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Binding of *S*-Adenosylhomocysteine to Various Domains of the Plasma Membrane and to the Endoplasmic Reticulum from Rat Liver: Relation between Binding and Phospholipid Methyltransferase Activity

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S-Adenosylhomocysteine (AdoHcy) binding to various membrane fractions of rat liver was determined at pH 7.4, using an oil centrifugation technique. The highest binding activity was found in the heavy microsomal (M-H) fraction enriched in endoplasmic reticulum, but high binding activity was also observed in the light microsomal fractions enriched in blood sinusoidal membranes (M-L fraction), and the heavy nuclear fraction (N-H fraction) containing the contiguous area. A substantial portion of AdoHcy binding activity in the M-L fraction may be ascribed to contamination of this fraction with endoplasmic reticulum, as indicated by the distribution of NADPH cytochrome *c* reductase activity. Binding activity was low in the light nuclear (N-L) fraction corresponding to the bile canaliculi. Phospholipid methyltransferase activity was determined in the same membrane fractions under similar conditions (pH 7.4), and in the absence and presence of added phospholipids. The distribution of the enzyme activity was dependent on the presence of exogenous phospholipids, and grossly similar to AdoHcy binding, the highest activities being observed in the M-H and the M-L fractions. The N-H fraction, rich in AdoHcy-binding activity, demonstrated, however, a very low phospholipid methyltransferase activity. It is concluded that AdoHcy-binding activity is not confined to the plasma membranes, and a major fraction of the binding activity resides on membranes derived from the endoplasmic reticulum. Also, the present results add to previous data suggesting that phospholipid methyltransferase does not totally account for the AdoHcy-binding sites on rat liver membranes.

The endogenous transmethylase inhibitor, *S*-adenosylhomocysteine (AdoHcy)[2] (1) binds to plasma membranes from rat liver (2, 3) and rat cerebral cortex (4). The binding of AdoHcy to rat hepatocytes and purified rat liver plasma membranes is characterized by saturability, reversibility, and specificity, and the kinetics are consistent with a heterogenous population of binding sites (3). The nature of these AdoHcy acceptors has not been established. Although the affinity for AdoHcy to the membrane sites is of the same order of magnitude as the inhibitor constant for AdoHcy of phospholipid methyltransferase, several lines of evidence suggest that this enzyme does not totally account for the AdoHcy-binding sites (3, 5). These observations, together with the recent reports suggesting that some 5′-alkylthionucleo-

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[2] Abbreviations used: AdoHcy, *S*-adenosylhomocysteine; M-L, microsomal light; M-H, microsomal heavy; N-L, nuclear light; N-H, nuclear heavy; Hepes, 4-(2-hydroxyethyl)-1-piperazineethane sulfonic acid.

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sides other than AdoHcy affect membrane transport and function (6, 7), warrant further investigation into the properties and functional role of membrane acceptors for AdoHcy and related compounds.

The present paper reports on the binding of AdoHcy to various domains of the rat liver plasma membrane (11) and endoplasmic reticulum. The binding activity is related to the distribution of marker enzyme activities for the subcellular fractions and the distribution of phospholipid methyltransferase activity.

MATERIALS AND METHODS

Chemicals. AdoHcy, L-α-phosphatidyl-*N*, *N*-dimethylethanolamine dipalmitoyl, L-α-phosphatidylcholine, adenosine deaminase (type I from calf intestinal mucosa), cytochrome *c* (type III), NADPH (type I or II), 5'-AMP, and 2'- and 3'-AMP were purchased from Sigma Chemical Company, (St. Louis, Mo.), and L-α-phosphatidyl-*N*-monomethylethanolamine dipalmitoyl was from Calbiochem-Boehring Corporation (La Jolla, Calif.). *S*-[8-^{14}C]Adenosylhomocysteine (59 mCi/nmol) was prepared according to a published procedure (8). *S*-Adenosylhomocysteine hydrolase was purified to apparent homogeneity from mouse liver as described (9). Glass-fiber filters (type GF/B) were from Whatman, UK.

