Protein kinase C catalyses the phosphorylation and activation of rat liver phospholipid methyltransferase

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When a partially purified rat liver phospholipid methyltransferase is incubated with [γ - 32 P]ATP and rat brain protein kinase C, phospholipid methyltransferase (M_r 50000, pI 4.75) becomes phosphorylated. Phosphorylation of the enzyme showed Ca^{2+} /lipid-dependency. Protein kinase C-dependent phosphorylation of phospholipid methyltransferase was accompanied by an approx. 2-fold activation of the enzyme activity. Activity changes and enzyme phosphorylation showed the same time course. Activation of the enzyme also showed Ca^{2+} /lipid-dependency. Protein kinase C mediates phosphorylation of predominantly serine residues of the methyltransferase. One major peak of phosphorylation was identified by analysis of tryptic phosphopeptides by isoelectrofocusing. This peak (pI 5.2) differs from that phosphorylated by the cyclic AMP-dependent protein kinase (pI 7.2), demonstrating the specificity of phosphorylation of protein kinase C. Tryptic-peptide mapping by h.p.l.c. of the methyltransferase phosphorylated by protein kinase C revealed one major peak of radioactivity, which could be resolved into two labelled phosphopeptides by t.l.c. The significance of protein kinase C-mediated phosphorylation of phospholipid methyltransferase is discussed.

INTRODUCTION

Phospholipids play an important role in maintaining membrane structure and function. The phospholipid composition of biomembranes is adjusted by a complicated network of biosynthetic reactions, of which relatively little is known. Many phospholipids can be converted into others by modification of their polar head groups. One of these reactions is the conversion of phosphatidylethanolamine into phosphatidylcholine by three successive *N*-methylations, *S*-adenosylmethionine being the methyl donor [1]. In rat liver, phospholipid methylation supplies up to 40% of the phosphatidylcholine used by this organ [2]. Since phosphatidylcholine is the most abundant phospholipid in rat liver membranes, this reaction is clearly of great importance in maintaining membrane structure and function in the liver (reviewed in [3] and [4]).

Rat liver phospholipid methyltransferase is activated by glucagon [5–7], isoprenaline [8], vasopressin [9] and angiotensin [9]. The rat liver enzyme has been purified to apparent homogeneity [10]. It can exist in two forms, as a M_r 25000 monomer and as a dimer of M_r 50000. The latter dimer is the most abundant form of the enzyme found during purification and is not dissociated by boiling in 2% SDS containing β -mercaptoethanol [10]. In agreement with these observations, in an abstract [11] it has been reported that purified rat liver phospholipid methyltransferase has a M_r of about 50000 and a pI between 4 and 5. Previously we had shown that the catalytic subunit of cyclic AMP-dependent protein kinase catalyses the activation and phosphorylation of rat liver phospholipid methyltransferase and that the phosphorylated enzyme has a M_r of 50000 and pI 4.75 [12]. This is also the only form of the enzyme which is found to be phosphorylated in hepatocytes prelabelled with 32 P and treated with glucagon [13] or vasopressin

[14]. Phosphorylation in response to vasopressin [14] and by the cyclic AMP-dependent protein kinase [15] are at different sites, predominantly on serine residues. We present evidence that protein kinase C catalyses the phosphorylation and activation of rat liver phospholipid methyltransferase at two different sites, predominantly on serine.

EXPERIMENTAL

Materials

[γ - 32 P]ATP (3000 Ci/mmol) and *S*-adenosyl-L-[3 H]methionine (15 Ci/mmol) were obtained from Amersham. Phosphatidylserine, diolein, lysine-rich histone III-S, ATP and phosphoamino acid standards were purchased from Sigma. Reagents for electrophoresis and markers for M_r determination were from Bio-Rad. Ampholines and standards for pI determination were purchased from LKB. Cellulose plates for t.l.c. and high-voltage electrophoresis were from Merck. The Nucleosil C18 column for h.p.l.c. was from Scharlau. Other chemicals used were all reagent grade.

