MOLECULAR PHARMACOLOGY, 21:108–113

Characterization of S-Adenosylhomocysteine Binding to Isolated Rat Hepatocytes and Purified Rat Liver Plasma Membranes

Effect of Analogues of S-Adenosylhomocysteine

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Received May 11, 1981; Accepted August 17, 1981

SUMMARY

Studies on the disposition of extracellular *S*-adenosylhomocysteine by isolated rat hepatocytes have shown that *S*-adenosyl-L-homocysteine is not taken up by cells, but binds to acceptor(s) on the cell surface. The Scatchard plots for the binding of *S*-adenosylhomocysteine to hepatocytes and purified rat liver membranes at 0° were nonlinear, and consistent with high-affinity components with K_d values of 0.4 μM and 0.7 μM, respectively. About 60% of the *S*-adenosylhomocysteine that was bound to cells and purified membranes dissociated rapidly from its binding sites. The rapid initial phase was followed by a second slow phase obeying first-order kinetics, corresponding to a dissociation rate constant of 0.09 min^{-1}. *S*-Tubercidinylhomocysteine and unlabeled *S*-adenosylhomocysteine were potent inhibitors of the binding of *S*-[^{14}C]adenosylhomocysteine, whereas *S*-3-deazaadenosylhomocysteine, *S*-adenosylmethionine, and *S*-adenosyl-D-homocysteine were less effective. A fraction of the *S*-adenosylhomocysteine that was bound to rat hepatocytes was displaced by low concentrations of sinefungin and its metabolite, A9145C, but these compounds were weak inhibitors of *S*-adenosylhomocysteine binding to purified membranes. 5′-Deoxy-5′-*S*-isobutylthioadenosine showed slight inhibitory activity against *S*-adenosylhomocysteine binding to both cells and purified membranes. In conclusion, the equilibrium binding, dissociation rate kinetics, and displacement curves in the presence of *S*-adenosylhomocysteine analogues show that *S*-adenosylhomocysteine binds to a heterogeneous population of binding sites of intact hepatocytes and purified liver plasma membranes.

INTRODUCTION

AdoHcy[3] is both a product from and a potent inhibitor of AdoMet-dependent transmethylation reactions (1). The following observations point to the possibility that the interaction of AdoHcy with membrane components plays a role in the metabolic fate and biological features of this metabolite. Extracellular AdoHcy is not taken up by cells (2–4), but a unidirectional flow of intracellular AdoHcy to the extracellular medium has been demonstrated (5). Furthermore, some AdoHcy analogues seem to be transported into cells (6), and among these compounds SIBA inhibits membrane transport of nucleosides and sugar (7). Finally, binding of AdoHcy to the surface of rat hepatocytes (4) and membranes from rat cerebral cortex (8) has recently been demonstrated.

During our studies on the disposition of extracellular AdoHcy by rat hepatocytes, we observed that AdoHcy binds to specific acceptor(s) on the cell surface. Characterization of the binding was hampered by rapid metabolism of AdoHcy catalyzed by AdoHcy hydrolase (EC 3.3.1.1) leaking out of the cells (4). In the present work, this problem was circumvented by measuring binding at low temperature (0°). Binding of AdoHcy to both rat hepatocytes and purified rat liver plasma membranes, characterized by specificity, saturability, and reversibility, could be demonstrated.

MATERIALS AND METHODS

Chemicals. AdoHcy, D-AdoHcy, Hepes, collagenase (Type I), albumin (Fraction V, defatted) and DL-homo-

This work was supported by grants from Nordisk Insulinfond, the Norwegian Research Council for Science and the Humanities, Langfeldts fond, and Norsk Medisinaldepot.