Animals. Male Wistar rats, 150–200 g, from Möllegaard Hansens avlslaboratorier, Ejby, Denmark, were used. The animals were fasted overnight before the experiments.

Preparation of subcellular fractions. Two procedures were used:

1) Various membrane fractions from rat liver were prepared by homogenization of the livers in an isotonic sucrose medium, and fractionation of the liver homogenate into a nuclear (N) and microsomal (M) fraction, followed by subfractionation in a discontinuous sucrose gradient (10). The membrane fractions were collected at the interface between different layers, and designated microsomal light (M-L) fraction, microsomal heavy (M-H) fraction, nuclear light (N-L) fraction, and nuclear heavy (N-H) fraction, according to Wisher and Evans (11).

2) Plasma membranes from rat liver were prepared according to a method (12) involving homogenization of the livers in a hypotonic medium, followed by differential centrifugation, flotation of the membranes in a discontinuous sucrose gradient, and, finally, washing of the membranes (12).

Assay for binding of [^{14}C]AdoHcy. [^{14}C]AdoHcy (10 μM) was incubated with rat liver membranes at 0°C for 10 min in 20 mM Hepes, pH 7.4, containing 60 mM KCl, in a shaking water bath. The incubation was performed in the absence and presence of excess unlabeled AdoHcy (2.5 mM), to determine total [^{14}C]AdoHcy binding and nonspecific binding, respectively (3). The specific binding refers to total binding minus nonspecific binding.

Two procedures were evaluated for the separation of bound and free radioactive AdoHcy:

1) A sample (90 μl) from the incubation mixture was layered upon oil (2, 3) and centrifuged at 9000*g* for 45 s in 250-μl polyethylene tubes, using a Beckman 152 Microfuge. The oil was a mixture of dinoylphthalate:dibutylphthalate, 2:3 (v/v) for N-L and M-L fractions; 3:7 (v/v) for N-H and M-H fractions, respectively. The bottom of the tubes, containing packed membranes, was cut off, and the pellet was dissolved in 2% sodium dodecyl sulfate and counted by liquid scintillation as described (2, 3).

2) Samples from the incubation mixtures were applied to glass-fiber filters, and the membranes were collected on the filters using a Titertec cell harvester (Skatron A/S, Norway). The filters were washed with 0.5 ml of ice-cold incubation buffer, pH 7.4, and then placed in scintillation vials containing 2 ml of 2% sodium dodecyl sulfate. After shaking for 18 h at room temperature, 7 ml of scintillation fluid was added.

Assay for phospholipid methyltransferase. The assay is based upon the determination of [^{3}H]methyl-group incorporation into phospholipids in the presence of [^{3}H]AdoMet. The incubation mixture contained 1 mM $MgCl_2$, 50 μM [^{3}H]AdoMet, and membranes. To trap the AdoHcy formed, the incubation mixture was supplemented with AdoHcy hydrolase (2.6×10^{-4} units/ml) and adenosine deaminase (10 units/ml). When indicated, exogenous phospholipids (L-α-phosphophatidyl-*N*-monomethylethanolamine (1 mg/ml) and L-α-phosphatidyl-*N*, *N*-dimethylethanolamine (1 mg/ml) were added to the incubation mixture. Suspensions of phospholipids were prepared in the presence of Triton X-100, which was carried over into the incubation mixture at a final concentration of 0.015%. This concentration of Triton inhibited the enzyme activity by about 30%. The temperature was 37°C.

The reaction was terminated by the addition of 100-μl samples to 1ml of 1.2 M HCl, and the radioactive phospholipids were extracted and determined as described previously (5, 13).

Identification of radioactive phospholipids. A chloroform extract of membrane fraction incubated with [^{3}H]AdoMet was subjected to thin-layer chromatography on silica plates, as described previously (5, 13). In the absence of exogenous lipid substrates, the radioactivity was distributed among the three methylated phospholipids. Phosphatidyl-*N*-monomethylethanolamine was the most abundant species after a short incubation whereas most of the radioactive

phospholipids were identified as phosphatidyl-*N*, *N*-dimethylethanolamine and phosphatidylcholine after 10 min of incubation. In the presence of exogenous phospholipids most radioactivity was identified as phosphatidylcholine.