Purification of protein kinase C

Protein kinase C was partially purified from rat brain homogenate by chromatography on DEAE-cellulose DE-52 (1.5 cm $\times$ 5 cm column; flow rate 20 ml/h) as described by Le Peuch *et al.* [16]. The specific activity of the enzyme was 7 nmol of phosphate incorporated into histone III-S/min per mg at 30 °C. The standard reaction mixture contained, in a final volume of 50 μ l, 20 mM-Hepes/NaOH, pH 7.5, 5 mM-magnesium acetate, 5 mM-dithiothreitol, 20 μ M-[γ - 32 P]ATP (containing about 4×10^5 c.p.m./50 μ l reaction mixture), 1 mg of lysine-rich histone III-S/ml, 100 μ g of phosphatidylserine/ml, 200 μ g of diolein/ml and 0.5 mM- Ca^{2+} (CaCl_2). The

reaction was started by the addition of ATP and was carried out for 5 min at 30 °C. The phosphorylation reaction was terminated by the addition of 50 μ l of 10% (w/v) trichloroacetic acid, followed by filtration through glass microfibre Whatman GF/C filters. After extensive washing with trichloroacetic acid, the filters were dried and counted for radioactivity. For electrophoresis, after the reaction was stopped with trichloroacetic acid, proteins were concentrated by centrifugation, washed, dissolved in 100 μ l of 2% SDS/10% glycerol/0.015% Bromophenol Blue/30 mM-dithiothreitol/0.1 M-Tris/HCl, pH 6.8, and boiled for 2 min. After boiling, samples were subjected to SDS/polyacrylamide-gel electrophoresis in 10% (w/v)-acrylamide slab gels in a Tris/HCl/SDS buffer as described in Bio-Rad Laboratories Bulletin 1024. After electrophoresis, gels were dried between two Cellophane paper sheets and then submitted to autoradiography.

Purification of phospholipid methyltransferase

Phospholipid methyltransferase was partially purified from rat liver microsomal fractions by successive solubilization with Chaps {[3-(cholamidopropyl)dimethylammonio]propane-1-sulphonate} and chromatography on DEAE-Sephacel and Ultrogel Aca-34 as described previously [10,17]. When analysed by SDS/polyacrylamide-gel electrophoresis, the purified enzyme contains both the M_r -25000 monomer and the M_r -50000 dimer (see [10] for a detailed description of the purification procedure). As shown by scans of the SDS/polyacrylamide gels, the enzyme is about 90% pure and the M_r -50000 form of the enzyme accounts for about 80% of the total protein. The specific activity of the purified enzyme was 3–4 nmol of methyl group incorporated into phospholipids/min per mg at 22 °C. Phospholipid methyltransferase activity was assayed as previously described [5]. Protein was determined as described by Bradford [18], with the concentrated reagents from Bio-Rad.

Phospholipid methyltransferase phosphorylation

Phospholipid methyltransferase was phosphorylated in a reaction mixture containing, in a final volume of 100 μ l, 20 mM-Hepes/NaOH, pH 7.5, 5 mM-magnesium acetate, 5 mM-dithiothreitol, 1 mM-KF, 100 μ M-[γ - 32 P]-ATP (containing about 10^6 c.p.m.), 25 μ l of phospholipid methyltransferase (10 μ g of protein), 10 μ l of protein kinase C (10 μ g of protein), 100 μ g of freshly sonicated phosphatidylserine/ml, 10 μ g of diolein/ml and 0.5 mM- Ca^{2+} (CaCl_2). The reaction was initiated by the addition of ATP and continued at 30 °C for up to 5 min. The reaction was stopped by the addition of 4 vol. of acetone at –20 °C. After 20 min at –20 °C, acetone was removed by centrifugation and the pellet washed twice with acetone. After washing, the pellet was dried with a stream of N_2 and dissolved in 9.2 M-urea/5% β -mercaptoethanol/2% Nonidet P-40, containing 4% Ampholines (pH range 5–7) and 1% Ampholines (pH range 3.5–10). Proteins were separated by isoelectrofocusing in 10% -acrylamide gels in a non-equilibrium pH gradient from 3.5 to 10.0 [19]. After electrophoresis, gels were cut into 2.5 mm pieces and the associated radioactivity was assayed by scintillation counting. Standards for pI determination were: amyloglucosidase (pI 3.50), soya-bean trypsin inhibitor (4.55), lactoglobulin (5.20), bovine carbonic anhydrase B (6.85), horse

