Effect of phospholipids on purified lipoamidase

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Summary. Phospholipids were added to purified lipoamidase from porcine brain microsomal membranes, and changes in lipoamidase activity were examined. Approximately twofold activation of lipoamidase activity occurred upon the addition of phosphatidylethanolamine. On the other hand, phosphosphatidylserine, cardiolipin, and phosphatidic acid reduced the enzyme activity by approximately 80%. This pattern of the activation of lipoamidase by phosphatidylethanolamine and its inhibition by phosphatidylserine is similar to the pattern for adenylate cyclase, and contrasts with the pattern for ATPase.
Key words. Porcine brain lipoamidase; activation and inhibition by phospholipids; phosphatidylethanolamine; phosphatidylserine; phospholipase A2; microsome.

The enzymic activity of integral membrane enzymes is usually activated or inhibited by phospholipids[1,2]. The reconstitution method is usually employed in order to clarify the role of phospholipids in the activity of membrane enzymes. The reconstitution of vesicles from protein and phospholipids has been performed by many researchers[3–6]. Various reconstitution methods have been described, such as sonic oscillation[3,6], shaking by hand[4], and dilution[5]; however, an optimum method has not yet been established.

Lipoamidase was recently recognized as a membrane enzyme, e.g. in guinea pig liver[7], and in porcine brain (submitted for publication), and purification of lipoamidase has also been achieved. In this paper, we studied the effect of various phospholipids on the isolated porcine brain enzyme by means of the sonic oscillation method.

Materials and methods

Chemicals and reagents. The following phospholipids were obtained from Sigma: L-alpha-phosphatidylethanolamine (L-alpha-cephalin, Type I) from bovine brain (P-9137), L-alpha-phosphatidylethanolamine (L-alpha-cephalin, Type II-S) from sheep brain (P-4264), cardiolipin from *Escherichia coli* (C-9138), L-alpha-phosphatidic acid from egg yolk lecithin (P-9511), L-alpha-phosphatidylcholine (L-alpha-lecithin, Type III-B) from bovine brain (P-6638), L-alpha-phosphatidyl-L-serine from bovine brain (P-6641). Crude egg-yolk lecithin (content of phosphatidylcholine; approximately 60%), Nonidet P-40 (NP-40), 2-mercaptoethanol (ME), trifluoroacetic acid (TFA, amino acid sequencing grade), methanol (HPLC grade), chloroform (HPLC grade), acetone (HPLC grade), and ethylether were obtained from Nacalai Tesque Co., Kyoto, Japan. Phospholipase A2 (phosphatide 2-acyl-hydrolase, EC 3.1.1.4) from venom of *Trimeresurum flavoviridis* (Habu snake) came from Takara Shuzo Co., Kyoto, Japan. Phospholipase C (phosphatidylcholine choline phosphohydrolase, EC 3.1.4.3) from *Bacillus cereus* and phospholipase D (phosphatidylcholine phosphatido hydrolase, EC 3.1.4.4) from cabbage came from Boehringer-Mannheim Yamanouchi Co., Tokyo, Japan.

Preparation of microsomes from porcine brain. Microsomes were prepared from porcine brain (Lot # 29410, Pel-Freez Biologicals, Rogers, Arkansas, USA) by differential centrifugation, essentially according to the method described by Albers et al.[8]. Brain tissue (80 g) was homogenized in a Potter-Elvehjem homogenizer using 800 ml of a 0.32 M solution of sucrose (pH 7.0) containing 1 mM sodium phosphate buffer and 1 mM EDTA. Differential centrifugation was carried out to obtain nuclear, mitochondrial, microsomal, and soluble fractions. Microsomes were obtained by ultracentrifugation in 0.32 M sucrose for 90 min at 105,000 × g (Beckman L7 Ultracentrifuge, Type 35 Rotor).

Purification of porcine brain lipoamidase from microsomes. Purification of porcine brain lipoamidase was carried out recently by the authors, who obtained a purification factor of 601-fold, and a single glycoprotein band of Mr = 140,000 (submitted for publication). In outline, purification was performed as follows. Brain microsomes (10.5 mg of protein/ml, 100 ml from 80 g of porcine brain) were dispersed in 0.5% NP-40 solution containing 5% glycerol, 5% acetone, 1 mM ME, 1 mM EDTA, and 50 mM sodium phosphate (pH 6.0). Then, dispersed membrane proteins were processed by adsorption on CM-cellulose, DEAE-Sephacel, Sephadex G-200, Sepharose CL-4B, wheat germ agglutinin agarose (Vector Lab. Inc., Burlingame, CA, USA), and finally a serotonin-HPLC column (Honen Corporation, Tokyo, Japan). Purified lipoyl-X hydrolase had a specific activity of 8.5 nmol/min per mg of protein as assayed by lipoyl 4-aminobenzoic acid (LPAB).

