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PHOSPHOLIPID METHYLTRANSFERASE PHOSPHORYLATION BY INTACT HEPATOCYTES: EFFECT OF GLUCAGON

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We have obtained a rabbit antiserum that specifically immunoprecipitates the 50K and 25K proteins of rat liver phospholipid methyltransferase. Exposure of intact rat hepatocytes preincubated with [32P]phosphate to glucagon induces a time-dependent phosphorylation of the 50K protein of phospholipid methyltransferase. The incorporation of ^{32}P into the 50K protein was only on phosphoserine. These data support the concept that the activation of rat liver phospholipid methyltransferase by glucagon is mediated by phosphorylation of the enzyme.

Phospholipid methyltransferase, the enzyme that converts phosphatidylethanolamine into phosphatidylcholine being S-adenosylmethionine the methyl donor (1,2), is activated in a variety of mammalian cells by signals which mediate their effects through binding to specific receptors (reviewed in 3,4). Whereas changes in phospholipid methylation have been related to a variety of biological functions, the biochemical events leading to activation of phospholipid methyltransferase during signal transduction are largely unknown (3,4). In isolated rat hepatocytes, conditions which are known to elevate the intracellular cyclic AMP content produce a fast activation of phospholipid methyltransferase (5-7). Phospholipid methylation by isolated rat liver microsomes can be activated by treatment with cyclic AMP plus ATP (8,9) or Ca^{2+} + ATP (10). Microsomal rat liver phospholipid methyltransferase has been solubilized with

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Chaps and partially purified to obtain a preparation that when analyzed by SDS-polyacrylamide gel electrophoresis is mainly composed of two proteins with Mr of respectively 50K and 25K (11,12). Phospholipid methyltransferase activity of the purified enzyme is activated by the addition of ATP in the presence of the catalytic subunit of the cAMP-dependent protein kinase (12). Under these conditions only the 50K protein of the methyltransferase is phosphorylated (12). High voltage electrophorsis of an acidic hydrolysate prepared from the ^{32}P-phosphorylated enzyme showed only phosphoserine (12). The present results indicate that in intact rat hepatocytes glucagon stimulates the phosphorylation of serine residues of the 50K protein of phospholipid methyltransferase.

MATERIALS AND METHODS

Materials. CM-Affi Gel Blue, reagents for electrophoresis and proteins markers were from Bio-Rad. CNBr-activated Sepharose 4B was purchased from Pharmacia Fine Chemicals. Triton X 100 and TLC plastic cellulose sheets were from Merck. Sodium deoxycholate, phenylmethanesulphonyl fluoride, benzamidine, leupeptine, nitrophenyl-4-phosphate and phosphoaminoacid markers were from Sigma. Glucagon was from Novo.

Methods. Rat liver phospholipid methyltransferase was purified as previously described (11,12). Anti-phospholipid methyltransferase serum was obtained in rabbits by i.m. injection of the purified enzyme (≃ 200 µg) once a week during six weeks. At the end of this period the rabbits were i.m. injected with the acetone powder of the purified enzyme (≃ 200 µg) once a week during two weeks. The gamma fraction of the antiserum was then purified by chromatography on CM-Affi Gel Blue (Bio-Rad bulletin 1061) and ammonium sulfate precipitation. This antibody recognizes the 50K and 25K proteins of rat liver phospholipid methyltransferase, using immunoblotting techniques (13). The antibody was then coupled to CNBr-activated Sepharose 4B as described by the manufactures (Pharmacia Fine Chemicals) and used to immunoprecipitate lysates of rat hepatocytes.

Rat hepatocytes were isolated from normally fed Wistar rats (250 to 300 g) and incubated 1h, in the presence of 0.1 mM [^{32}P]-phosphate (0.2 mCi/ml, Amersham/Searle) (14), as previously described (5). Additions of glucagon (dissolved in 0.9% NaCl containing 1 mg/100 ml BSA), or an equal volume of the same solution were made directly to the cell suspension. Cells (1 ml containing about 20 mg wet weight/ml) were lysed by addition of 4 ml cold acetone and incubated at -20°C for 20 min. After