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[3] The abbreviations used are: AdoHcy, *S*-adenosyl-L-homocysteine; AdoMet, *S*-adenosyl-L-methionine; SIBA, 5′-deoxy-5′-*S*-isobutylthioadenosine; *d*-AdoHcy, *S*-adenosyl-D-homocysteine; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; c^7AdoHcy, *S*-tubercidinyl-L-homocysteine; c^3AdoHcy, *S*-3-deazaadenosyl-L-homocysteine.

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cysteine were obtained from Sigma Chemical Company (St. Louis, Mo.). 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), c^7AdoHcy (Dr. J. Coward, Rensselaer Polytechnic Institute, Troy, N. Y.). AdoMet was obtained from Koch-Light Laboratories Ltd. (Colnbrooks, Bucks, England), and [8-^{14}C]adenosine (59 mCi/mmole) from the Radiochemical Centre (Amersham, England). *S*-[8-^{14}C] Adenosylhomocysteine (59 mCi/mmole) and c^3AdoHcy were synthesized enzymatically by condensation of homocysteine with [^{14}C]adenosine and 3-deazaadenosine, respectively. The reactions were catalyzed by homogeneous AdoHcy hydrolase from mouse liver. [^{14}]AdoHcy and c^3AdoHcy were purified by high-pressure liquid chromatography on a Nucleosile 50-5 μM silica column eluted isocratically with 68% acetonitril in 1.7 mM potassium phosphate buffer (pH 6.0) at a flow rate of 2 ml/min. Phosphate was removed from [^{14}C]AdoHcy and c^3AdoHcy by chromatography on a Sephadex G-10 column (0.5 × 15 cm) equilibrated and eluted with distilled water.

Preparation of isolated hepatocytes. Liver cells were prepared by a slight modification (9) of the collagenase perfusion method described by Berry and Friend (10). Following collagenase perfusion, the livers were perfused for 1 min with collagenase-free solution at 20°. Hepatocytes were separated from nonparenchymal cells by centrifugation (9), and the cells were washed twice with the incubation medium. The initial cell viability was 97 ± 2%.

Preparation of rat liver plasma membranes. Livers were homogenized in ice-cold 0.25 M sucrose containing 100 mM Tris-HCl (pH 8.0) with two strokes in a Potter-Elvehjem homogenizer at 1000 rpm. After fractionating the total liver homogenate (11), the microsomal fraction was subfractionated using discontinuous sucrose gradient centrifugation (11). The light microsomal subfraction was collected at the interface between the two upper layers of sucrose, diluted with 4 volumes of cold distilled water, and centrifuged; the membrane pellet was resuspended in 20 mM Hepes buffer (pH 7.4) containing 60 mM KCl (Buffer A) to a protein concentration of about 15 mg/ml.

Incubation conditions. All incubations were performed at 0°. Cells were incubated in 20 mM Hepes buffer (pH 7.4) containing 137 mM NaCl, 5.37 mM KCl, 0.81 mM $MgSO_4$, 4 mM $CaCl_2$, 0.34 mM Na_2HPO_4, 0.35 mM KH_2PO_4, 2% bovine serum albumin, 5.5 mM glucose, streptomycin sulfate (250 μg/ml) and benzyl penicillin (30 μg/ml). The membranes were incubated in Buffer A.

Determination of AdoHcy binding to hepatocytes and purified membranes. Suspensions of rat hepatocytes or plasma membranes were incubated at 0° in a shaking water bath in the presence of [^{14}C]AdoHcy at concentrations indicated in the legends to the separate figures. [^{14}C]AdoHcy associated with the cells or membranes (bound fraction) was separated from the incubation medium (free fraction) by centrifugation of cells or membranes through oil, the composition of which was somewhat different for cells and membranes. The method allowed the detection of AdoHcy-acceptor complexes characterized by a half-life of less than 15 sec (see Results).

