Phosphorylation of diacylglycerol kinase *in vitro* by protein kinase C

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We investigated the effects of enzyme phosphorylation *in vitro* on the properties of diacylglycerol kinase. Diacylglycerol kinase and protein kinase C, both present as M_r -80000 proteins, were highly purified from pig thymus cytosol. Protein kinase C phosphorylated diacylglycerol kinase (up to 1 mol of ^{32}P /mol of enzyme) much more actively than did cyclic AMP-dependent protein kinase. Phosphorylated and non-phosphorylated diacylglycerol kinase showed a similar pI , approx. 6.8. Diacylglycerol kinase phosphorylated by either protein kinase C or cyclic AMP-dependent protein kinase was almost exclusively associated with phosphatidylserine membranes. In contrast, soluble kinase consisted of the non-phosphorylated form. The catalytic properties of the lipid kinase were not much affected by phosphorylation, although phosphorylation-linked binding with phosphatidylserine vesicles resulted in stabilization of the enzyme activity.

INTRODUCTION

In agonist-stimulated cells, diacylglycerol kinase initiates the resynthesis of phosphatidylinositols by phosphorylating diacylglycerol released by the action of phospholipase C [1,2]. This lipid kinase is considered to play a major role in the control of the intracellular concentration of the second messenger, diacylglycerol. Reflecting this, the effects of diacylglycerol kinase inhibitors [3–8] have been interpreted in several types of cells as being due to potentiation of protein kinase C action caused by increased diacylglycerol concentration. The inhibition of this lipid kinase appeared to be involved in the pathogenesis of spontaneous hypertension in rats [9], and in the mitogenesis of endothelial cells induced by hydroxyeicosatetraenoates [10]. In non-stimulated cells, the kinase is likely to regulate normal lipid metabolism by controlling the concentrations of diacylglycerol and phosphatidate, both of which serve as precursors of a variety of glycerolipids.

Detailed studies on the regulatory mechanisms of diacylglycerol kinase are needed to elucidate its function in stimulated as well as in non-stimulated cells. So far, several observations have been presented as likely candidates for its regulatory mechanisms. A translocation of soluble kinase to the membranes has been observed in rat brain [11], human neutrophils [12] and *Amoeba* [13]. In accord with these findings, we showed, by using anti-diacylglycerol kinase antibody [14], that the same enzyme protein is distributed in the cytosol and membrane fractions of pig brain. By analogy with protein kinase C activation [2,15], the enzyme translocation may be one of the activation mechanisms of diacylglycerol kinase. However, recent studies with fibroblasts stimulated with platelet-derived growth factor [16,17] showed that the membrane-bound diacylglycerol kinase, which is distinct from the soluble enzyme, is responsible for phosphorylating the stearoyl/arachidonoyl type of

diacylglycerol released in the stimulated cells. In this case, the translocation of soluble enzyme is not required to use membrane-bound diacylglycerol. We recently showed [18] that there are several immunologically distinct kinase isoforms exhibiting a markedly tissue-characteristic occurrence. It is not yet known whether there are variable regulatory mechanisms operating in different cell types or for different diacylglycerol kinase isoforms.

Previously [19] we found that diacylglycerol kinase can be phosphorylated by an endogenous protein kinase present in crude pig brain extracts. This observation suggested that the kinase could be a phosphoprotein, although no detailed studies were made on the nature of the protein kinase and on the effects of enzyme phosphorylation. In view of certain similarities so far described for the action of diacylglycerol kinase and protein kinase C, such as utilization of diacylglycerol as substrate or activator, intracellular translocation and phosphatidylserine-dependencies, we decided to address the question of whether protein kinase C can regulate the lipid kinase by phosphorylation. The present work shows, by using highly purified enzymes, that diacylglycerol kinase is actively phosphorylated by protein kinase C, and that the protein phosphorylation enhances binding of the lipid kinase to phosphatidylserine membranes.

EXPERIMENTAL

Materials

$[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (4000 Ci/mmol) was bought from ICN Radiochemicals. 12-*O*-Tetradecanoylphorbol 13-acetate (TPA) and phosphatidylserine were bought from P-L Biochemicals and Avanti Polar Lipids respectively. The catalytic subunit of cyclic AMP-dependent protein kinase (protein kinase A) and histone type III-S were

Abbreviations used: PAGE, polyacrylamide-gel electrophoresis; protein kinase A, the catalytic subunit of cyclic AMP-dependent protein kinase; TPA, 12-*O*-tetradecanoylphorbol 13-acetate.