centrifugation, the dried pellet was treated with 0.5 ml of a mixture of detergents (1% Triton X 100, 0.1% sodium deoxycholate and 0.1% SDS) containing 10 mM Tris-HCl buffer, pH 7.0, 20 mM sucrose, 150 mM KF, 15 mM EDTA, 2 mM EGTA, 50 mM beta-mercaptoethanol, 1 mM phenylmethanesulphonyl fluoride, 1 mM benzamidine, 50 µg/ml leupeptine and 1 mM nitrophenil-4-phosphate. The mixture was clarified at 10,000 x g for 2 min. The antibody bound to Sepharose was added (150 µl of Sepharose beads). After 20 min at 4° C the immunoprecipitates were centrifuged and washed three times in a buffer containing 50 mM Hepes pH 7.0, KF 100 mM and EGTA 15 mM, once in NaCl 1.5 M in the same buffer, and once in 0.1% SDS. After washing, the immunoprecipitates were treated with 5% TCA centrifuged and dissociated by incubation at 100°C for 2 min in 2% SDS and 100 µl of each sample analysed by SDS-polyacrylamide gel electrophoresis (10% acrylamide) (15). Gels were stained with Coomassie Blue R to visualize the markers and dried. Phosphoproteins were localyzed by autoradiography, using a Kodak XOmat-GRS film and intensifier screens, at - 70 °C during 6 days. Autoradiograms were quantitated by densitometry using a Beckman DU-8 spectrophotometer.

For the analysis of phosphoaminoacids, after staining the gel weakly, the piece of gel containing the 50K protein was cut and subjected to acid hydrolysis in 6N HCl during 2h at 110°C. After hydrolysis, phosphoaminoacids were separated by high voltage electrophoresis on cellulose plates by the procedure of Hunter and Sefton (16) as previously described (12). Phosphoaminoacids were localyzed by autoradiography using intensifier screens at - 70°C during 6 days.

RESULTS AND DISCUSSION

We have obtained a rabbit antiserum specific for rat liver phospholipid methyltransferase. The gamma fraction of this antiserum recognizes the 50K and the 25K proteins of phospholipid methyltransferase, using immunoblotting techniques. This gamma fraction was coupled to Sepharose beads and used to immunoprecipitate detergent-solublized acetone pellets obtained from isolated rat hepatocytes. Analysis by SDS-polyacrylamide gel electrophoresis of the immunoprecipitate reveals two main proteins with Mr of respectively 50K and 25K (Fig.1).

Isolated rat hepatocytes were preincubated with [^{32}P]phosphate and exposed to 0.001 µM glucagon for 10 min before homogenization. The 50K protein of phospholipid methyltransferase was then purified by immunoprecipitation, with the specific

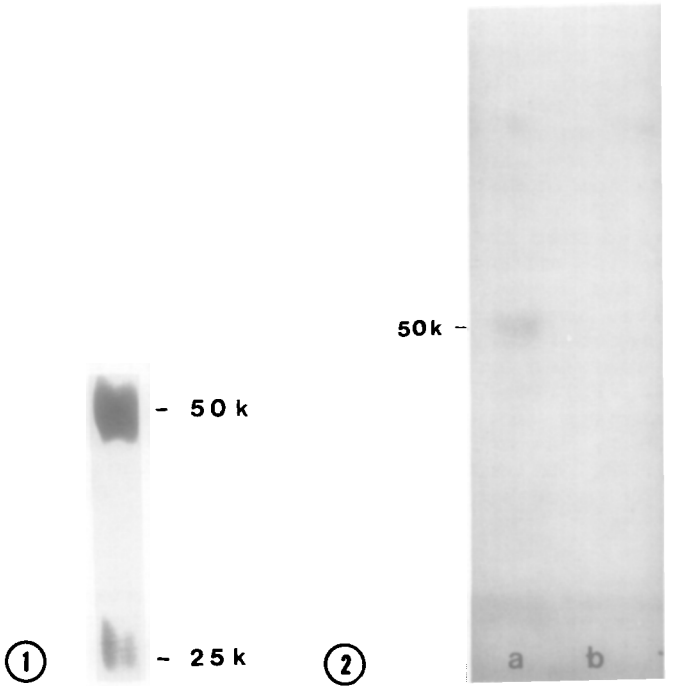
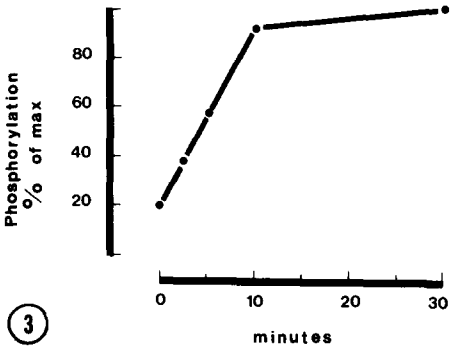


Figure 1. Specificity of the Immunoprecipitate. Isolated rat hepatocytes were lysed with acetone and the dried pellet solubilized in the mixture of detergents as described under Materials and Methods. After incubation with the antibody coupled to Sepharose, the immunoprecipitate was centrifuged, washed and after dissociation by boiling with 2% SDS subjected to SDS-polyacrylamide gel electrophoresis. After electrophoresis proteins were visualized with Coomassie-Blue R.