Samples of 250 μl from the cell suspension were layered onto a mixture (100 μl) of dinonyl phthalate and dibutyl phthalate (1:3, v/v) in 400-μl polyethylene microcentrifuge tubes, and centrifuged at 7000 rpm for 15 sec in a Beckman 152 Microfuge. The membranes were separated from the medium by the following modification of the procedure described above: samples of 90 μl were analyzed; the oil was composed of dinonyl phthalate and dibutyl phthalate, 2:3 (v/v), and the centrifugation time was increased to 45 sec. The bottom of the tubes, containing packed cells or membranes, was cut off, wiped dry of oil, and placed in scintillation vials containing 1 ml of 2% sodium dodecyl sulfate. After 18 hr at 23° (overnight) 6 ml of Dilusolve (Packard) were added.

The radioactivity associated with the cells or membranes incubated with [^{14}C]AdoHcy represents total binding of AdoHcy. Nonspecific binding of [^{14}C]AdoHcy was determined in a parallel run by incubating cells or membranes with the same concentration of radioactive AdoHcy in the presence of 2.5 mM unlabeled AdoHcy. Specific binding of AdoHcy refers to total binding minus nonspecific binding (see also Results).

Determination of protein. Protein was determined by the method of Lowry *et al.* (12) using bovine serum albumin as standard.

RESULTS

Specific and nonspecific binding of AdoHcy. The binding of AdoHcy to hepatocytes and purified membranes was determined at 0°, conditions under which nearly no degradation of AdoHcy takes place (4). A fraction of [^{14}C]AdoHcy bound to cells and membranes was not suppressed by high concentrations of unlabeled AdoHcy (2.5 mM), and this fraction is referred to as nonspecific binding of AdoHcy. This fraction increased linearly as a function of the concentrations of cells or membranes (Fig. 1) and [^{14}C]AdoHcy (data not shown). Specific binding of [^{14}C]AdoHcy represents the total binding corrected for nonspecific binding, and increased proportionally to the concentrations of cells or membranes (Fig. 1) but was saturable with respect to AdoHcy (see the following paragraph).

Equilibrium binding. The progress curves for the (specific) binding of AdoHcy to isolated rat hepatocytes and rat liver plasma membranes are shown in Fig. 2. At low concentration (0.05 μM) of [^{14}C]AdoHcy, the binding of [^{14}C]AdoHcy to the cells increased for the first few minutes, after which a plateau was obtained. Equilibrium was attained after shorter times at higher concentration (1 μM) of AdoHcy. Binding of [^{14}C]AdoHcy (1 μM) to purified membranes reached a plateau within a few minutes (Fig. 2). On the basis of these data, equilibrium binding was routinely determined after 10 min of incubation.

Equilibrium binding of [^{14}C]AdoHcy to cells and membranes was determined at various concentration (0.2–100 μM) of radioactive ligand. A Scatchard plot for the binding of [^{14}C]AdoHcy to hepatocytes was hyperbolic. The