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purchased from Sigma. Sigma also supplied 1,2-diolein, alkaline phosphatase and proteinase inhibitors (aprotinin, leupeptin and soya-bean trypsin inhibitor). Other materials have been described previously [18].

Purification of diacylglycerol kinase and protein kinase C

Both enzymes were purified from the cytosol of frozen pig thymus, which was collected on solid CO₂ at the local slaughter-house. All operations were conducted at or below 4 °C.

Diacylglycerol kinase was purified as 80 kDa protein by essentially the same methods as described for pig brain [20,21]. The enzyme preparation was more than 90% pure, as judged by densitometric scanning after SDS/PAGE and staining with Coomassie Brilliant Blue. Specific enzyme activity was 1.5 μ mol of phosphatidate formed/min per mg of protein when assayed under the conditions described below. The enzyme could be stored at -80 °C, but some inactivation occurred during a prolonged storage (30% loss after 3 months).

Protein kinase C was purified by the procedures designed in view of the results described by Woodget & Hunter [22] and Jaken & Kiley [23] for rat brain. The thymus (100 g) was homogenized with 300 ml of 20 mM-Tris/HCl (pH 7.4)/0.25 M-sucrose/5 mM-EDTA/10 mM-EGTA/0.1% (v/v) β -mercaptoethanol containing 0.1% aprotinin, 0.1 mM-phenylmethanesulphonyl fluoride, 4 μ g of leupeptin/ml and 4 μ g of soya-bean trypsin inhibitor/ml. The cytosol obtained by ultracentrifugation (100 000 g for 30 min) was applied to a column of DEAE-cellulose DE-52 (Whatman; 5.6 cm $\times$ 9 cm) pre-equilibrated with 20 mM-Tris/HCl (pH 7.4)/1 mM-EDTA/0.1% β -mercaptoethanol. Protein kinase C was eluted with 1 litre of a linear NaCl gradient (25–300 mM) in the equilibration buffer. The enzyme was precipitated with solid (NH₄)₂SO₄ (80% satn.), and, after dialysis against 20 mM-potassium phosphate (pH 7.5)/2 mM-EDTA/0.1% β -mercaptoethanol/10% (w/v) glycerol, was applied to a hydroxyapatite (Bio-Rad) column (2 cm $\times$ 19 cm) equilibrated with the same buffer. Elution with 250 ml of a linear 20–500 mM-potassium phosphate gradient yielded two enzyme peaks, at 90 and 125 mM phosphate. The peak at 125 mM-phosphate, which represented approx. 70% of the recovered enzyme activity, was further purified by phosphatidylserine affinity chromatography as described by Uchida & Filburn [24]. The eluted enzyme was concentrated with solid poly(ethylene glycol 6000) and dialysed against 20 mM-Tris / HCl (pH 7.4) / 1 mM-EDTA / 0.1% β -mercaptoethanol/100 mM-NaCl/10% glycerol. The final enzyme preparation was stabilized by Triton X-100 at a final concn. of 0.05% [25]. The enzyme was stored at -80 °C. The purified protein kinase C had a specific enzyme activity of 400 nmol/min per mg of protein when assayed with type III-S histone as described below. In the presence of EGTA instead of Ca²⁺, the activity was decreased to 20–30 nmol/min per mg of protein. On SDS/PAGE, the enzyme preparation contained several bands in addition to 80 kDa protein (about 30% of total protein), which was judged to be an intact protein kinase C from the results of autophosphorylation experiments (see the Results section).

Enzyme assays

The reaction mixture (100 μ l) for assaying diacylglycerol kinase contained 100 mM-Tris/HCl (pH 7.4), 20 mM-

NaF, 1 mM-dithiothreitol, 10 mM-MgCl₂, 100 mM-NaCl, 2 mM-EGTA, 0.5 mM-diolein, 0.2 mM-phosphatidylserine, 1 mg of bovine serum albumin/ml, 2 mM-[γ -³²P]-ATP (5000–10 000 c.p.m./nmol) and purified enzyme (10–100 ng of protein). The required amounts of phosphatidylserine and diolein in chloroform were mixed, dried under N₂, and sonicated in 10 mM-Tris/HCl (pH 7.4)/150 mM-NaCl before use. After incubation of the mixture at 30 °C for 3 min, labelled phosphatidate was extracted as described previously [18].