Enzyme assay. Lipoamidase was determined by measuring the product of *p*-aminobenzoate (PAB) from the synthetic substrate LPAB by the HPLC-fluorometric method[9]. The substrate LPAB was synthesized as described recently[10]. The reaction mixture contained, per 0.1 ml:

sodium phosphate buffer, pH 7.0, 10 μmol; LPAB, 0.2 μmol; EDTA, 0.1 μmol; ME, 1.0 μmol; enzyme, 0.56 μg (40 pmol; calculated using Mr = 140,000), or microsomes, 48 μg of protein. After incubation at 37 °C for 1 h, 0.2 ml of methanol was added to stop the reaction. A portion of supernatant (0.01 ml), obtained by centrifugation at 1500 × g for 15 min, was injected into the HPLC system.

Proteoliposomes with purified enzyme and phospholipid. Proteoliposome preparation was performed according to the procedure described previously[6]. Phospholipids were dissolved in a chloroform solution (10 mg/ml) and stored at − 80 °C. LPAB (0.2 μmol) and phospholipid (usually 1 mg) were dried under a stream of nitrogen gas in a test tube (12 × 75 mm I.D.). Then, isolated lipoamidase (0.56 μg) and 0.1 ml of sodium phosphate buffer (pH 7.0) containing 1 mM EDTA, 0.1 mM calcium chloride, 10 mM ME, and 5% glycerol were mixed and sonicated at 37 °C for 3 min under the nitrogen gas. The prepared mixture was sealed with aluminium foil, and the reaction was started by incubation at 37 °C. Liberated PAB was assayed as described above.

Protein content. Protein concentration was determined by Lowry's method[11].

Results and discussion

Isolated microsomal membranes were treated with phospholipase A2, phospholipase C, and phospholipase D. The results are shown in the table. Phospholipase A2 from snake venom exhibited approximately 40% inhibition of native microsomal enzyme activity. However, phospholipases C and D had no effect on enzyme activity. A similar hydrolase enzyme, biotinidase, which cleaves biotinyl 4-aminobenzoate to biotin and PAB, was also incubated with phospholipase A2. No inhibition of biotinidase was detected. Phospholipid hydrolyzed by phospholipase A2 may induce the inhibition of enzyme activity.

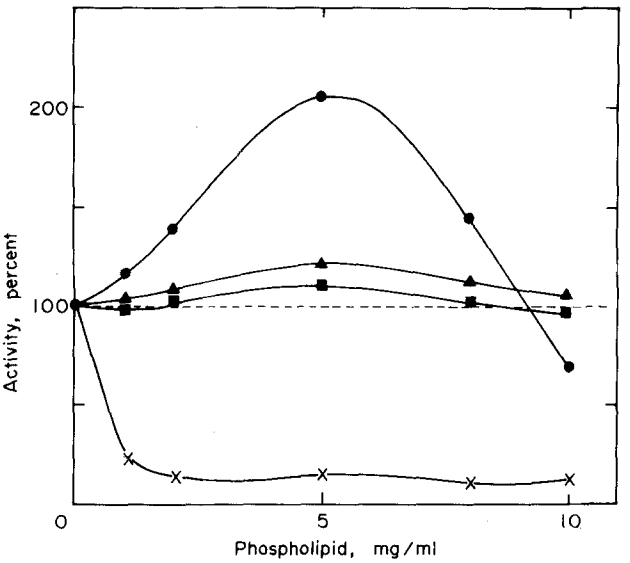
In order to elucidate the effect of particular phospholipids on lipoamidase, we purified the enzyme as described in 'Materials and methods'. Lipoamidase (0.56 μg) was added to phospholipids by the sonic oscillation method, as previously described[6]. The results are shown in the figure. Phosphatidylethanolamine from bovine brain activated the enzyme activity 2.1-fold (CV = 8%) at an optimum phospholipid concentration of 5 mg/ml. Higher concentrations of phosphatidylethanolamine were slightly inhibitory. On the other hand, phosphatidylserine inhibited lipoamidase (fig.), and cardiolipin and phosphatidic acid also inhibited the enzyme activity. Either the acidic or the anionic moiety of phospholipid seems to be inhibitory to lipoamidase.

The activation effect that phosphatidylethanolamine was found to have on lipoamidase was similar to that found recently by Hebdon et al.[5] on rat brain adenylate cyclase.

Effect of phospholipase treatment on microsomal porcine brain lipoamidase activity*

Phospholipase	Residual activity (%)
Without	100
Phospholipase A2	58
Phospholipase C	98
Phospholipase D	97

*Microsomes (10.5 mg/ml, 0.02 ml) were treated for 2 h at 37 °C by the addition of phospholipase (dissolved at 1 mg/ml, 0.02 ml) at pH 7.0. A portion of 0.02 ml of treated microsomes was added to 0.08 ml of substrate solution containing LPAB. Controls without phospholipase were also run and taken as 100% activity. Control activity was 63.0 pmol/min per mg of protein. Phospholipase alone did not hydrolyze the substrate of LPAB.