myoglobin acidic band (6.85), horse myoglobin basic band (7.35), lentil lectin acidic band (8.15), lentil lectin middle band (8.45), lentil lectin basic band (8.65) and trypsinogen (9.30).

Analysis of 32 P-labelled tryptic peptides

Purified phospholipid methyltransferase was phosphorylated with protein kinase C as described above. Analysis of 32 P-labelled peptides was carried out as previously described [15]. Briefly, after phosphorylation the enzyme was precipitated with acetone at –20 °C. Acetone was then removed by centrifugation and the pellet dried under a stream of N_2 . After drying, the pellet was resuspended in 50 mM- NH_4HCO_3 (pH 7.7) and dialysed overnight against the same buffer. After dialysis, trypsin was added (1 part/20 parts of protein, w/w) and incubated for 1 h at 37 °C. After this incubation, trypsin was again added (1 part/40 parts of protein) and incubated for 3 h at 37 °C. After trypsin treatment the solution was freeze-dried, and phosphopeptides were analysed by isoelectrofocusing as described above. The preparation of samples for tryptic-peptide mapping by h.p.l.c. was the same as that described for isoelectrofocusing, and resolution of the 32 P-labelled tryptic peptides was performed on a Scharlau Nucleosil C18 column as described [15]; 1.5 min fractions were collected and radioactivity was determined by Čerenkov counting. The peak fractions were pooled, freeze-dried, dissolved in a small volume of water and applied to a cellulose plate for t.l.c. The plates were then developed with butan-1-ol/pyridine/water/acetic acid (13:10:10:2, by vol.) and 32 P-labelled peptides were localized by autoradiography as described [15].

Phosphoamino acid analyses

Purified phospholipid methyltransferase was phosphorylated as described above. After phosphorylation, samples were precipitated with acetone and their phosphoamino acid contents were analysed as previously described [12] by the techniques of Hunter & Sefton [20]. The phosphoamino acid-associated radioactivity was determined by scintillation counting of the scraped spots [15].

RESULTS

Phospholipid methyltransferase is a substrate of protein kinase C *in vitro*

Histone phosphorylation by the partially purified protein kinase C showed Ca^{2+} /lipid-dependency (Fig. 1), and was not affected by the addition of the inhibitor protein of cyclic AMP-dependent protein kinase or in the presence of Ca^{2+} /calmodulin (results not shown), demonstrating that its properties are the same as those of the fully purified protein kinase C [16].

Direct evidence that protein kinase C could phosphorylate phospholipid methyltransferase was obtained by incubating our preparation of protein kinase C with purified phospholipid methyltransferase in the presence of [γ - 32 P]ATP. Phosphorylated proteins were resolved by isoelectrofocusing in 10% -acrylamide gels, and radioactivity was assayed by cutting and counting the gels (Fig. 2). Like the phosphorylation of histone, phosphorylation of phospholipid methyltransferase showed Ca^{2+} /lipid-dependency (Fig. 2), indicating that its pro-