Protein kinase C activity was measured in the reaction mixture (30 μ l) containing 20 mM-Tris/HCl (pH 7.4), 10 mM-MgCl₂, 0.5 mM-CaCl₂, 0.5 mg of histone type III-S/ml, 50 nM-TPA, 100 μ g of phosphatidylserine/ml, 100 μ M-[γ -³²P]ATP (500–5000 c.p.m./pmol) and enzyme. The reaction was done in the linear range with respect to incubation time and enzyme amounts. After incubation of the mixture at 30 °C for 5 min, the reaction was stopped by adding 1 ml of 10% (w/v) trichloroacetic acid containing 5 mM-potassium phosphate and 1 mM-ATP together with 100 μ l of 1% (w/v) bovine serum albumin solution. The acid-precipitable materials were collected on glass-fibre filters (GC 50; Toyo Roshi, Tokyo, Japan), washed, and counted for radioactivity in toluene-based scintillator. The Ca²⁺-independent activity was assayed by replacing Ca²⁺ with 2 mM-EGTA in the above reaction mixture. To measure protein kinase A activity, TPA, phosphatidylserine and Ca²⁺ were omitted from the mixture. Other assay conditions were the same as described for protein kinase C.

Phosphorylation of diacylglycerol kinase by protein kinases

Diacylglycerol kinase (100–400 ng of protein) was incubated with protein kinases in the reaction mixture described for the enzyme assay. For protein kinase A, the specific radioactivity of [³²P]ATP was raised to 5000–15 000 c.p.m./pmol. The reaction was stopped by adding 8 μ l of 5-times-concentrated Laemmli sample buffer [26], followed immediately by heating at 90 °C for 5 min. Usually, 20 μ l samples were then subjected to SDS/PAGE as described by Laemmli [26]. The gels were stained with Coomassie Brilliant Blue, dried, and autoradiographed (Fuji X-ray film) with an intensifying screen at -80 °C. The radioactive bands were cut out and counted for radioactivity in toluene scintillator. The enzyme phosphorylation was expressed as pmol of ³²P incorporated into the 80 kDa protein band. In the present study, both protein kinase C and diacylglycerol kinase migrated as a 80 kDa band upon SDS/PAGE. Therefore the phosphorylation of diacylglycerol kinase was estimated by subtracting the 80 kDa radioactivity observed in parallel incubations of protein kinase C alone.

When diacylglycerol kinase activity was assayed after preincubation with protein kinase C, [³²P]ATP was replaced with the same concentration of non-radioactive ATP, and the reaction was stopped by adding 3 μ l each of 200 mM-EGTA and 1% bovine serum albumin. Samples (usually 10 μ l) were taken for measuring the activity under the standard assay conditions. In some experiments, diacylglycerol kinase activity and phosphorylated proteins were analysed after sedimenting phosphatidylserine liposomes. In this case, the preincubated mixture was added with 30 μ l of 2 mM-phosphatidylserine suspension in 10 mM-Tris/HCl (pH 7.4)/

150 mM-NaCl in addition to EGTA and bovine serum albumin. After vortex-mixing, the mixture was spun down at 4 °C in a micro-centrifuge (Kubota, Tokyo, Japan) at 15000 rev./min for 5 min. The supernatant was carefully aspirated, and the phosphatidylserine pellets were suspended by a brief sonication in 50 μ l of 25 mM-Tris/HCl (pH 7.4)/0.25 M-sucrose/1 mM-EDTA/0.5 mM-dithiothreitol containing 1 mg of bovine serum albumin/ml. Both supernatant and pellet were subjected to enzyme assay or SDS/PAGE analysis.

Other analytical procedures

Phosphorylated diacylglycerol kinase was immunoprecipitated by using antibody against pig brain enzyme as described previously [18,19]. Before addition of immune IgG, the phosphorylation mixture was adjusted to 1% (w/v) Triton X-100 to solubilize phosphatidylserine-bound proteins. Phosphoamino acid analysis was done by the method of Hunter & Sefton [27], as described previously [19]. Two-dimensional PAGE (isoelectrofocusing followed by SDS/PAGE) was performed by O'Farrell's method [28]. V8 protease (*Staphylococcus aureus*, ICN Immunobiologicals) peptide mapping was performed by the method of Cleveland *et al.* [29].

