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Biochemistry

Phosphatidate-dependent protein phosphorylation

(protein kinases/lipid second messengers/protein kinase C)

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ABSTRACT **Phosphatidate-dependent protein phosphorylation was observed in soluble extracts from rat liver, brain, lung, and testis. The phosphorylation was stimulated by free Ca^{2+} in the range of 360–800 nM. Incubation mixtures containing phosphatidate provided markedly different profiles of protein phosphorylation from those with phosphatidylserine plus 1,2-diolein. Phosphatidate-dependent phosphorylation of a 30-kDa protein in the soluble fraction from heart was also observed. This phosphorylation did not require Ca^{2+}. Soluble fractions from liver, testis, brain, and lung phosphorylated the 30-kDa heart protein in a phosphatidate-dependent Ca^{2+}-independent manner. We propose that part of the action of phosphatidate in cells may be mediated by a protein kinase(s).**

Phosphatidic acid (PA) has been suggested to be a second messenger (1, 2) and recently has been the object of much study. In a wide variety of cell types, a phospholipase D acting on phosphatidylcholine has been shown to be activated rapidly by diverse agonists (for review, see ref. 3). Agonists can also elicit PA through the phosphorylation of diacylglycerol and through a phospholipase D acting on phosphatidylinositol (4–6). In studies designed to investigate the cellular actions of PA, workers have incubated cells with exogenous PA or with microbial phospholipase D. These studies have implicated PA in the regulation of DNA synthesis (7–10); platelet aggregation (11); superoxide formation by neutrophils (12); the induction of c-*myc* (7), c-*fos* (7), and platelet-derived growth factor (10); the release of insulin (13), parathyroid hormone (14), and aldosterone (15); steroidogenesis (16); and cAMP formation (17, 18). The mechanism of action of PA is unknown, but by analogy with many well-characterized second messenger systems, PA may act through a protein kinase. We show in this study that PA-dependent protein phosphorylation occurs in soluble preparations from rat liver, heart, brain, spleen, lung, and testis.

MATERIALS AND METHODS

Materials. Lipids were purchased from Avanti Polar Lipids. PA was derived from egg lecithin and phosphatidylserine (PS) was purified from bovine brain. Protein substrates, ATP, sucrose, and EGTA were from Sigma. Protease inhibitors were from Transformation Research (Framingham, MA) and isotopes were from New England Nuclear/DuPont. Peptide protein kinase substrates were from Peninsula Laboratories. Protogel acrylamide was purchased from National Diagnostics (Manville, NJ). SDS, *N,N,N',N'*-tetramethylethylenediamine, Coomassie brilliant blue R-250, and ammonium persulfate were from BRL. Protein molecular weight standards were from Bio-Rad.

Preparation of Cytosol. Male 200-g Sprague–Dawley rats (Harlan–Sprague–Dawley) were killed by cervical dislocation. Their tissues were removed immediately and placed in ice-cold 250 mM sucrose/1 mM EGTA/5 mM NaHepes, pH 7.5, containing leupeptin (40 μg/ml) and antipain (40 μg/ml). The tissues were minced in this buffer, washed several times by decantation, and homogenized in this buffer (28 ml/g of tissue) using a Dounce homogenizer. The homogenates were centrifuged at 37,000 × g for 20 min and the supernatant fraction was removed for assay.

Assay of Protein Phosphorylation. Samples (10–40 μl) of tissue supernatant fractions were incubated with 200 μM [γ-^{32}P]ATP (2000–4000 cpm/pmol)/2 mM $MgCl_2$/1 mM EGTA (total)/50 mM NaHepes, pH 7.2, in a final volume of 100 μl. $CaCl_2$ and lipids were included at the concentrations indicated in the figure legends. Lipids were sonicated at 10 times the final concentration in H_2O for 15 min in a bath-type sonicator. Reaction mixtures were incubated at 37°C with shaking for 4 min, and reactions were terminated by the addition of 100 μl of 2× concentrated SDS sample buffer (35). Samples were boiled immediately for 3 min and analyzed by SDS/PAGE using 10% polyacrylamide gels 32 cm long (Hoefer). Gels were stained with Coomassie blue, destained, and subjected to autoradiography with X-OMAT AR (Kodak) or Cronex (DuPont) films using intensifier screens except when densitometric measurements were performed. Exposures were usually overnight.

In some experiments, radioactive bands were excised from the gels and subjected to liquid scintillation counting; in others, films were quantitated by densitometry using an LKB 2202 Ultroscan laser densitometer and a Varian DS 650 integrator. Phosphorylation of peptide substrates was assessed using the conditions above with the peptides at 200 μM. Incubations were terminated by the addition of 25 μl of 300 mM NaF/100 mM EDTA, pH 7.5, and 70 μl was applied to a phosphocellulose paper (Whatman) and processed as described by Roskoski (19). Proteins were assayed by the bicinchoninic acid method (Pierce). Free Ca^{2+} concentrations in Ca–EGTA buffers were calculated using the COMICS program (20).

