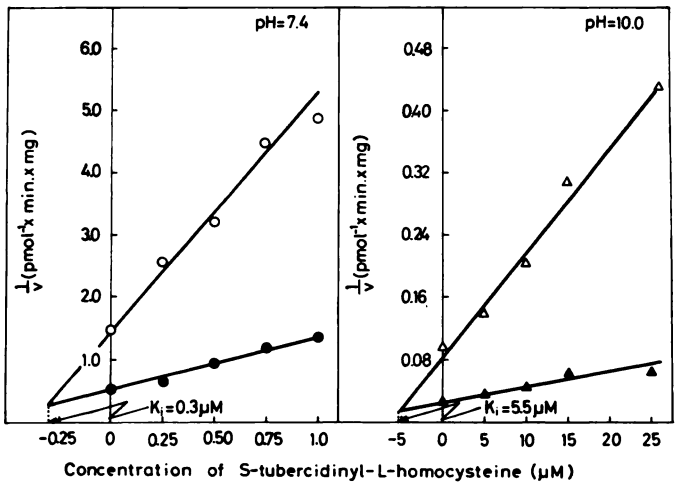


FIG. 3. *Inhibition of phospholipid methyltransferase activity by S-tubercidinyl-L-homocysteine*

The initial velocities (v) of the incorporation of [^{3}H]methyl groups from [^{3}H]AdoMet into phospholipids in rat liver plasma membranes (0.8 mg of protein per milliliter) were determined in the presence of 1 mM $MgCl_2$ at pH 7.4 (*left panel*) and at pH 10.0 (*right panel*). The concentrations of *S*-tubercidinyl-L-homocysteine are indicated on the figure, and the concentrations of [^{3}H]AdoMet were 1 μM (○); 5 μM (●), 20 μM (△), and 100 μM (▲). The data are plotted according to Dixon (21). The inhibitor constants (K_i) were determined directly from the graphs.

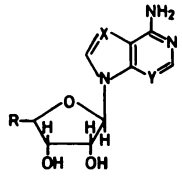
At physiological pH (7.4), linear competitive inhibition of phospholipid methylation was observed in the presence of c^{7}AdoHcy (Fig. 3), AdoHcy, c^{3}AdoHcy, D-AdoHcy, and sinefungin, and the inhibitor constants increased in the other mentioned. The sinefungin metabolite, A9145C, and SIBA were essentially without effect (Table 2). The inhibitor constants for c^{7}AdoHcy and AdoHcy were one order of magnitude lower than the apparent K_m value for AdoMet. Similar results were obtained at pH 10.0, but the inhibitor constants were about 10-fold higher than at pH 7.4 (Table 2).

DISCUSSION

Inhibition of AdoMet-dependent transmethylation by AdoHcy was first demonstrated by Gibson *et al.* (22), who used phospholipid methyltransferase from rat liver. Inhibition of this enzyme by AdoHcy was later confirmed by others (18, 19, 23). These data are in accordance with the finding that AdoHcy is a potent inhibitor of phospholipid methyltransferase(s) from rat liver plasma mem-

TABLE 1

AdoHcy analogues tested and their structural formulae



Name	Abbreviation	X	Y	R-group
S-adenosyl-L-homocysteine	AdoHcy	-N-	-N-	HOOC-CH(NH$_2$)-(CH$_2$)$_2$-S-CH$_2$-
S-adenosyl-D-homocysteine	D-AdoHcy	-N-	-N-	
S-tubercidinyl-L-homocysteine	c^{7}AdoHcy	-C-	-N-	
S-3-deazaadenosyl-L-homocysteine	c^{3}AdoHcy	-N-	-C-	
Sinefungin		-N-	-N-	HOOC-CH(NH$_2$)-(CH$_2$)$_2$-CH(NH$_2$)-CH$_2$-
A9145C		-N-	-N-	HOOC-CH(NH$_2$)-(CH$_2$)$_2$-CH(NH$_2$)-CH=
5'-deoxy-5'-S-isobutyl-thioadenosine	SIBA	-N-	-N-	(CH$_3$)$_2$CH-CH$_2$-S-CH$_2$-
S-adenosyl-L-methionine	AdoMet	-N-	-N-	HOOC-CH(NH$_2$)-(CH$_2$)$_2$-S(CH$_3$)$^+$-CH$_2$-

TABLE 2

Inhibitor constants for AdoHcy and AdoHcy analogues

The inhibitor constants for AdoHcy and the analogues listed at left were determined from Dixon plots, as shown for c^{7}AdoHcy in Fig. 3. The inhibition of phospholipid methylation was determined in the presence of magnesium at pH 7.4 and at pH 10.0 (in the absence of magnesium). For comparison, K_m values are given for AdoMet under the same conditions.