Fig. 1. Phosphorylation of histone by a purified preparation of protein kinase C

This was done during 5 min as described in the Experimental section. After phosphorylation, samples were electrophoresed on SDS/10%-acrylamide gels, and phosphoproteins were detected by autoradiography. The sample used in lane (a) contained 100 μ g of phosphatidylserine/ml, 200 μ g of dioleoin/ml, 4 mM-EGTA and no CaCl_2 . The sample in lane (b) contained 0.5 mM- Ca^{2+} but no phosphatidylserine or dioleoin. The sample in lane (c) contained 100 μ g of phosphatidylserine/ml, 200 μ g of dioleoin/ml and 0.5 mM- Ca^{2+} .

perties are those of a phosphorylation mediated by protein kinase C. The pI of the phosphorylated protein was 4.75, which is the same as that previously reported for cyclic AMP-dependent phosphorylation of phospholipid methyltransferase [12] and for the purified enzyme [11]. When ^{32}P -labelled proteins were analysed by SDS/polyacrylamide-gel electrophoresis and autoradiography, about 90% of the protein-bound ^{32}P had a M_r of 50000 (results not shown). The M_r of this protein is the same as that previously reported for cyclic AMP-dependent phosphorylation of phospholipid methyltransferase [10,12]. When the phosphorylation assay was carried out in the absence of phospholipid methyltransferase but in the presence of kinase, phosphatidylserine, dioleoin and Ca^{2+} , no protein-bound ^{32}P was detected by SDS/polyacrylamide-gel electrophoresis or isoelectrofocusing. This indicates that the protein with pI 4.75 and M_r 50000 is not a contaminant present in the protein kinase C preparation. The ^{32}P content of the M_r -50000 protein varied between experiments from 0.5 to 0.7 mol of phosphate/mol of M_r -50000 protein. This value was calculated by considering that the M_r -50000

Fig. 2. Phosphorylation of phospholipid methyltransferase by a purified preparation of protein kinase C

This was done during 5 min as described in the Experimental section. After phosphorylation, samples were analysed by isoelectrofocusing on 10%-acrylamide gels. The sample used in (●) was incubated with 100 μ g of phosphatidylserine/ml, 200 μ g of dioleoin/ml and 0.5 mM- Ca^{2+} . The sample used in (▲) was incubated in the absence of exogenous lipids but in the presence of 0.5 mM- Ca^{2+} , and the sample in (○) was incubated in the presence of 100 μ g of phosphatidylserine/ml, 200 μ g of dioleoin/ml and 1 mM-EGTA but in the presence of CaCl_2 . After electrophoresis, gels were sliced into 2.5 mm pieces and the radioactivity associated with each piece determined. The pI values of protein standards are shown.

form of the enzyme represents 80% of the total protein. To identify the amino acid in phospholipid methyltransferase phosphorylated by protein kinase C after hydrolysis, phosphoamino acids were separated by one-dimensional high-voltage electrophoresis, identified with ninhydrin, and the associated radioactivity was determined by scintillation counting. Under these conditions, more than 90% of the amino acid-bound ^{32}P was associated with phosphoserine, and 5–8% was associated with phosphothreonine. No radioactivity was detected associated with phosphotyrosine.

Protein kinase C phosphorylates phospholipid methyltransferase at two different sites

To identify the site(s) in phospholipid methyltransferase phosphorylated by protein kinase C, after phosphorylation the enzyme was digested with trypsin, and phosphopeptides were separated by isoelectrofocusing in 10%-acrylamide gels. As shown in Fig. 3, protein kinase C phosphorylates phospholipid methyltransferase at one major site, of pI 5.2. As previously described [10,15], cyclic AMP-dependent protein kinase also phosphorylates phospholipid methyltransferase (Fig. 3), but at a different site (pI 7.2), demonstrating the specificity of phosphorylation by protein kinase C. Experiments were also performed to examine the site(s) of phosphorylation of phospholipid methyltransferase, by separating the tryptic peptides by h.p.l.c. followed by t.l.c. With this procedure, our previous studies [15] have indicated that there is one major site of cyclic AMP-dependent phosphorylation in the methyltransferase. By h.p.l.c. a