RESULTS AND DISCUSSION

Characteristics of diacylglycerol kinase phosphorylation

In the initial experiments, we attempted to confirm that the phosphorylation of diacylglycerol kinase did occur by protein kinase C. As shown in Fig. 1, incubation of diacylglycerol kinase alone did not give radioactive proteins, in agreement with our previous observation with pig brain enzyme [19]. Incubation of the protein kinase C preparation gave a radioactive 80 kDa band, and this radioactivity was markedly enhanced when diacylglycerol kinase was incubated together with it. The 80 kDa radioactive material almost disappeared when Ca^{2+} was omitted from the reaction mixture (Fig. 1, lane d). Since protein kinase C was purified by phosphatidylserine affinity chromatography, the results indicated that diacylglycerol kinase could serve as substrate for protein kinase C. To confirm this, phosphorylated proteins were immunoprecipitated with anti-diacylglycerol kinase antibody, which was shown to be reactive with pig thymus 80 kDa enzyme [18] (Fig. 2). The antibody precipitated 75–80% of the 80 kDa radioactivity from the mixture containing the two enzymes, but failed to react with autophosphorylated protein kinase C. Since both of the two enzymes gave a radioactive 80 kDa band, the extent of phosphorylation of diacylglycerol kinase was estimated from the radioactivity subtracted with that obtained from incubations of protein kinase C alone. Such assessment of the enzyme phosphorylation was verified by the results of immunoprecipitation experiments using 100 ng of diacylglycerol kinase and various amounts of protein kinase C (activity of 10–100 pmol/min). In this case, the precipitated radioactivity could always account for 80–90% of the enzyme phosphorylation, which was calculated in a parallel experiment by subtracting the radioactivity due to protein kinase C autophosphorylation.

Fig. 3 shows that protein kinase C actively phosphorylated diacylglycerol kinase, reaching a stoichiometry of 1 mol of ^{32}P /mol of the enzyme (on the basis of

Fig. 1. Phosphorylation of diacylglycerol kinase by protein kinase C

Diacylglycerol kinase (100 ng of protein) and protein kinase C (75 ng) were incubated separately or together for 10 min as described in the text. After boiling the mixture in SDS-sample buffer, equal samples were analysed by SDS/PAGE, followed by autoradiography: (a), diacylglycerol kinase alone; (b), protein kinase C alone; (c), diacylglycerol kinase plus protein kinase C; (d), the same as in lane (c), except for the presence of 2 mM-EGTA instead of Ca^{2+} .

a molecular mass of 80 kDa). In contrast, the enzyme phosphorylation by protein kinase A proceeded to a very limited extent. In these experiments, a pretreatment of diacylglycerol kinase with alkaline phosphatase did not affect the enzyme phosphorylation. This may suggest that the purified enzyme had not been significantly phosphorylated.

When the concentration of protein kinase C was increased, the autophosphorylation was accelerated in a sigmoidal curve, and at higher concentrations phosphorylation of diacylglycerol kinase was unexpectedly inhibited (Fig. 4). This observation was not due to an interaction between the two enzymes, since the use of a wide range of diacylglycerol kinase concentrations (50–400 ng of protein) gave a similar inhibition of the enzyme phosphorylation. It appears that protein kinase C became autophosphorylated in preference to phosphorylating diacylglycerol kinase, and that the autophosphorylated enzyme could not phosphorylate the lipid kinase. In practice it was necessary to use a low concentration of protein kinase C (less than 100 ng of protein) to study the enzyme phosphorylation in the present work. The concentration-dependency of protein kinase C labelling appeared to fit in a concept of intermolecular mechanisms for its autophosphorylation. However, the autophosphorylation was reported to be

Fig. 2. Immunoprecipitation of phosphorylated proteins with antibody against diacylglycerol kinase

Diacylglycerol kinase and protein kinase C were incubated as described for Fig. 1. The reaction was stopped by adding 3 μ l each of 200 mM-EGTA and bovine serum albumin (10 mg/ml). The mixture was treated successively with immune IgG (50 μ g) and 50 μ l of 10% (w/v) Pansorbin (Calbiochem). Immunoprecipitates were collected by a brief centrifugation, and then were boiled in SDS-sample buffer. Equal samples of the original reaction mixture (lanes *a* and *b*) were analysed by SDS/PAGE in parallel with immunoprecipitates (lanes *c* and *d*). Lanes (*a*) and (*c*), incubation of protein kinase C alone; lanes (*b*) and (*d*), incubation of diacylglycerol kinase plus protein kinase C.

due to an intrapeptide process [30,31]. We do not know the reason for this discrepancy.