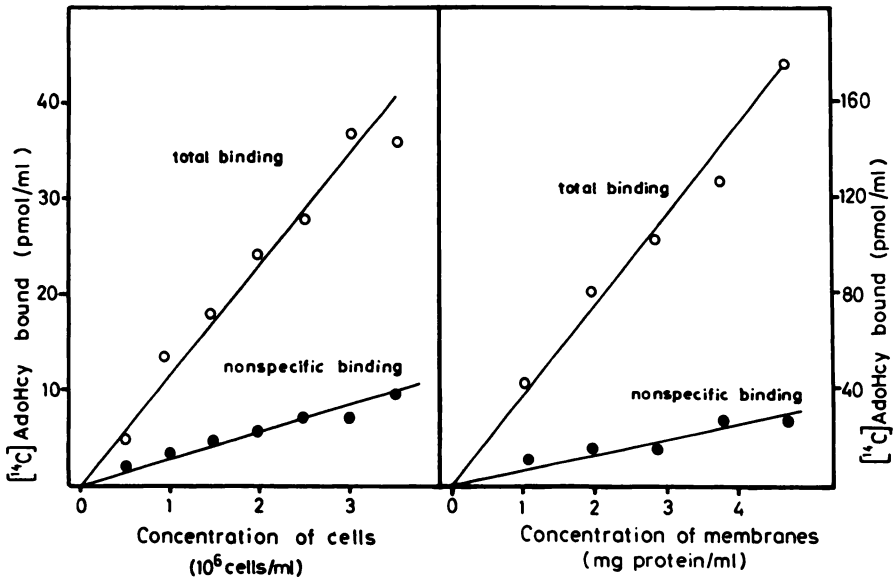

FIG. 1. *Specific and nonspecific binding of AdoHcy to rat hepatocytes and purified rat liver plasma membranes*
[^{14}C]AdoHcy (1 μM) was incubated at 0° for 10 min with increasing concentrations of rat hepatocytes (*left*) and purified membranes (*right*) in the absence (○) and presence (●) of 2.5 mM unlabeled AdoHcy.

dissociation constant (K_d) corresponding to the high-affinity component of the plot was 0.7 μM (Fig. 3A), and the intersection of this component with the abscissa of the graph showed a binding capacity of about 12 pmoles/10^6 cells for the high-affinity site(s). Similar results were obtained for the binding of [^{14}C]AdoHcy to purified plasma membranes. High-affinity binding characterized by a K_d of 0.4 μM could be demonstrated, and the binding capacity of the high-affinity component was about 75 pmoles/mg of membrane protein (Fig. 3B).

Dissociation rate kinetics. The dissociation rate kinetics of the AdoHcy-acceptor complexes were determined both for cells and purified membranes. Cells or membranes were incubated with [^{14}C]AdoHcy for 10 min, and the incubation mixture was then made 2.5 mM in unlabeled AdoHcy, to prevent rebinding of radioactive ligand. The dissociation rate curves for [^{14}C]AdoHcy bound to cells and membranes were almost identical. About 60% of [^{14}C]AdoHcy dissociated rapidly, and the kinetics of the process was not assessable. The rapid phase was followed by a second slow phase of dissociation obeying first-order kinetics, consistent with a dissociation rate constant of 0.09 min^{-1} (Fig. 4).

Effect of analogues of AdoHcy. Inhibition of the binding of [^{14}C]AdoHcy (at a total concentration of 1 μM) to isolated rat hepatocytes by increasing concentrations of

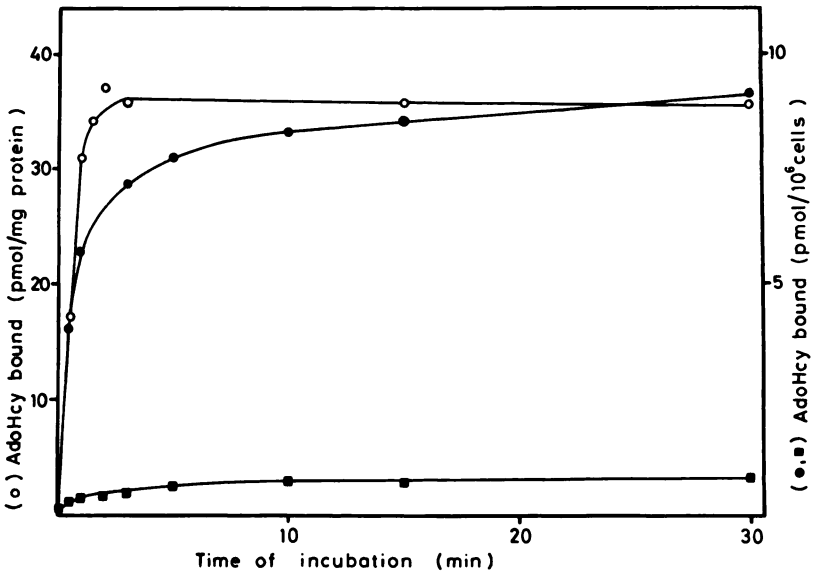

FIG. 2. *Time course for the binding of AdoHcy to hepatocytes and purified rat liver plasma membranes*
[^{14}C]AdoHcy at concentrations of 0.05 μM (■) and 1 μM (●) was incubated at 0° with rat hepatocytes (5.6×10^6 cells/ml) and the binding of [^{14}C]AdoHcy to the cells was determined at various time points. In a parallel experiment, the binding of [^{14}C]AdoHcy (1 μM) to purified membranes (4.5 mg of protein per milliliter) as a function of time (○) was determined.

