Vasopressin-stimulated phosphorylation of rat liver phospholipid methyltransferase in isolated hepatocytes

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Addition of vasopressin (1 μM) to isolated rat hepatocytes prelabeled with [^{32}P]phosphate was accompanied by a 250% increase in the phosphorylation of phospholipid methyltransferase. Vasopressin-stimulated phospholipid methyltransferase phosphorylation was time- and dose-dependent. ^{32}P-labeled phospholipid methyltransferase was recovered by immunoprecipitation and SDS-polyacrylamide gel electrophoresis. After electrophoresis, phospholipid methyltransferase was electroeluted from the polyacrylamide gel and subjected to tryptic digestion or HCl hydrolysis. Analysis of ^{32}P-labeled peptides reveals only one site of phosphorylation and the analysis of [^{32}P]phosphoamino acids indicates that phosphoserine is the only labeled amino acid.

Vasopressin *Phospholipid methyltransferase* *Protein phosphorylation* *Electroelution* *Hepatocyte* *Phosphoamino acid*

1. INTRODUCTION

Phospholipid methyltransferase, the enzyme that converts phosphatidylethanolamine into phosphatidylcholine *S*-adenosylmethionine being the methyl donor, was first described by Bremer and Greenberg [1]. During the last years, evidence has accumulated which indicates that this reaction plays a role in transmembrane signaling [2–4]. A variety of signals, including glucagon [5,6], isoproterenol [7], gonadotropin [8] and adrenocorticotropin [9], stimulate phospholipid methyltransferase probably via a cyclic AMP-dependent mechanism. Partially purified phospholipid methyltransferase from rat liver microsomes is activated by cyclic AMP-dependent phosphorylation of a 50 kDa protein [10] at a single serine residue [11]. The rat liver methyltransferase has been purified to apparent homogeneity (Pajares, M.A. et al., unpublished) and has a molecular mass of about 50 kDa being phosphorylated by the cyclic AMP-dependent protein kinase. This same 50 kDa protein has been phosphorylated in isolated rat hepatocytes stimulated by glucagon [12]. These results indicate that phospholipid methyltransferase exists in a high-activity phosphorylated form and in a low-activity dephosphorylated form. In addition to this cyclic AMP-dependent mechanism of regulation of rat liver phospholipid methyltransferase there is also evidence in favor of a Ca^{2+}-dependent phosphorylation mechanism. Vasopressin and angiotensin II, two hormones which in rat liver mediate their effects by increasing the intracellular Ca^{2+} content [13], stimulate phospholipid methyltransferase [14]. Furthermore, phospholipid methyltransferase in isolated rat liver microsomes is activated by Ca^{2+} and ATP in the presence of calmodulin [15]. The present results show that vasopressin induces phospholipid methyltransferase phosphorylation in isolated rat hepatocytes. Phosphorylation of the methyltransferase is time- and dose-dependent and only on phosphoserine. Analysis of ^{32}P-labeled peptides of phospholipid methyltransferase reveals that vasopressin induces the phosphorylation of one site.

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2. MATERIALS AND METHODS

2.1. *Materials*

Vasopressin, collagenase, sodium deoxycholate, phenylmethanesulphonyl fluoride, benzamidine, leupeptin, nitrophenyl-4-phosphate and phosphoamino acid markers were from Sigma. [^{32}P]Phosphate (carrier free) was from Amersham, cellulose-coated thin layer plates and Triton X-100 from Merck and reagents for electrophoresis and protein markers from BioRad.

2.2. *Methods*

Hepatocytes from normally fed Wistar rats (250–300 g) were prepared as described [5] and incubated for 1 h in the presence of 0.1 mM [^{32}P]phosphate (0.2 mCi/ml) according to the published methods [12]. Addition of vasopressin (dissolved in 0.9% NaCl containing 1 mg/ml bovine serum albumin), or an equal volume of the same solution were made directly to the cell suspension. Cells (1 ml containing about 20 mg wet wt/ml) were lysed by addition of 4 ml ice-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% SDS and 0.1% sodium deoxycholate) containing 10 mM Tris-HCl, pH 7.0, 20 mM sucrose, 150 mM KF, 15 mM EDTA, 2 mM EGTA, 50 mM β-mercaptoethanol, 1 mM phenylmethanesulphonyl fluoride, 1 mM benzamidine, 50 μg/ml leupeptin and 1 mM nitrophenyl-4-phosphate as described [12]. The mixture was then clarified by centrifugation at 10000 × g for 2 min in a Beckman microfuge. Phospholipid methyltransferase was then immunoprecipitated with an antiserum against rat liver phospholipid methyltransferase coupled to Sepharose according to the published methods [12]. The immunoprecipitates were 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) [12]. Gels were stained with Coomassie blue R, dried and [^{32}P]phospholipid methyltransferase localized by autoradiography, using a Kodak X Omat-GRS film and intensifier screens at −70°C during 6 days [12]. The radioactivities present in the methyltransferase from control and vasopressin-treated cells were compared by scanning the autoradiogram and the stained gel in a densitometer (Beckman DU-8). The area of the peak of radioactivity is then divided by the area of the correspondent peak of protein and the resulting values from control and vasopressin-treated cells compared. Results are expressed as percentage increase in the phosphorylation of phospholipid methyltransferase caused by vasopressin.