As shown in Fig. 5, purified diacylglycerol kinase gave a broad band around pI of 6.8 in a two-dimensional PAGE analysis. The pI values of the enzyme phosphorylated by either protein kinase C or protein kinase A were not much different from that of non-phosphorylated enzyme. In repeated experiments, enzyme phosphorylated to different extents (0.2–0.6 mol of 32 P/mol) gave a similar pI value. Why the pI values were not affected by phosphorylation remains unknown, though a similar observation has been made for phosphorylated phosphoinositide-specific phospholipase C [32]. A very similar peptide-mapping pattern was obtained by V8-protease treatment of the enzyme labelled by the two protein kinases (results not shown). Both protein kinases phosphorylated exclusively serine residues (results not shown). Therefore we could not detect significant differences in the mode of enzyme phosphorylation by the two protein kinases. In a variety of cells and tissues, acidic 80 kDa protein (pI approx. 4.4) has been shown to be a major substrate of protein kinase C (see, e.g., [33–35]). The function of this protein is unknown, and this protein was confirmed to be distinct from diacylglycerol kinase in the present work.

Fig. 3. Time course of diacylglycerol kinase phosphorylation by protein kinases

Diacylglycerol kinase (105 ng) was incubated with either protein kinase C (●) or protein kinase A (▲). After incubation for various time periods, radioactivity incorporated into 80 kDa protein was determined by SDS/PAGE. The amount of the two protein kinases was adjusted to an activity of 30 pmol/min, based on their activity toward type III-S histone. Incubation of protein kinase C alone (○) was done in parallel, and the 80 kDa radioactivity corresponding to autophosphorylation was subtracted from that found when diacylglycerol kinase was present (△). This radioactivity was considered to represent diacylglycerol kinase phosphorylation.

Properties of phosphorylated diacylglycerol kinase

The influence of enzyme phosphorylation on diacylglycerol kinase activity was difficult to assess, owing to the thermal lability of the enzyme. As presented in Fig. 6, preincubation without protein kinase C caused a considerable loss of enzyme activity, and this inactivation did not occur when protein kinase C was present. Enzyme degradation was not responsible for the inactivation, since immunoblotting of the preincubated enzyme did not show significant loss of the 80 kDa protein (result not shown). Further, the 80 kDa protein was the sole radioactive product even after incubation for 1 h with protein kinase C. Thermal instability of diacylglycerol kinase has also been noted with crude enzymes from different sources [17,18,36]. Kinetic

Fig. 4. Effect of protein kinase concentrations on the phosphorylation of diacylglycerol kinase

Diacylglycerol kinase (350 ng) was incubated for 10 min with various concentrations of protein kinase C (●) or protein kinase A (▲). The incubation of protein kinase C alone (○) was also included to estimate the phosphorylation of diacylglycerol kinase (△). The amounts of protein kinases are expressed as pmol/min, based on their activity toward histone.

studies showed that the $V_{\max}$ of the enzyme after a 10 min pre-incubation (0.4 mol of phosphate/mol) was not much different from, or sometimes less than, that estimated for non-incubated enzyme (1.0 μ mol/min per mg). Further incubation of the enzyme with protein kinase C failed to increase the $V_{\max}$ value. In these studies, the optimal concentration of phosphatidylserine (0.2 mM) remained unchanged by the phosphorylation. The present assay conditions could not define the micellar condition of diacylglycerol added in different concentrations. Therefore the kinetic studies described here provided only apparent K_m values for diacylglycerol. In this respect, a mixed micellar assay using octyl glucoside was developed for *Escherichia coli* diacylglycerol kinase [36,37] and has recently been applied to the Swiss 3T3 fibroblast enzyme [16,17]. However, octyl glucoside was extremely inhibitory for the thymus 80 kDa diacylglycerol kinase (more than 95% inhibition at 55 mM), and we could not use this assay system. In spite of these difficulties, comparison of the kinetic data between phosphorylated and non-phosphorylated enzymes enabled us to conclude that the major effect of protein phosphorylation was an enzyme stabilization rather than activation.