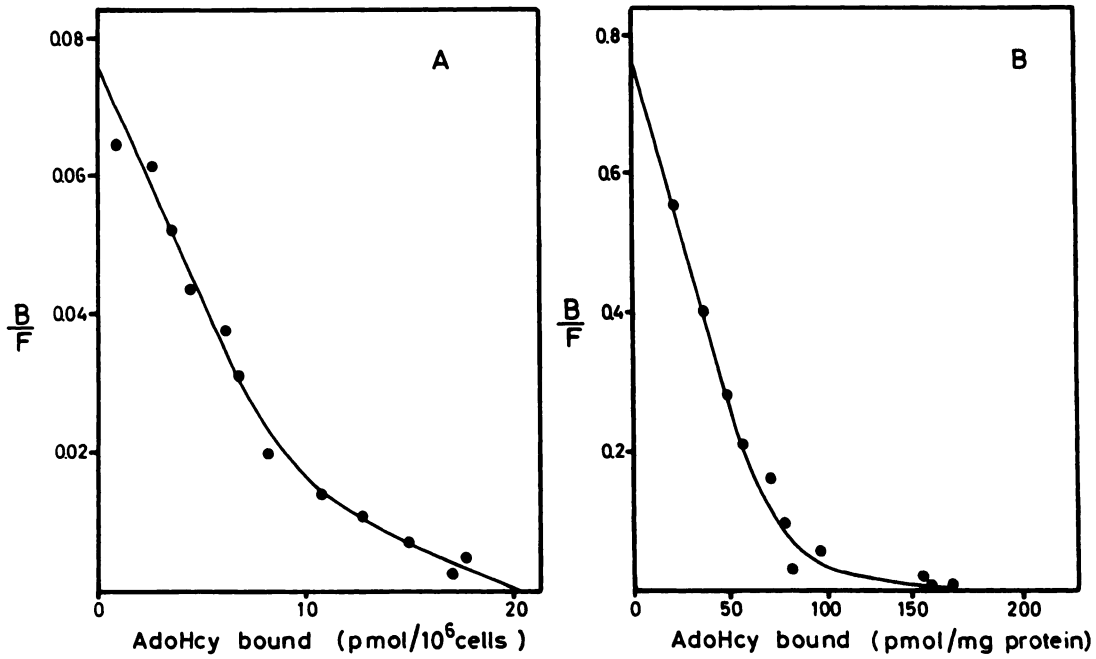

FIG. 3. *Scatchard plots for the binding of AdoHcy to rat hepatocytes (A) and purified rat liver plasma membranes (B)*
The binding of [^{14}C]AdoHcy to rat hepatocytes (4.7 × 10^6 cells/ml) or purified membranes (4 mg of protein per milliliter) was determined at various concentrations of [^{14}C]AdoHcy. The temperature was 0° and time of incubation 10 min.

unlabeled AdoHcy and analogues of AdoHcy was determined. The data of Fig. 5A show that AdoHcy (IC_{50} = 3.8 μM) was the most potent inhibitor, followed by c^7AdoHcy (IC_{50} = 6.1 μM), c^3AdoHcy (IC_{50} = 10 μM), AdoMet (IC_{50} = 14 μM), D-AdoHcy (IC_{50} = 21 μM) and SIBA (IC_{50} > 500 μM). The displacement curves obtained in the presence of sinefungin and its metabolite, A9145C, were biphasic. These compounds were relatively potent inhibitors at low concentrations (<5 μM), but the displacement curves leveled off at high concentrations of inhibitor (Fig. 5A).