Inhibitor	Inhibitor constants (K_i)	
	pH 7.4	pH 10.0
	μM	
AdoHcy	0.5	7.5
c^{7}AdoHcy	0.3	5.5
c^{3}AdoHcy	1.5	20
Sinefungin	20	89
A9145C	NI[a]	NI
D-AdoHcy	15	125
SIBA	NI	NI
	K_m values for AdoMet	
	μM	
	4.1	92

[a] NI, Not inhibitory.

branes, and the K_i for AdoHcy is one order of magnitude lower than the apparent K_m for AdoMet (Table 2). In addition, several analogues of AdoHcy, known to be effective in other biological systems (11, 24), are potent inhibitors of the enzyme(s) (Fig. 3 and Table 2).

The metabolically stable AdoHcy analogue, c^{7}AdoHcy, is an inhibitor of various transmethylases in both cell-free systems and intact cells (24). Among the compounds tested by us, this analogue was the most potent inhibitor of phospholipid methylation in rat liver plasma membranes (Table 2 and Fig. 3). c^{7}AdoHcy may be a useful tool for studying the role of phospholipid methylation in various biological processes.

The K_i values for c^{3}AdoHcy are about 3-fold higher than those for AdoHcy (Table 2). This observation is particularly interesting in the light of the effect of 3-deazaadenosine on phospholipid methylation *in vivo* reported by Chiang *et al.* (25). This adenosine analogue functions as a good substrate for the enzyme *S*-adenosylhomocysteine hydrolase (EC 3.3.1.1), but it also blocks the cellular degradation of AdoHcy catalyzed by this enzyme. The administration of 3-deazaadenosine to animals results in a massive cellular buildup of AdoHcy and c^{3}AdoHcy, which in turn inhibits a number of transmethylation reactions (10). 3-Deazaadenosine is a potent inhibitor of phospholipid methylation in livers of rat and hamster, but whether the effects stem from cellular accumulation of AdoHcy or c^{3}AdoHcy, or both, has been questioned (25). The present report is in agreement with the finding that 3-deazaadenosine is an effective inhibitor of phospholipid methylation (25), and extends this observation by providing data suggesting that the effect of 3-deazaadenosine is mediated by the accumulation of both c^{3}AdoHcy and AdoHcy.

Sinefungin and its metabolite, A9145C, are naturally occurring "carba" analogues of AdoHcy (Table 1) isolated from *Streptomyces griseolus*; sinefungin has antifungal, antiviral, and oncostatic properties and is an extremely potent inhibitor of virion mRNA methyltransferase and protein *O*-methyltransferase (11, 26). Both phospholipid methyltransferase and protein *O*-methyltransferase have been assigned a role in processes such as chemotaxis and secretion (12). The inhibitor constants for sinefungin of phospholipid methyltransferase(s) from rat liver membranes (Table 2) are several order of magnitude higher than that reported for protein *O*-methyltransferase (11). This observation suggests that sinefungin is a rather specific tool for the investigation of the role of the latter enzyme in chemotaxis and secretion, as originally proposed by Borchardt *et al.* (26).

SIBA has biological properties similar to those reported for sinefungin, but this analogue of AdoHcy is a weak inhibitor of transmethylases in cell-free systems (11). The finding of no inhibition of phospholipid methylation by SIBA (Table 2) is in agreement with this general statement. A SIBA receptor on the plasma membrane has recently been postulated on the basis of the finding that this compound is an inhibitor of cellular transport of sugar and nucleosides (11). Lack of inhibition of phospholipid methyltransferase by SIBA suggests that the effects of this compound on the membrane are not mediated by a direct effect on this enzyme.

The existence of specific AdoHcy binding sites on the surface of rat hepatocytes has recently been demonstrated in our laboratory (27). The effect of AdoHcy analogues on the binding of AdoHcy to intact hepatocytes and purified rat liver membranes shows similarities to the inhibition of phospholipid methyltransferase(s) by these compounds, but some differences were noted. Both sinefungin and its metabolite, A9145C, inhibit the binding of AdoHcy to the membrane acceptor, and the displacement curves in the presence of these inhibitors are biphasic (28). Furthermore, the high binding capacity for AdoHcy of the rat liver membranes (28) contrasts to the low specific activity of phospholipid methyltransferase (Figs. 1–3; ref. 29). Thus, binding of AdoHcy to phospholipid methyltransferase does not totally account for the membrane binding of AdoHcy.

The inhibition studies (Table 2) suggest that the structural requirements for the interaction of AdoHcy and its analogues with the catalytic site(s) of phospholipid methyltransferase(s) at pH 7.4 and pH 10.0 are similar. No analogue tested specifically inhibits one of the catalytic processes involved in the stepwise methylation of phosphatidylethanolamine to phosphatidylcholine. Thus, the present report does not provide any data related to the existence of one or two enzymes participating in the methylation of phospholipids.

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