Protein kinase C is known to bind to phosphatidylserine vesicles containing diacylglycerol or phorbol esters [2,15]. Besterman *et al.* [11] showed that diacylglycerol kinase can also bind to phospho-

Fig. 5. Two-dimensional electrophoresis of non-phosphorylated and phosphorylated forms of diacylglycerol kinase

Purified diacylglycerol kinase (5 μ g of protein, *a*) and the enzyme labelled by protein kinase A (*b*) or protein kinase C (*c*) were analysed by two-dimensional PAGE. The enzyme phosphorylation was conducted under the conditions described in Fig. 3. Only the portions of the Coomassie Brilliant Blue-stained gel (*a*) and autoradiographs (*b,c*) containing 80 kDa protein are shown. In phosphorylation experiments, labelled enzyme was immunoprecipitated as described in Fig. 2. Before analysis, the enzyme was dissociated from the immune complex with urea [28]. The pH of electrofocusing gels is also presented.

tidylserine/diacylglycerol membranes, although phorbol ester-containing membranes were not tested. In the immunoprecipitation experiments (Fig. 2), we noticed that, without added Triton X-100, both protein kinase C and diacylglycerol kinase were pelleted upon centrifugation even in the absence of the antibody. On the basis of this observation, radioactive proteins were analysed separately for soluble and pelleted fractions in the experiments shown in Fig. 7. After being phosphorylated, both enzymes were almost exclusively recovered in phosphatidylserine pellets, with very little remaining in the supernatant. Diacylglycerol kinase labelled by protein kinase A, which would not interact with phospholipids or phorbol esters, was also sedimented mostly in phosphatidylserine pellets. This indicated that an association of protein kinase with membranes was not a prerequisite to the finding. Incubation with protein kinase A yielded several radioactive proteins in addition to the 80 kDa band. This was not due to the degradation of diacylglycerol kinase, since these proteins were labelled to a similar extent even in the incubation of protein kinase A alone. Two possibilities arise from the present finding: firstly, diacylglycerol kinase exists, irrespective of phosphorylation status, exclusively as a membrane-bound form in the reaction mixture. Alternatively, the phosphorylation is a required or favourable condition for the kinase to become membrane-associated. In order to test these possibilities, the kinase activity was monitored during the incubation in both soluble and pelleted fractions. The extent of enzyme phosphorylation was simultaneously assessed in a parallel incubation. Fig. 8 shows that the loss of soluble activity occurring in the presence of protein kinase C was accounted for by an

Fig. 6. Effect of enzyme phosphorylation on diacylglycerol kinase activity

Expt. (a): diacylglycerol kinase (350 ng) was incubated alone (●) or with protein kinase C (activity 50 pmol/min; ○) for 0–20 min. The reaction was stopped with EGTA and bovine serum albumin as described in the text. For 0 min incubation, EGTA and albumin were added before protein kinase C, and the mixture was kept in an ice bath. The lipid kinase activity was measured with 10 μ l samples under the standard assay conditions. Expt. (b): diacylglycerol kinase (50 ng) preincubated with (○) or without (●) protein kinase C for 10 min in Expt. (a) was assayed with various concentrations of diacylglycerol. The concentrations of other reaction components were the same as described in the text. Separate experiments showed that the kinase incorporated 0.4 mol of phosphate/mol. Double-reciprocal plots of the kinase activity versus diacylglycerol concentrations are presented.

Fig. 7. Analysis of phosphorylated diacylglycerol kinase in phosphatidylserine vesicle and soluble fractions

Diacylglycerol kinase was incubated for 10 min with protein kinase C (lanes c and d) and protein kinase A (lanes e and f) as described for Fig. 3. Protein kinase C was also incubated alone (lanes a and b). Exceptionally, the phosphorylation by protein kinase A was conducted under the reaction conditions for protein kinase C (in the presence of Ca^{2+} , phosphatidylserine and TPA), to make a comparison between the two enzymes. The reaction was stopped as described in the text, and soluble and phosphatidylserine pellet fractions were separated by a centrifugation. Half aliquots of both fractions were analysed by SDS/PAGE. Lanes (a), (c) and (e), soluble fraction; lanes (b), (d) and (f), phosphatidylserine pellets.