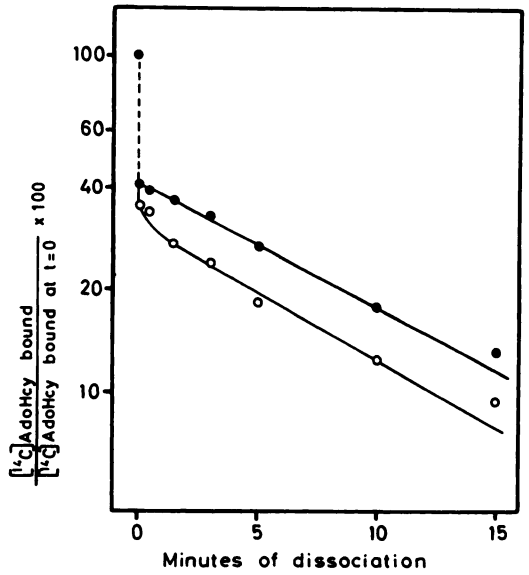

FIG. 4. *Dissociation of [^{14}C]AdoHcy bound to rat hepatocytes and purified rat liver plasma membranes*
[^{14}C]AdoHcy (1 μM) was incubated with rat hepatocytes (4.2 × 10^6 cells/ml) or purified plasma membranes (5.2 mg of protein per milliliter) for 10 min at 0°, and the amount of [^{14}C]AdoHcy bound at this time point was defined as 100% (⊙). After 10 min of incubation, the incubation mixture was made 2.5 mM in unlabeled AdoHcy, and the amount of [^{14}C]AdoHcy bound to cells (●) or membranes (○) was determined at various time points. The data are plotted as the logarithm of [^{14}C] AdoHcy bound (as percentage versus time of dissociation (time elapsed after addition of unlabeled AdoHcy).

The experiment described above was conducted with purified plasma membranes, and slightly different results were obtained. c^7AdoHcy was equally as effective as unlabeled AdoHcy (IC_{50} = 3.0 μM) in inhibiting [^{14}C] AdoHcy binding. The efficiency of the other analogues decreased in the following order: c^3AdoHcy (IC_{50} = 16μM), AdoMet (IC_{50} = 32 μM), D-AdoHcy (IC_{50} = 41 μM), sinefungin (IC_{50} = 180 μM), and SIBA (IC_{50} > 500 μM). A9145C (IC_{50} > 1000 μM) was essentially without effect (Fig. 5B).

DISCUSSION

AdoHcy seems to bind to a heterogeneous population of binding sites on both hepatocytes and purified membranes. This statement is based on the nonlinear Scatchard plots (Fig. 3), dissociation rate curves (Fig. 4), and the biphasic displacement curves obtained for hepatocytes in the presence of sinefungin and A9145C (Fig. 5).

The characteristics of the AdoHcy binding to hepatocytes and purified membranes are grossly similar, but some differences were noted. The affinity of AdoHcy to membranes was somewhat higher than the affinity to intact hepatocytes (Fig. 3). Furthermore, some differences in the structural requirements for the interaction of AdoHcy analogues with the AdoHcy binding sites of hepatocytes and purified plasma membranes are indicated by the data presented in Fig. 5. Several explanations to these findings can be offered. AdoHcy binding to