Fig. 3. Tryptic-peptide mapping by isoelectrofocusing of the phospholipid methyltransferase phosphorylated by protein kinase C

Purified phospholipid methyltransferase was phosphorylated for 5 min by protein kinase C (b) or cyclic AMP-dependent protein kinase (a). Conditions for phosphorylation with cyclic AMP-dependent protein kinase were as previously described [15]. Phosphorylated phospholipid methyltransferase was subjected to tryptic digestion as described in the Experimental section. After tryptic digestion, samples were subjected to isoelectrofocusing on 10%-acrylamide gels. The radioactivity in each gel fraction was determined by cutting the gel into 2.5 mm pieces and determining the radioactivity associated with each piece by scintillation counting. The pI values of protein standards are shown.

major peak of radioactivity with a retention time of 16 min was obtained (Fig. 4a). The peak fractions were then pooled, freeze-dried, applied to a cellulose plate, and ^{32}P -labelled peptides were separated by t.l.c. As shown in Fig. 4(b), two major labelled phosphopeptides were identified by autoradiography after t.l.c.

Phosphorylation of phospholipid methyltransferase by protein kinase C stimulates its activity

The possible role of protein kinase C in activating phospholipid methyltransferase was evaluated by measuring the activity of the purified enzyme at various times after the addition of the kinase. As shown in Fig. 5, phospholipid methyltransferase is activated 2-fold by the addition of protein kinase C. Fig. 5 also shows that phosphorylation of phospholipid methyltransferase and the changes in enzyme activity have the same time course. Protein kinase C-mediated phospholipid methyltransferase activation showed Ca^{2+} /lipid-dependency. No effect on phospholipid methyltransferase activity was observed when ATP was omitted from the assay mixture or replaced by the non-hydrolysable analogue adenosine 5'-[$\beta\gamma$ -imido]triphosphate.

Fig. 4. Tryptic-peptide mapping, by h.p.l.c. and t.l.c., of phospholipid methyltransferase phosphorylated by protein kinase C

Purified phospholipid methyltransferase was phosphorylated for 5 min by protein kinase C and subjected to tryptic digestion as described in the legend to Fig. 3. The digest was resolved on a Scharlau Nucleosil C18 column as described in the Experimental section (a). The radioactivity in each fraction was determined by Cerenkov counting. The peak fractions were pooled, freeze-dried, dissolved in a small volume of water and applied to a cellulose plate for t.l.c. The plate was then developed with butan-1-ol/pyridine/water/acetic acid (13:10:8:2, by vol.), and ^{32}P -labelled peptides were localized by autoradiography (b). Radioactivity in the first h.p.l.c. fractions is due to [γ - ^{32}P]ATP.

Fig. 5. Time course of phospholipid methyltransferase phosphorylation and activation in the presence of protein kinase C

Phospholipid methyltransferase was incubated with a purified preparation of protein kinase C as described in the Experimental section. At various times, a sample was taken and phospholipid methyltransferase activity was assayed (○) or the incorporation of ^{32}P into the protein band of pI 4.75 was determined (●). For the determination of phospholipid methyltransferase activity, the samples were incubated in the presence of 5 mM-EGTA, to stop the phosphorylation reaction, and 175 μM -*S*-adenosyl[*Me*- ^3H]methionine as described in the Experimental section. Results are averages of three independent determinations.