Protein hydrolysis and tryptic digestion were carried out as described [10,11] using phosphorylated phospholipid methyltransferase electroeluted from the SDS-polyacrylamide gel. Elution of phospholipid methyltransferase from the gels was carried out using an ISCO model 1750 electrophoretic sample concentrator. After electrophoresis, phospholipid methyltransferase was located by comparing against a stained gel. Then the gel which contains the 50 kDa protein was cut out and placed in the concentration well of the sample cup of the apparatus. A buffer containing 0.04 M Tris/acetate, pH 8.36, 0.002 M EDTA was added to the sample cup and electroelution carried out during 5.5 h at 1 W in 0.1 M Tris/acetate, pH 8.36, 0.002 M EDTA. Phospholipid methyltransferase was then removed from the concentration well, lyophilized and subjected to tryptic digestion [11] or to acid hydrolysis in 6 N HCl [10]. Tryptic peptides were separated by thin-layer chromatography on cellulose plates developed with 1-butanol/pyridine/water/acetic acid (65:50:40:10, v/v) [11]. ^{32}P-labeled peptides were localized by autoradiography as described above. After HCl hydrolysis phosphoamino acid standards were added and separated by high voltage electrophoresis on cellulose plates [16]. Phosphoamino acids were then localized with ninhydrin, scrapped from the cellulose plate and counted [10].

3. RESULTS AND DISCUSSION

Addition of 0.1 μM vasopressin to a suspension of rat hepatocytes prelabeled with [^{32}P]phosphate results in an increase in ^{32}P incorporation into phospholipid methyltransferase (fig.1). Maximal phosphorylation was observed about 5 min after the addition of vasopressin. This pattern is similar to that reported [14] for vasopressin-mediated stimulation of rat hepatocytes phospholipid methyltransferase.

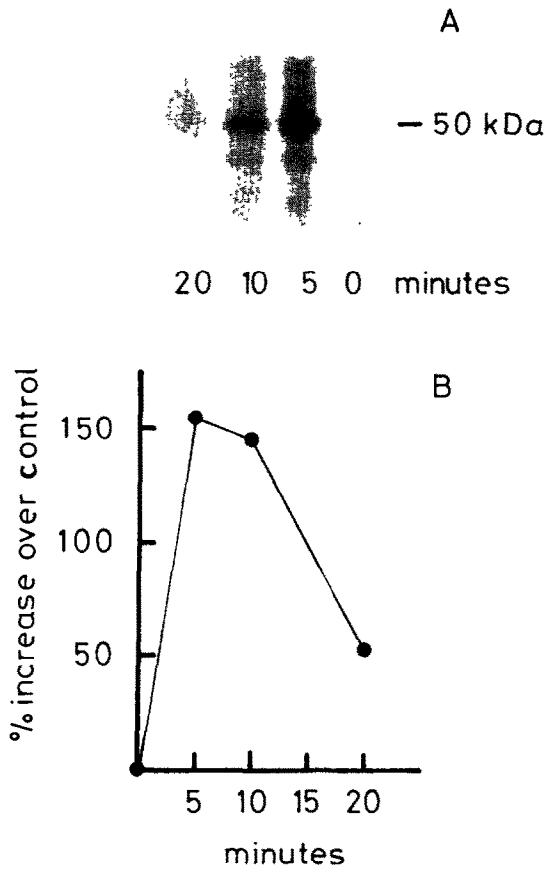

Fig.1. Vasopressin-stimulated phosphorylation of rat liver phospholipid methyltransferase in isolated rat hepatocytes. Hepatocytes were prelabeled with [^{32}P]phosphate for 60 min. These cells were then incubated in the presence or absence of vasopressin (0.1 μM). At various times, samples were taken and the incorporation of ^{32}P into phospholipid methyltransferase determined by immunoprecipitation and SDS-polyacrylamide gel electrophoresis as described in section 2. The increase in phospholipid methyltransferase phosphorylation was determined by comparing the radioactivity in the 50 kDa protein between control and vasopressin-stimulated samples. Panel A shows a typical SDS-gel autoradiogram comparing phospholipid methyltransferase phosphorylation at various times after vasopressin addition. The results in B are the average of 3 independent experiments.