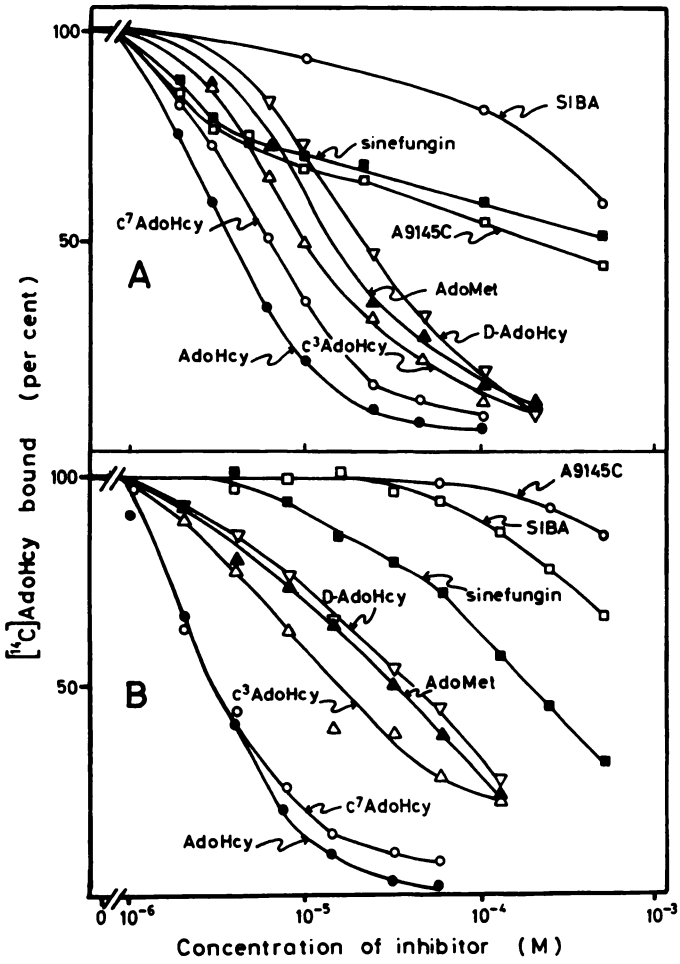

FIG. 5. *Displacement of [14C]AdoHcy binding by AdoHcy analogues*

[14C]AdoHcy (1 μM) was incubated with hepatocytes (4.5×10^6 cells/ml) (A) or purified plasma membranes (4.8 mg of protein per milliliter) (B) in the presence of increasing concentrations of unlabeled AdoHcy or AdoHcy analogues as indicated. The amount of [14C]AdoHcy bound is plotted versus the concentration of inhibitor.

intact hepatocytes takes place at the outer surface of the plasma membrane, whereas AdoHcy may gain access to both the outer surface and cytoplasmic side of purified plasma membranes. Some differences may also be attributed to the slightly different incubation conditions used.

The hepatocytes were incubated in an isotonic medium containing bovine serum albumin. The medium was composed so as to preserve the integrity of the cells. The oil-centrifugation technique used for separation of cells from the medium was adapted to the experiments with membranes by decreasing the density of the oil (see Materials and Methods), to allow the sedimentation of the membranes through the oil layer. The suspension of membranes was layered upon the oil, and the density of the incubation medium had to be reduced accordingly. These are the reasons why hepatocytes and membranes were not incubated in the same medium. However, it should be noted that equilibrium binding of [14C]AdoHcy to isolated hepatocytes was not affected by the presence of bovine serum albumin in the cellular medium.[4]

The binding capacity of the AdoHcy acceptors on the surface of rat hepatocytes (12 pmoles/10^6 cells; Fig. 3) is about one-half of the cellular content of AdoHcy in these cells.[5] As already pointed out, AdoHcy may also bind to the cytoplasmic side of the plasma membrane. Thus, the possibility exists that a substantial fraction of AdoHcy in the intact cell is bound to cellular acceptors. This suggestion is reinforced by the observation that AdoHcy forms a stable complex with *S*-adenosylhomocysteine hydrolase (13) and hydroxyindole *O*-methyltransferase (14). The possibility of cellular sequestration of AdoHcy should be taken into account when relating the cellular content of this metabolite to its effect on transmethylation reactions in intact cells.