Assays for marker enzyme. NADPH cytochrome *c* reductase and 5'-nucleotidase activities were assayed by modifications of published methods (14-16).

NADPH cytochrome *c* reductase activity was measured as the reduction of cytochrome *c* by recording the absorbance at 550 nm using a Beckman 25 recording spectrophotometer. The assay mixture contained 0.1 mM cytochrome *c*, 0.2 mM NADPH, 13-150 μg protein/ml in 0.33 M potassium phosphate buffer, pH 7.6. The temperature was 30°C. 5'-Nucleotidase activity was determined as liberated phosphate from 5'-AMP. The liberation of phosphate from 2'- and 3'-AMP was subtracted as a correction for nonspecific phosphatase. The assay mixture contained 5 mM of either 5'-AMP or 2'-AMP and 3'-AMP, 10 mM KCl, 5 mM $MgCl_2$, 10 μg/ml of protein in 75 mM Tris-HCl buffer pH 9.0. Incubation time was 15 min and the temperature 37°C. The reaction was stopped by the addition of an equal volume of 0.66 M H_2SO_4, and phosphate was measured by a molybdate/malachite green method (17).

Determination of protein. This was performed by the method of Albro (18).

RESULTS

Evaluation of the Methods for Separation of Free and Bound Ligand

Published data (3) on the binding of AdoHcy to membranes of rat liver are based on an oil centrifugation method for the separation of free and bound ligand. We compared this method with a binding assay involving the retention of the membranes on glass fibers. The oil centrifugation technique gave values for the specific binding of AdoHcy which are about twice those obtained with the filtration method for all the membrane subfractions. This may be explained by the failure to detect AdoHcy binding corresponding to the high dissociation rate constant (3) by the filtration method.

The oil centrifugation method was further evaluated by measuring marker enzymes for plasma membrane (5'-nucleotidase) and endoplasmic (NADPH cytochrome *c* reductase) in the supernatant obtained by centrifugation of the incubation mixture. Only 10% of 5'-nucleotidase activity in the M-L fraction and 30% of NADPH cytochrome *c* reductase activity in the M-H fraction remained in the supernatant, indicating that the major part of the plasma membranes and endoplasmic reticulum was separated from the incubation medium during centrifugation.

The results reported below are based on the oil centrifugation technique.

Characterization of the Membrane Subfractions

The distribution of marker enzymes was similar to that observed in previous investigations (10, 11, 19). The results from a typical experiment is shown in Table I. The marker for the plasma membranes, 5'-

TABLE I

DISTRIBUTION AND RECOVERY OF MARKER ENZYMES IN VARIOUS MEMBRANE SUBFRACTIONS OF RAT LIVER[a]

Marker enzyme	Specific activity				Amount in fraction (%)				Recovery (%)	RSA			
	M-L	M-H	N-L	N-H	M-L	M-H	N-L	N-H		M-L	M-H	N-L	N-H
5'-Nucleotidase	450	67	370	13	38.9	17.0	14.2	10.7	116.0	18.5	2.3	23.6	2.3
NADPH cytochrome *c* reductase	110	510	20	13	9.4	33.9	0.1	1.3	96.8	4.5	4.4	0.2	0.3

[a] 5'-Nucleotidase and NADPH cytochrome *c* reductase activities were determined in membrane fractions from rat liver prepared according to Aronson and Touster (10). The enzyme activity is given in nmol/mg/min RSA, relative specific activity is the ratio of percentage of total activity in fraction/percentage of protein in fraction. The subfractions are named according to Wisher and Evans (11): M-L fraction, microsomal light fraction; M-H fraction, microsomal heavy fraction; N-L fraction, nuclear light fraction; and N-H fraction, nuclear heavy fraction.

nucleotidase, is mainly localized to the M-L (containing the blood sinusoidal membranes) and N-L fractions (enriched in bile canaliculi). The marker enzyme for the endoplasmic reticulum, cytochrome *c* reductase, was largely confined to the M-H fraction. A substantial amount of this enzyme activity was also recovered in the M-L fraction, whereas the N-L and N-H fractions showed low NADPH cytochrome *c* reductase activity. The recovery of these two marker enzymes was almost complete (Table I).

