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EVIDENCE FOR THE EXISTENCE OF PROTEIN INHIBITORS FOR S-ADENOSYL-L-METHIONINE-MEDIATED METHYLATION OF PHOSPHATIDYLETHANOLAMINE IN RAT LIVER CYTOSOL

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SUMMARY. The effect of rat liver cytosol on phosphatidylcholine biosynthesis via the N-methylation of phosphatidylethanolamine has been studied. The purified rat liver plasma membrane was used as an enzyme source. The rat liver 105,000 x g supernatant(cytosol) contained two different inhibitors for S-adenosyl-L-methionine-mediated methylation of phosphatidylethanolamine to phophatidyl-N-mono-methylethanolamine. The inhibitors were inactivated by pretreatment with trypsin or heating at 96°C, but were not inactivated by RNase. The inhibitors did not inhibit the synthesis of phosphatidylcholine from phosphatidyl-N-mono-methylethanolamine. The results indicate that two different protein inhibitors for methylation are present in rat liver cytosol fraction and that the inhibition by these proteins may have a role for the regulation of phosphatidylcholine synthesis in the plasma membrane.

Phosphatidylcholine is synthesized via the CDP-choline pathway or via the methylation of phosphatidylethanolamine(1,2). The methylation pathway has important roles for biological actions of cell, such as a maintenance of membrane fluidity(3), regulation of the number of beta-adrenergic receptors(4), regulation of human growth hormone binding to the membranes(5), Ca^{2+}-ATPase activity(6), monocyte chemotaxis(7), lymphocyte mitogenesis(8), and mast cell histamine release(9). The synthesis of phosphatidylcholine via the methylation pathway is known to be regulated by several small molecules, such as S-adenosyl-L-homocysteine and S-3-deazaadenosyl-L-homocysteine (10). These small molecules, which can inhibit N-methylation of phosphatidylethanolamine, are transformed from 3-deazaadenosine which is

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administered to experimental animals(10). These substances, however, enhance the synthesis of phosphatidylcholine by activation of CTP:phosphocholine cytidyltransferase which is known to be a rate-limiting step for phosphatidylcholine synthesis via the CDP-choline pathway(11). And it is postulated that there is a coordinate regulation of phosphatidylcholine biosynthesis via the CDP-choline pathway and N-methylation pathway.

Although the coordinate regulation must be important to maintain the biological activity of cell, the endogenous regulator for synthesis of phosphatidylcholine has not been isolated. In this study, we examined whether the endogenous regulator for the N-methylation exists or not in rat liver.

MATERIALS AND METHODS

Materials: S-adenosyl-L-[methyl-^{3}H]methionine(14 Ci/mmol) was purchased from New England Nuclear(Boston MA). Ribonuclease type II-A, trypsin type I, trypsin inhibitor and phosphatidyl-N-mono-methylethanolamine were purchased from Sigma Chemical Co.(St.Louis,MO).

Preparation of liver plasma membrane: Male Wistar rats, weighing 50 - 75 g, were killed by decapitation. The liver was preperfused with ice cold saline for 10 min. The tissue was rapidly removed, cooled and weighed. All subsequent steps were performed at 4°C. The tissue was homogenized in 20 mM Tris-Cl pH 7.5, containing 0.25 M sucrose, 0.5 mM EGTA and 0.5 mM $MgCl_2$. The homogenate was centrifuged at 150 x g for 10 min, and the supernatant was recentrifuged at 2,000 x g for 30 min. The precipitate was dissolved with the same buffer. The plasma membrane was purified by centrifugation through a discontinuous sucrose density gradient(9 ml 45.8%, 9 ml 37.0%, 8 ml 25.0%(weight/volume), and 0.25 M sucrose). The tubes were centrifuged at 24,000 rpm for 120 min at 4°C using sw 25.1 rotor(Hitachi, Tokyo,Japan). The fraction that appeared at the interface of 37.0%/25.0% sucrose was collected. This plasma membrane fraction was diluted in equal volume of 50 mM Tris-Cl pH 7.5, containing 0.5 mM EGTA and was centrifuged at 105,000 x g for 60 min. The final precipitate was suspended with the same buffer and was used as a plasma membrane fraction. Protein concentration was measured by the method of Lowry et al.(12).

Preparation of N-methyltransferase inhibitors: Rat liver cytosol, prepared by centrifugation of homogenate at 105,000 x g, was used as one of the sources of methyltransferase inhibitors. Gel filtration of inhibitors was carried out on a column of Sephacryl S-200 (Pharmacia), 60 x 2.0 cm, and the inhibitors were eluted with 50 mM sodium acetate buffer pH 6.5, containing 10 mM $MgCl_2$ and 0.1 mM EGTA.