The effect of varying concentrations of vasopressin on phospholipid methyltransferase phosphorylation was also investigated. As shown in fig.2, phospholipid methyltransferase phosphorylation by vasopressin was a dose-dependent process. Maximal response in methyltransferase phosphorylation (about 250% over the control value) was observed at 1 μM vasopressin. This dose-response curve is also similar to that previously reported [14] for vasopressin-mediated stimulation of rat hepatocytes phospholipid methyltransferase.

The ^{32}P-labeled phospholipid methyltransferase recovered in the immunoprecipitate and purified by SDS-polyacrylamide gel electrophoresis was electroeluted and subjected to HCl hydrolysis and phosphoamino acid analysis or to extensive tryptic digestion and peptide mapping. Vasopressin stimulates the incorporation of ^{32}P only into phosphoserine (table 1) and enhances the phosphorylation of one single peptide (fig.3). The R_f of this phosphopeptide (0.35) differs from that previously reported (0.30) for cyclic AMP-dependent phospholipid methyltransferase phosphorylation [11].

The results presented here provide evidence that vasopressin-dependent stimulation of phospholipid methyltransferase in intact hepatocytes is mediated by phosphorylation. Previously [12] we have observed that glucagon-dependent stimula-

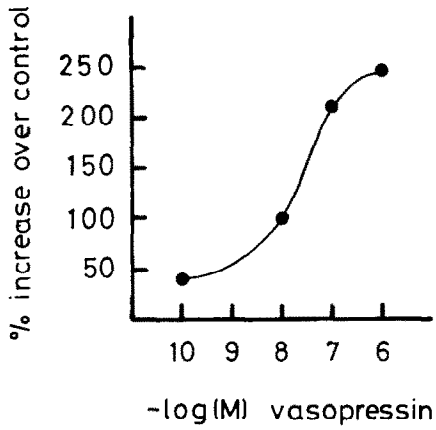

Fig.2. Effect of different doses of vasopressin on phospholipid methyltransferase phosphorylation. Rat hepatocytes prelabeled with [^{32}P]phosphate were exposed to various doses of vasopressin for 5 min before lysis with acetone. After lysis, phospholipid methyltransferase was purified and its ^{32}P content determined as described in section 2. The results are the average of 2 independent experiments.

Table 1

Analysis by high voltage electrophoresis of ^{32}P-labeled phosphoamino acids from phospholipid methyltransferase isolated from vasopressin-treated rat hepatocytes

	^{32}P (cpm)
Phosphoserine	790
Phosphothreonine	ND
Phosphotyrosine	ND

^{32}P-labeled phospholipid methyltransferase in the SDS-gels derived from vasopressin-stimulated hepatocytes (0.1 μM during 5 min) was electroeluted and incubated at 110°C for 2 h in 6 N HCl. After hydrolysis, phosphoamino acid standards were added and separated by high voltage electrophoresis on a cellulose plate with acetic acid/pyridine/water (50:5:945, v/v) as described [10]. After electrophoresis the phosphoamino acids were visualized with ninhydrin, scraped from the plate and counted. ND, non-detectable

tion of phospholipid methyltransferase in intact hepatocytes is also mediated by phosphorylation. Whereas glucagon mediates its effects via a cyclic AMP-dependent protein kinase, vasopressin probably acts via a Ca^{2+}-dependent protein kinase. We have also reported the activation of phospholipid methyltransferase in rat liver microsomes by the presence of Ca^{2+}, ATP and calmodulin [15]. These results suggest the participation of a Ca^{2+}/calmodulin-dependent protein kinase in the activation of phospholipid methyltransferase by vasopressin. Furthermore, vasopressin-dependent phosphorylation of phospholipid methyltransferase occurs at only one site whose R_f differs from that previously observed [11] for cyclic AMP-dependent phosphorylation of phospholipid methyltransferase. These results indicate the existence of two sites of phosphorylation in rat liver phospholipid methyltransferase, one modulated by a Ca^{2+}-dependent protein kinase and a second one modulated by a cyclic AMP-dependent protein kinase.

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Fig.3. Analysis by TLC of ^{32}P-labeled tryptic peptides from phospholipid methyltransferase isolated from vasopressin-treated rat hepatocytes. ^{32}P-labeled phospholipid methyltransferase in the SDS-gels derived from vasopressin-stimulated hepatocytes (0.1 μM during 5 min) was electroeluted and digested with trypsin as described in section 2. After trypsinization, peptides were separated by TLC in 1-butanol/pyridine/water/acetic acid (130:100:80:20, v/v) and phosphopeptides localized by autoradiography. Only one phosphopeptide (site 1) is observed under these conditions.

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