Binding of AdoHcy to Membranes

The binding of AdoHcy to homogenate and membrane fractions from rat liver was compared with the distribution of 5'-nucleotidase and NADPH cytochrome *c* reductase (Table II). The highest binding activity was observed in the M-H fraction, but significant activity was also seen in the fractions enriched in the blood sinusoidal membranes (M-L fraction) and contiguous area (N-H fraction). The membrane fraction (N-L) containing bile canaliculi showed low AdoHcy-binding activity. A substantial part of the AdoHcy-binding activity in the M-L fraction may be ascribed to endoplasmic reticulum because of a high relative specific activity of NADPH cytochrome *c* reductase in this fraction (Tables I and II). In contrast, NADPH cytochrome *c* reductase activity in the membrane fraction (N-H) enriched in the contiguous membrane area was low, but a high binding activity in this fraction was observed (Table II). Binding activity of membranes prepared in isotonic medium was similar to that of the M-L and N-H fractions.

Phospholipid Methyltransferase

The phospholipid methyltransferase activity was determined in the membrane subfractions under conditions similar to those used for the measurement of binding activity (pH 7.4). In the absence of exogenous phospholipids, the highest activity was found in the M-H fraction, intermediary activity in the fraction (M-L) containing blood sinusoidal membranes, and low activity in the N-L and N-H fractions. Supplementing the incubation mixture with phospholipids greatly enhanced (about 20-fold) the activity in the M-L fraction, but the activity in the M-H and N-L fractions was also stimulated markedly (about 10-fold). In contrast, only a twofold stimulation was observed with the N-H fraction and with plasma membranes isolated in hypotonic medium (Table II).

TABLE II

S-ADENOSYLHOMOCYSTEINE BINDING AND THE ACTIVITIES OF PHOSPHOLIPID METHYLTRANSFERASE AND SOME MARKER ENZYMES IN VARIOUS MEMBRANE FRACTIONS FROM RAT LIVER[a]

Fraction	*S*-Adenosylhomocysteine binding (pmol/mg protein)	Phospholipid methyltransferase (pmol/mg/min)		NADPH cytochrome *c* reductase (nmol/mg/min)	5'-Nucleotidase (nmol/mg/min)
		(−)	(+)		
Homogenate	9.8 ± 6.8	23.9 ± 9.9	42.2 ± 10.1	67.0 ± 26.0	32.0 ± 11.0
M-L	39.8 ± 21.2	8.8 ± 3.7	215.0 ± 88	138.7 ± 27.6	790.0 ± 413.0
M-H	92.4 ± 22.4	28.2 ± 10.7	287.0 ± 52	409.3 ± 149.1	142.0 ± 71.8
N-L	7.9 ± 4.9	3.6 ± 2.5	28.9 ± 8.4	20.0 ± 8.0	1050.0 ± 847.0
N-H	30.8 ± 4.2	2.5 ± 1.0	17.0 ± 5.9	9.0 ± 4.6	40.7 ± 32.9
Membranes prepared in isotonic medium	28.7 ± 10.2	9.9 ± 0.7	29.6 ± 7.4	61.0 ± 11	250.0 ± 80

[a] M-L, M-H, N-L, and N-H fractions were prepared [10] and named as ascribed in Table I. Values are means ± SD (n = 3). Phospholipid methyltransferase activity is measured in the absence (−) or presence (+) of exogneous phospholipid substrates.

DISCUSSION

The results presented in this report show that the AdoHcy-binding sites are not confined to the plasma membrane. High binding activities were demonstrated in the fractions enriched in endoplasmic reticulum (M-H fraction) and blood sinusoidal membranes (M-L fraction). These fractions also contained high phospholipid methyltransferase activity. However, in the N-H fraction high binding activity and low phospholipid methyltransferase activity were observed. This finding adds to previous data (3) suggesting that phospholipid methyltransferase does not totally account for the AdoHcy-binding activity of rat liver membranes.