One essential question which arises is the relationship of the AdoHcy binding sites of the plasma membrane to known membrane components participating in the metabolism of AdoMet, AdoHcy, or its analogues. The inhibition of AdoHcy binding by AdoMet (Fig. 5) suggests that membrane components interacting with this metabolite may contribute to the AdoHcy binding sites of the plasma membranne. A permease for AdoMet of the plasma membrane has been postulated (15), but data on the interaction of this membrane component with AdoHcy and AdoHcy analogues are not available, and speculations of its relationship to membrane binding of AdoHcy would be premature.

AdoMet-dependent transmethylases localized in the plasma membrane may account for AdoHcy binding. This possibility is supported by the following observations. The affinity of transmethylases for AdoHcy is often higher than the affinity for its substrate, AdoMet (1). Second, the AdoHcy-analogues found to be potent inhibitors of the binding of AdoHcy (c7AdoHcy, c3AdoHcy, and sinefungin; Fig. 5) are potent inhibitors of various transmethylases (7, 16, 17), whereas SIBA, which is an ineffective inhibitor of AdoHcy binding (Fig. 5), has very weak inhibitory activity against any transmethylase *in vitro* (7).

Two membrane-associated transmethylases catalyzing the stepwise methylation of phosphatidylethanolamine to phosphatidylcholine are ubiquitously distributed in mammalian tissues. The enzyme catalyzing the incorporation of the first methyl group into phosphatidylethanolamine seems to be localized on the cytoplasmic side of the membrane, whereas the enzyme catalyzing the incorporation of the second and third methyl groups probably faces the outer surface of the cell (18).

Several lines of evidence suggest that phospholipid methyltransferases of the plasma membrane do not totally account for the membrane binding of AdoHcy, at least in rat liver. Phospholipid methylation in rat liver plasma membranes is not inhibited by the sinefungin metabolite, A9145C, and c7AdoHcy is even more potent than AdoHcy itself (19). Thus, the structural requirements for the interaction of AdoHcy analogues with the catalytic site of phospholipid methyltransferase and the AdoHcy acceptors on the surface of rat hepatocytes (Fig. 5A) are somewhat different. Furthermore, the high binding capacity of the AdoHcy-binding sites of rat liver

[4] J. Aarbakke and A. Bessesen, unpublished results.

[5] S. Helland and P. M. Ueland, unpublished results.

plasma membrane (Fig. 3) contrasts to the low specific activity of phospholipid methyltransferase (19, 20).

The AdoHcy analogue, SIBA, inhibits nucleoside and sugar transport into chick and mouse cells, and a SIBA receptor of the membrane has been postulated as an explanation of these findings (7). SIBA is a weak inhibitor of AdoHcy binding to hepatocytes and purified plasma membranes (Fig. 5). Thus, there is no obvious reason to relate the hypothetical SIBA receptor to the AdoHcy acceptors on the plasma membrane of rat hepatocytes.

The possibility exists that membrane acceptors for AdoHcy and its analogues may be a hitherto unrecognized target for these compounds. The interaction of AdoHcy with these acceptors may modulate membrane function or structure by mechanism(s) not involving AdoMet-dependent transmethylation reactions. Further studies are required to give an answer to this question. During the preparation of the manuscript, a paper on AdoHcy binding to membranes from rat cerebral cortex by Fonlupt *et al.* (8) appeared. The kinetic parameters of the binding show striking similarities with those of the AdoHcy binding to rat liver membranes. These data point to the possibility that membrane binding of AdoHcy is a general phenomenon.

ACKNOWLEDGMENTS

The authors wish to thank Ivar Bergesen (Lilly Laboratories), Dr. James Coward, and Dr. Edgar Lederer for generous gifts of AdoHcy analogues used in this work. The expert technical assistance of Halvard Bergesen and Audun Høylandskjær is highly appreciated. We also wish to thank Dr. Nils Erik Huseby for helpful discussions on the preparation of membranes.

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