Enzyme assays: The methylation of phosphatidylethanolamine to phosphatidyl-N-mono-methylethanolamine was assayed by measuring incorporation of methyl group from S-adenosyl-L-[methyl-^{3}H]methionine into

phospholipids in the membrane(13). The assay medium contained 4 uM S-adenosyl-L-[methyl-^{3}H]methionine, 10 mM $MgCl_2$, 0.1 mM EGTA, 50 mM sodium acetate buffer(pH 6.5) and plasma membrane fraction(0.1 mg of protein) in a total volume of 300 ul. The reaction was performed at 37°C for 30 min. The reaction was terminated by adding 3 ml of chloroform/methanol/hydrochloric acid(2/1/0.02, vol/vol). After the addition of 2 ml of 0.1 M KCl in 50% methanol, the tubes were vigorously shaken and centrifuged at 2,000 x g for 10 min. The aqueous phase was aspirated, and the chloroform phase was washed with 2 ml of 0.1 M KCl in 50% methanol. 1 ml of the final chloroform phase was transfered to a vial. After the solvent was evaporated, 10 ml of scintilation liquid(0.4% 2,5-diphenyloxazol in toluene and Triton X-100 (2:1)) was added, and radioactivity was measured.

The methylation of phosphatidyl-N-mono-methylethanolamine to phosphatidylcholine was assayed with 50 mM sodium borate buffer(pH 9.0), 1 uM S-adenosyl-L-[methyl-^{3}H]methionine and 100 ug of phosphatidyl-N-mono-methylethanolamine by the procedure described above.

RESULTS

Prasad and Edwards(13) reported that optimal pH for the methylation of phosphatidylethanolamine to phosphatidyl-N-mono-methylethanolamine is 6.5 and that of phosphatidyl-N-mono-methylethanolamine to phosphatidyl-di-methylethanolamine or phosphatidylcholine is 9.0. In our studies, the presence of two pH optima for phospholipid methylation was observed. At pH 6.5, methylation of phosphtidyl-N-mono-methylethanolamine was not observed and the methylation of it was activated at pH 9.0. On the other hand, the methylation of phosphatidylethanolamine was observed at pH 6.5, but the methylation of it was not observed at pH 9.0(Fig.1). The presence of two pH optima was also observed

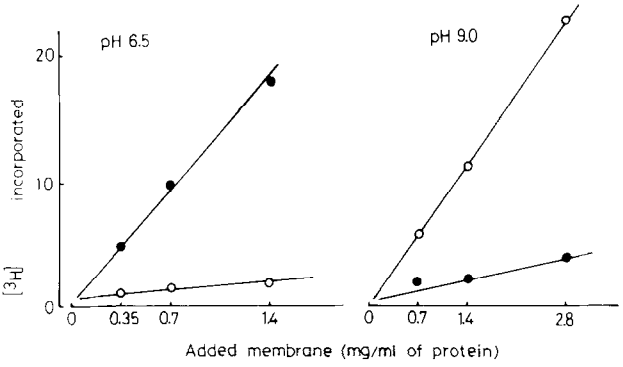


Fig.1 Effects of pHs on S-adenosyl-L-methionine-mediated N-methylation of phosphatidylethanolamine or phosphatidyl-N-mono-methylethanolamine. —●—;methylation of phosphatidylethanolamine, —o—;methylation of phosphatidyl-N-mono-methylethanolamine. [methyl-^{3}H]-incorporated(cpm x 10^{-3}) are indicated.

in other tissues, such as adrenal medulla(14), pituitary(13) and erythrocyte(14,15).

The amount of [^{3}H]methyl radioactivity incorporated into phospholipids was significantly inhibited in the presence of rat liver cytosol fraction at pH 6.5. At pH 9.0, however, the cytosol did not inhibit [^{3}H]methyl incorporation into phosphatidyl-N-mono-methylethanolamine. Microsomal or mitochondrial fractions also inhibited methylation at pH 6.5, but their inhibitory activity was not greater than that of cytosol(Table 1).

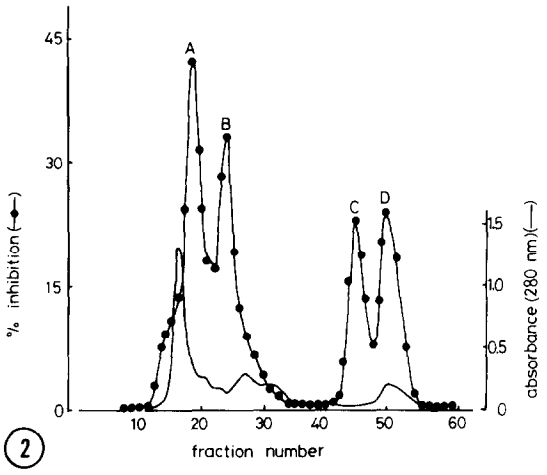
In studies with gel filtration of cytosolic inhibitors, two main inhibitory activities were separated. One of them was eluted as two different peaks just after the void volume of the column. Another inhibitory activity was eluted as two different peaks near the elution position of salt(Fig.2). The latters were not inactivated by heating at 96°C. However, the inhibitory activity of the formers was inactivated by heating at 96°C for 10 min. Moreover, these inhibitory activities were also inactivated by preincubation at 34°C for 120 min(Fig. 3). The inhibitory activity of these inhibitors was inactivated by preincubation with trypsin but not inactivated by RNase(Table 2). The results indicate that these big inhibitory modulators for phospholipid methylation contain a structure as that of protein.