Figure 2. Autoradiogram showing the effect of glucagon on the phosphorylation of the 50K protein of phospholipid methyltransferase by intact rat hepatocytes. Isolated rat hepatocytes preincubated with [^{32}P] were exposed for 10 min to glucagon (0.001 µM) (a) or to the disolvent of glucagon (b) before lysis with acetone. Samples were then treated as mentioned under Materials and Methods and subjected to SDS-polyacrylamide gel electrophoresis. The gel was then dried and autoradiographed during 6 days at -70°C.

antiserum coupled to Sepharose, followed by SDS-polyacrylamide gel electrophoresis. Exposure of isolated rat hepatocytes to glucagon markedly enhances the phosphorylation of the 50K protein (Fig.2). Treatment of isolated rat hepatocytes with a saturating dose of glucagon (1 µM) produces a time-dependent stimulation of phospholipid methyltransferase phosphorylation (Fig.3). Maximal phosphorylation is attained about 10 min and sustained for at least 30 min after the addition of the hormone. The time course



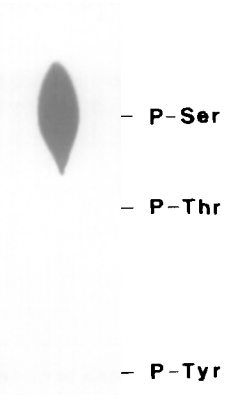


Figure 3. Effect of glucagon on the time-course of phosphorylation of the 50K protein of phospholipid methyltransferase by intact rat hepatocytes. At time zero, isolated rat hepatocytes, preincubated with [32P], were exposed to glucagon (1 μM) and at various times the 50K protein of phospholipid methyltransferase purified by immunoprecipitation as described under Materials and Methods. After electrophoresis and autoradiography the incorporation of [^{32}P] into the 50K protein was quantitated by densitometry. Results are the average of three independent experiments.

Figure 4. Autoradiogram showing that serine is the only aminoacid of the 50K protein of phospholipid methyltransferase phosphorylated by intact rat hepatocytes exposed to glucagon. Rat hepatocytes preincubated with [^{32}P] were exposed to glucagon for 10 min before lysis with acetone. After lysis the 50K protein of phospholipid methyltransferase was purified by immunoprecipitation and SDS electrophoresis. After electrophoresis proteins were weakly visualized with Coomassie-Blue R. The 50K protein was cut and subjected to acidic hydrolysis. After hydrolysis, phosphoaminoacids were separated by high voltage electrophoresis on a cellulose plate and identified by autoradiography. P-Ser, phosphoserine; P-Thr, phosphothreonine; P-Tyr, phosphotyrosine.

of phosphorylation in response to a saturating dose of glucagon agrees with the time course of activation of phospholipid methyltransferase by glucagon in rat hepatocytes previously reported (5).

Autoradiograms of high-voltage cellulose electrophoresis of an acidic hydrolysate of the 50K protein of phospholipid methyltransferase isolated from hepatocytes treated with 1 μM glucagon showed only phosphoserine (Fig.4). The incorporation of ^{32}P into the 50K protein of non-stimulated hepatocytes was also only on phosphoserine (Table 1). These results agree with our previous observation showing that the cyclic AMP-dependent

TABLE 1

Phosphorylation of serine residues of the 50K protein of phospholipid methyltransferase by intact rat hepatocytes

	Non-stimulated	1 μM glucagon
Phosphoserine (cpm)	450	2530

Isolated rat hepatocytes were preincubated with [^{32}P] and exposed to 1 uM glucagon or to the disolvent of glucagon for 10 min before lysis with acetone. The 50K protein of phospholipid methyltransferase was then purified and subjected to acidic hydrolysis as described under Materials and Methods Phosphoaminoacids were visualized by autoradiography, scratched from the cellulose plate and the amount of [^{32}P] determined by scintillation counting. No detectable [^{32}P] incorporation was observed into phosphothreonine or phosphotyrosine. Results are the average of three independent experiments.

protein kinase phosphorylates the 50K protein of phospholipid methyltransferase only on phosphoserine (12). It remains to be determined whether basal phosphorylation occurs at the same site(s) than that phosphorylated in response to glucagon, and whether this site(s) is the same than that obtained by incubating the enzyme with the cyclic AMP-dependent protein kinase.

From the present results it can be concluded that in intact rat hepatocytes glucagon stimulates the phosphorylation of the 50K protein of phospholipid methyltransferase at serine residues. Glucagon, and other conditions which are known to elevate the intracellular content of cyclic AMP (5-7), activates phospholipid methyltransferase in rat hepatocytes. Furthermore, phosphorylation of the 50K protein of the purified enzyme at serine residues activates its methylating activity (12). Therefore, the available evidence indicates that phosphorylation of phospholipid methyltransferase in rat hepatocytes is an important mechanism for the short-term hormonal control of the

synthesis of phosphatidylcholine by the transmethylation pathway. It remains to be determined whether phospholipid methyltransferase in cells other than hepatocytes is also phosphorylated and whether in these systems phosphorylation modulates the enzyme activity.

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