increase of membrane-bound activity. On the other hand, incubation of diacylglycerol kinase alone resulted in a loss of soluble activity, which could not be explained by enzyme translocation. The results of the enzyme-phosphorylation studies showed that the kinase remaining in the supernatant was little phosphorylated. Some radioactivity in the supernatant could be due to unilamellar phospholipid vesicles, which could not be completely sedimented. We failed to define exact stoichiometric relationships between the enzyme translocation and the extent of enzyme phosphorylation. For instance, the decrease in soluble activity in the presence of protein kinase C should have been greater than that observed with control incubations, owing to simultaneously occurring inactivation and membrane association. It was difficult to account for this discrepancy. The use of nanogram amounts of the enzyme might have contributed to the inactivation, and the addition of protein kinase C might have stabilized diacylglycerol kinase non-specifically through the effects of increased protein concentration. In this respect, the addition of bovine serum albumin considerably stabilized the lipid kinase during the control incubation, but suppressed the activity of protein kinase C utilizing histone or diacylglycerol kinase. In spite of this discrepancy, the results show that the phosphorylation greatly enhances the enzyme association with membranes, resulting in enzyme stabilization. The finding is in accord with the reported stabilization of rat brain diacylglycerol kinase by the presence of phosphatidyl-

Fig. 8. Phosphatidylserine-bound and soluble forms of diacylglycerol kinase after phosphorylation by protein kinase C

Expt. (a): diacylglycerol kinase (160 ng) was incubated alone (○) or with protein kinase C (activity 30 pmol/min; ●) for 0–20 min in the presence of 100 μ M-ATP (unlabelled). Soluble (----) and phospholipid-pellet (—) fractions were separated as described for Fig. 7, and the enzyme activity was assayed under the standard reaction conditions. Expt. (b): the same incubations as in Expt. (a) were done, except for the use of [γ - 32 P]ATP (4000 c.p.m./pmol). The 80 kDa radioactivity in soluble (----) and phosphatidylserine-pellet (—) fractions was determined by SDS/PAGE. ○, Protein kinase C alone; ●, diacylglycerol kinase plus protein kinase C.

serine [11]. However, the membrane association of non-phosphorylated enzyme occurred to a much more limited extent in comparison with rat brain enzyme. We do not know whether this difference is due to different properties of rat brain and pig thymus enzyme, or to the use of phosphatidylserine membranes containing phorbol ester instead of diacylglycerol. We avoided the use of diacylglycerol as protein kinase C activator, since it was difficult to assess the influence of phosphatidate accumulated in the reaction mixture.

The functional role of soluble diacylglycerol kinase has not been precisely defined. The soluble enzyme requires phospholipids as activators, and its substrate, diacylglycerol, would be generated in membranes of agonist-stimulated cells. We have noted (K. Yamada, F. Sakane & H. Kanoh, unpublished work) that the distribution patterns of the 80 kDa enzyme between soluble and membrane fractions are quite variable, depending on the cell type. In thymocytes and other lymphoid cells, this enzyme species is present predominantly as soluble form, whereas in the brain, almost equal amounts of the enzyme are distributed in soluble and particulate fractions. Our present findings suggest the intriguing possibility that the membrane-bound 80 kDa kinase could be a phosphorylated species. This can be investigated by using intact lymphocytes. We could not detect marked differences in the enzyme-phosphorylation patterns catalysed by protein kinases A and C. Both protein kinases phosphorylated the lipid kinase in a very similar manner, as detected by peptide mapping, two-dimensional PAGE and phosphoamino acid analysis. Both enzymes similarly gave membrane-bound lipid kinase after phosphorylation. Although detailed studies with intact cells are needed to confirm the physiological significance of the present findings, it seems likely that the translocation of soluble kinase to membranes could be caused by any

types of different serine-protein kinases in addition to the protein kinases A and C tested herein.

Recently, the related enzyme phosphatidylinositol kinase has been shown to be associated with platelet-derived growth factor receptor [38] and oncogene products [38–40]. For diacylglycerol kinase, there is circumstantial evidence indicating its functional association with oncogene products [41–43]. The effects of tyrosine phosphorylation on diacylglycerol kinase would be an interesting subject for further investigation.

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