The inhibitory activity of these proteins was related to the protein concentration in the methylation of phosphatidylethanolamine. How-

Table 1. Effect of cytosol, microsomal and mitochondrial fractions on the methylation of phosphatidylethanolamine.

Added fraction(50 μl)	[^{3}H] incorporated(cpm)
Buffer	22,450
Cytosol (40 μg of protein)	2,670
Microsomal(40 μg of protein)	19,770
Mitochondrial(40 μg of protein)	20,200

Each datum indicates the mean of five determinations.



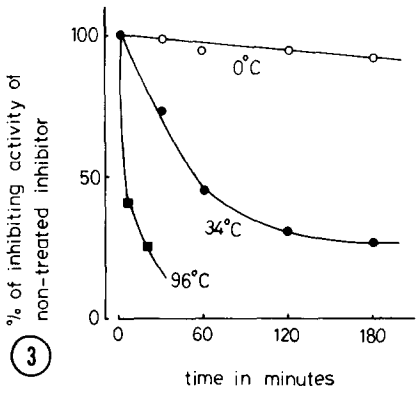


Fig.2 Gel filtration profile of cytosolic inhibitors for methylation of phosphatidylethanolamine. Cytosol fraction was applied onto a Sephacryl S-200 column and inhibitors were eluted. The inhibitory activity in each fraction was assayed.

Fig.3 Inactivation of the inhibitory activity of the inhibitor during the incubation. Inhibitor B, which was obtained by gel filtration study(Fig.2), was used as an inhibitor. The inhibitor protein(100 ug) was preincubated at 0°C, 34°C and 96°C for appropriate times. After the preincubation the inhibitory activity was measured.

ever, S-adenosyl-L-methionine-mediated methylation of phosphatidyl-N-mono-methylethanolamine was not inhibited by these proteins(Fig.4).

DISCUSSION

We have demonstrated that several inhibitors for S-adenosyl-L-methionine-mediated N-methylation of phosphatidylethanolamine are present

Table 2. Effect of pretreatment of inhibitor B with trypsin or RNase on its inhibitory activity.

Pretreatment(30 min)	% inhibition
Incubation at 0°C	100.0
Incubation at 34°C	22.4
Incubation at 34°C in the presence of 100 μg/ml of trypsin	98.9
Incubation at 34°C in the presence of 40 μg/ml of RNase	23.6

Each datum indicates the mean of triplicate determinations. Inhibitor B was obtained by gel filtration study(Fig.2). The methylation was observed at pH 6.5.

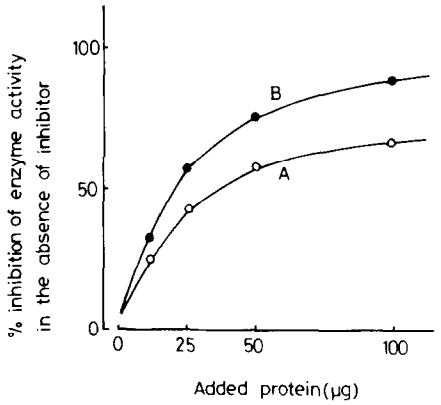


Fig.4 Inhibitory activity of inhibitors A and B on the methylation of phosphatidylethanolamine. Inhibitors A and B were obtained from a gel filtration study(Fig.2).

in rat liver cytosol fraction. One of them, which is separated as two different peaks in gel filtration study, is heat-stable and relatively small in molecular size. These small molecule inhibitors enhance the synthesis of phosphatidylcholine from [^{3}H]-choline(Data not shown). Based on these data, these small molecules, as suggested by Pritchard et al.(10), may have an important role for the coordinate regulation of phosphatidylcholine synthesis via CDP-choline pathway and via N-methylation pathway.

On the other hand, we found two different protein inhibitors for N-methylation in rat liver cytosol fraction. The substances may also have a role for the regulation of phosphatidylcholine synthesis via N-methylation pathway. These inhibitory proteins have no effect on the methylation of phosphatidyl-N-mono-methylethanolamine, suggesting that the proteins may bind to the inner face of the plasma membrane and modify the synthesis of phosphatidyl-N-mono-methylethanolamine, since the enzyme, which catalyzes synthesis of phosphatidyl-N-mono-methylethanolamine from phosphatidylethanolamine, localized to the inner face of the plasma membrane and the phosphatidylcholine synthesis from phosphatidyl-N-mono-methylethanolamine is held in outer face of the membrane(14). The inhibitory activity of these protein inhibitors was inactivated during incubation of these proteins at 34°C for 120

min. indicates that these inhibitory activities may be unstable in physiological condition.

While the role of protein inhibitors for methylation of phospholipids is no doubt important, the actual role in the cell function remains to be elucidated.

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