DISCUSSION

The M_r -50000 form of phospholipid methyltransferase is phosphorylated *in vitro* by the cyclic AMP-dependent protein kinase at one single site on serine [10,12,15]. Phosphorylation of this site activates phospholipid methyltransferase about 4-fold [12]. Here we have provided evidence that, as with the cyclic AMP-dependent kinase, only the M_r -50000 form of the enzyme is phosphorylated by protein kinase C. This is also the only form of the enzyme that is phosphorylated in ^{32}P -labelled hepatocytes treated with glucagon [13] or with vasopressin [14]. It therefore seems that only when the enzyme is forming a M_r -50000 dimer have the kinases access to the sites of phosphorylation in the enzyme. Protein kinase C catalyses the phosphorylation of phospholipid methyltransferase on serine at two different sites. These two sites are different from that previously reported [15] for cyclic AMP-dependent phosphorylation of phospholipid methyltransferase. This conclusion is based on the following observations. (1) When tryptic digests of phospholipid methyltransferase phosphorylated by cyclic AMP-dependent protein kinase are analysed by isoelectrofocusing, one major site of phosphorylation, with pI 7.2, is detected. After the same treatment, phospholipid methyltransferase phosphorylated by protein kinase C gave one major peak of phosphorylation, with pI 5.2. (2) Analysis of tryptic digests of phospholipid methyltransferase phosphorylated by cyclic AMP-dependent protein kinase by h.p.l.c. and t.l.c. gave one major labelled phosphopeptide with a retention time of 43 min and R_F of 0.3 [15]. Under the same conditions, phospholipid methyltransferase phos-

phorylated by protein kinase C gave after h.p.l.c. one major labelled peak with a retention time of 16 min. This labelled peak could be further separated by t.l.c. into two major labelled phosphopeptides, with R_F values of 0.2 and 0.05.

The present results also indicate that phospholipid methyltransferase can be activated by protein kinase C and that phosphorylation seems to be responsible for the activity changes. This conclusion is based on the following two observations. (1) Protein kinase C-catalysed phospholipid methyltransferase activation and phosphorylation showed the same time course. In a previous experiment [12], using cyclic AMP-dependent protein kinase, phosphorylation and activation of phospholipid methyltransferase showed a maximum, owing to the presence of endogenous phosphoprotein phosphatase activity in the preparation of phospholipid methyltransferase. The present experiments were carried out in the presence of 1 mM-KF to prevent methyltransferase dephosphorylation, and both phosphorylation and activity changes reached a plateau about 5 min after the addition of the kinase and ATP. (2) Activation and phosphorylation by protein kinase C of phospholipid methyltransferase activity showed Ca^{2+} /lipid-dependency. Furthermore, increased methyltransferase activity was not observed if ATP was omitted from the assay mixture or replaced by the non-hydrolysable analogue adenosine 5'-[β -imido]triphosphate. We do not yet have values for the relative rates of phosphorylation of the two sites shown in Fig. 4(b). Therefore, at present we do not know if phosphorylation of both sites is necessary to obtain changes in phospholipid methyltransferase activity. The stoichiometry of phosphorylation is about 0.6 mol of phosphate/mol of M_r -50000 protein, which is similar to the stoichiometry of phospholipid methyltransferase phosphorylation observed with cyclic AMP-dependent protein kinase [15].

In intact rat hepatocytes, glucagon [13] and vasopressin [14] phosphorylate phospholipid methyltransferase on serine residues. Little is known about the sites of phosphorylation in intact cells. One major site of phosphorylation has been identified, in rat hepatocytes stimulated with vasopressin, by tryptic-phosphopeptide analysis by t.l.c. [14], and glucagon seems to phosphorylate the same site as the cyclic AMP-dependent protein kinase (I. Merida & J. M. Mato, unpublished work). The site of phosphorylation in phospholipid methyltransferase observed in vasopressin-treated hepatocytes, as detected by t.l.c., coincides with the site of protein kinase C phosphorylation, which moves further from the origin after t.l.c. Whether those two sites are identical needs further evidence.

It has been shown [21] that in isolated rat adipocytes the tumour promoter phorbol 12-myristate 13-acetate stimulates phospholipid methyltransferase. Since tumour promoters have been found to activate protein kinase C [22], these results, together with our present evidence indicating that protein kinase C catalyses the phosphorylation and activation of rat liver phospholipid methyltransferase, suggest that protein kinase C may function to modulate phospholipid methylation.

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