Effects of several phospholipids on purified lipoamidase activity. Purified lipoamidase was added to phospholipids according to the method described in the Materials and methods section. Circles: phosphatidylethanolamine. Triangles: crude egg-yolk lecithin. Squares: phosphatidylcholine. Crosses: phosphatidylserine. Each point is the average of three determinations.

They found that phosphatidylethanolamine specifically activated adenylate cyclase[5]. On the other hand, phosphatidylserine, phosphatidylinositol, phosphatidylglycerol, and phosphatidic acid were not effective, and inhibited the activation effect of phosphatidylethanolamine[5].

Our finding is in contrast to the results for ATPases; i.e. Mg-ATPase from the procaryotic microbe *Acholeplasma laidlawii*[4] was activated by phosphatidylgylcerol, phosphatidylserine, or phosphatidic acid. Na-K-ATPase from the rabbit kidney outer medulla[12] was also activated by phosphatidylserine and phosphatidylinositol.

Phospholipids may play a regulatory role in lipoamidase activity in the porcine brain that is similar to that on adenylate cyclase of the rat brain in vivo.

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Inhibition of in vitro RNA synthesis by hycanthone, oxamniquine and praziquantel

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Summary. The schistosomicides, hycanthone, oxamniquine and praziquantel, were found to inhibit the in vitro RNA synthesis using isolated hamster liver nuclei. Preincubation of the nuclei with these drugs revealed that the inhibitory effect of oxamniquine was irreversible and progressed with time, whereas that of hycanthone and parziquantel was reversible. On the other hand, hycanthone and praziquantel have a high affinity for DNA but oxamniquine does not. The data indicate that the mechanism of inhibition by oxamniquine is different from that of hycanthone and praziquantel.
Key words. Schistosomicides; hycanthone; oxamniquine; praziquantel; RNA synthesis; drug resistance.

Hycanthone, oxamniquine and praziquantel have been used as therapeutic drugs against schistosomiasis, although the use of hycanthone has recently been discontinued[1,2]. The rapid absorption and distribution of the drugs throughout all tissues contributed to the extremely fast onset of drug action against the three important strains of human schistosomes, *S. hematobium*, *S. mansoni* and *S. japonicum*[3,4]. A single intramuscular or oral dose is generally effective[2]. However, continued usage of hycanthone and oxamniquine has been found to induce drug resistance in *S. mansoni*[5,6].

Despite the routine use of these drugs for clinical treatment, none of them are without side effects. Symptoms like coughing, headaches, vomiting, nausea, anorexia, gastrointestinal disturbances, skin rashes, arthralgia and myalgia are common[2]. Hycanthone, for example, is a hepatotoxic drug which causes severe hepatic injury[1]. In addition to these toxic side effects, the antischistosomal agents are also carcinogenic and mutagenic[7,8]. The mutagenicity of hycanthone was detected in Salmonella, Drosophila, *E. coli* T4 bacteriophage and various cell cultures[9–11]. Oxamniquine, on the other hand, produced a weak response in the frame-shift mutant TA1538 of *Salmonella typhimurium*[11].

The mechanism of action of hycanthone against *S. mansoni* has been investigated. Mattocci et al.[12,13] reported that hycanthone inhibited the macromolecular synthesis of *S. mansoni*, both in vitro and in vivo. We[14] have found enzymatic differences between hycanthone resistant and sensitive strains of *S. mansoni*, suggesting the important effect of hycanthone in altering gene expression. In this paper we report the inhibition of hycanthone on RNA synthesis in a mammalian system and compare its effects to that of oxamniquine and praziquantel.

Materials and methods

Materials. Calf thymus DNA, CTP, GTP, UTP, ATP, dithiothreitol and sucrose were purchased from Sigma Chemical Co. [5,6-^{3}H]UTP (36 Ci/mmole) was from ICN Chemical Co. Hycanthone was generous gift of Dr. Ernest Bueding, Johns Hopkins University. Oxamniquine was obtained from Pfizer Pharmaceuticals Company and praziquantel from Miles Pharmaceuticals. Hamster liver nuclei were isolated according to the modified procedure of Ernest et al.[15]. All other chemicals used were of reagent grade.

In vitro RNA synthesis. The procedure used for in vitro RNA synthesis was that of Reeder and Roeder[16] and Marzluff et al.[17]. The reaction mixture contained 25 mM tris-chloride, pH 7.6, 0.25 M sucrose, 0.25 mM calcium acetate, 5 mM $MgCl_2$, 1 mM $MnCl_2$, 0.05 mM EDTA, 2.5 mM dithiothreitol, 0.15 M KCl, 0.4 mM of each of ATP, CTP and GTP, 0.05 mM [5,6-^{3}H]UTP and about 10^7 isolated liver nuclei. After incubation at ambient temperature for 30 min, the reaction was stopped by adding equal volume of 20% trichloroacetic acid. An