The kinetic parameters of the AdoHcy binding to membranes from rat cerebral cortex resemble those of the binding to rat hepatocytes and membranes (3, 4). Based on studies with various AdoHcy analogs modified in the amino acid residue, it was concluded that the AdoHcy-binding sites of membranes from rat cerebral cortex have similar structural requirements as phospholipid methyltransferase (20). In contrast, there is disparity between the inhibitory effect of some AdoHcy analogs on AdoHcy binding to rat liver plasma membrane and phospholipid methyltransferase (3, 5). However, the results obtained with membranes from rat brain are based on measurement of AdoHcy binding by retention of membranes on glass-fiber filters (4, 20). With this method we were able to measure only one-half of the binding activity, probably because of the failure to detect the AdoHcy binding characterized by a high dissociation rate (3). Therefore, some differences in results may be related to different methods used for assessment of binding activity.

The highest binding activity for AdoHcy was demonstrated in the M-H fraction. Thus, the total amount of AdoHcy that might be bound to membrane structures in the liver *in vivo* may far exceed the amount bound to the outer surface of the plasma membrane (3). About 12 pmol/10^6 cells of AdoHcy are bound to intact rat hepatocytes, which is about 1/3 of the AdoHcy content of these cells (3, 13). This finding is in accordance with the hypothesis that a substantial fraction of intracellular AdoHcy is compartmentalized *in vivo* (3), and is not available as an inhibitor of methyltransfer reactions. However, the AdoHcy-binding sites might be occupied by other metabolites, for example, AdoMet, in the intact cells.

The observation that the AdoHcy-binding sites are not exclusively localized to the plasma membrane argues against the possibility that these sites represent AdoHcy receptors mediating effects of AdoHcy serving as an extracellular signal. More likely, the AdoHcy-binding sites are general constituents of membrane structure. These acceptors may be identical to the catalytic site(s) of AdoMet-dependent transmethylase(s). Alternatively, the AdoHcy-binding sites play a role in membrane function, not mediated by AdoMet-dependent transmethylation. Whether the high binding activity in membranes corresponding to the contiguous face (Table II) may be a clue to the functional role of these acceptors, remains to be established.

The binding of AdoHcy was determined at physiological pH (pH 7.4). For comparison, phospholipid methylation was determined at the same pH, even though the pH optimum for this enzymatic reaction has been reported to be in the alkaline range (21–23). The high pH optimum of this reaction *in vitro* may be explained by changes in the lipid structure induced by alkaline pH (24).

Data presented in this paper also provide some information on the distribution of phospholipid methyltransferase in the rat liver membrane fraction. The interpretation of these data, however, is difficult in the light of the divergent opinions (22, 23) on the properties of phospholipid methyltransferase(s).

It has been reported that the stepwise methylation of phosphatidylethanolamine to phosphatidylcholine is carried out by two separate enzymes, designated methyltransferase I and II. The former enzyme is responsible for the incorporation of the first methyl group, whereas the latter catalyzes the incorporation of two methyl

groups into phosphatidylethanolamine to form a final product, phosphatidylcholine. These hypothetical enzymes seem to be distinguishable on the basis of pH optimum, ionic requirements, K_m for AdoMet, and association with membranes (21, 22). However, Schneider and Vance (23) partially purified phospholipid methyltransferase from rat liver microsomes and reported that both enzyme activities seem to reside on the same protein molecule. The existence of one enzyme catalyzing the formation of phosphatidylcholine from phosphatidylethanolamine has also been suggested by others (25, 26).

We have observed that phospholipid methylation was stimulated by the exogenous phospholipids to different degrees in different membrane subfractions (Table II), an effect which was suggested by the finding of Schneider and Vance (23) in rat liver microsome, and Colard and Breton (27) in rat liver plasma membrane. This may be explained by the partial solubilization of one methyltransferase during preparation of the fractions (28). Alternatively, the phospholipid composition of various membrane subfractions varies (29), and this may result in different availabilities of endogenous substrates for the enzyme. Thus, the existence of one or two phospholipid methyltransferase(s) is a question which is critical for the interpretation of data obtained with membrane fractions. Further studies on this subject should await detailed knowledge of the properties of phospholipid methyltransferases.

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