RESULTS

Phosphorylation of Soluble Proteins. In preliminary experiments (data not shown), we observed PA-dependent protein phosphorylation in the soluble but not in the particulate fraction from rat liver. Fig. 1 demonstrates that soluble fractions from rat liver (Fig. 1*A*) and heart (Fig. 1*B*) have a PA-dependent phosphorylation activity. PA-dependent phosphorylation was evident at 30 sec after the initiation of the reaction (data not shown). The substrates were proteins, because the phosphorylated material was sensitive to digestion with Pronase. In the liver, much of the PA-dependent phosphorylation was dependent on the presence of Ca^{2+},

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Abbreviations: PA, phosphatidic acid; PS, phosphatidylserine.
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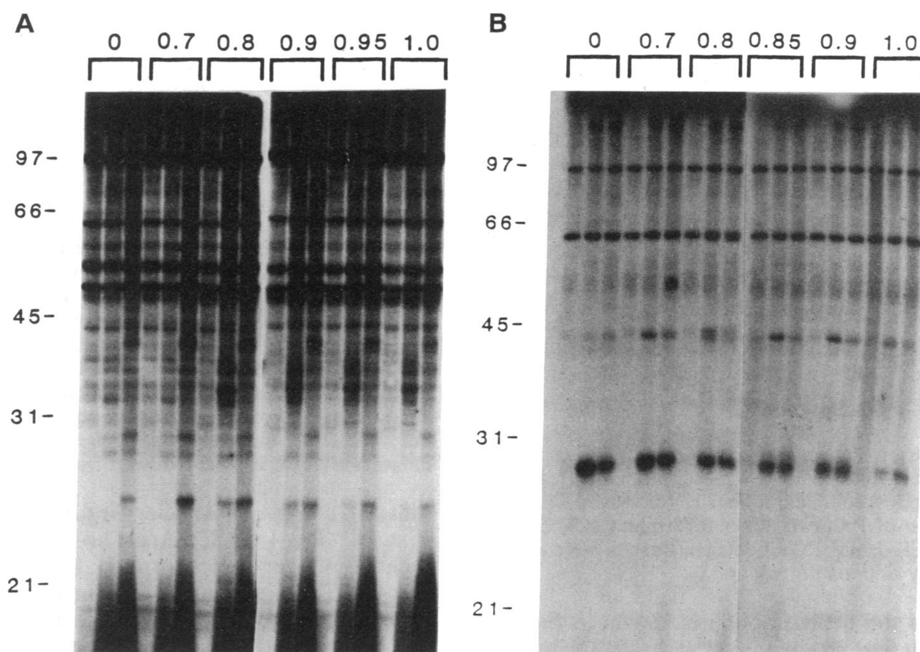

FIG. 1. Lipid and Ca^{2+} dependency of protein phosphorylation in soluble fractions. Liver (*A*) and heart (*B*) soluble fractions were prepared and 40 μl was assayed for protein phosphorylation. Incubation mixtures contained the indicated concentrations of $CaCl_2$ (0–1.0 mM $CaCl_2$, in the presence of 1 mM EGTA). At each concentration of $CaCl_2$, the incubations were carried out under three conditions: namely, no lipid added (left lane in each group), PA at 100 μg/ml (middle lane in each group), and PS at 100 μg/ml plus 1,2-diolein at 10 μg/ml (right lane in each group).

with calculated free Ca^{2+} at 360–800 nM stimulating and higher levels of Ca^{2+} inhibiting activity. When PA was included in the incubation mixture, proteins at 33 and 35 kDa were phosphorylated. These proteins were not phosphorylated in incubation mixtures containing PS plus diolein, activators of protein kinase C. Conversely, proteins were phosphorylated by protein kinase C that were not phosphorylated in incubation mixtures containing PA.

The soluble fraction from heart provided a very different phosphorylation patterns (Fig. 1*B*). In the presence of the Ca^{2+} chelator EGTA, a protein of 30 kDa was heavily phosphorylated in response to PA, whereas free Ca^{2+} at 9 μM acted as an inhibitor. This protein also was phosphorylated in incubation mixtures containing PS plus diolein and so is probably also a substrate for protein kinase C. The relative amounts of 30-kDa phosphorylation due to PA or to PS plus diolein varied with the concentration of the heart supernatant fraction, with more dilute fractions providing a reproducibly higher ratio of PA-dependent activity to PS- plus diolein-dependent activity (data not shown). When soluble fractions from rat brain, testis, and lung were assayed, PA-dependent phosphorylation was observed (data not shown) that had a Ca^{2+} dependency similar to that observed with rat liver (Fig. 1*A*).

The effect of the major phospholipids on protein phosphorylation in the soluble preparations from liver (Fig. 2*A*) and heart (Fig. 2*B*) was assessed. In both tissues, phosphatidylcholine and phosphatidylethanolamine were without effect. PS, which activates protein kinase C, stimulated the phosphorylation of proteins of 24, 28, 36, 45, 52, and 58 kDa in the liver and of 30 kDa in the heart fraction. Phosphatidylinositol was much less efficacious than PS in the liver fraction but also supported the phosphorylation of most of these proteins. PA stimulated the phosphorylation of proteins of 33 and 35 kDa in the liver and of many of the proteins phosphorylated in the presence of PS. The extent of phosphorylation of the 30-kDa heart protein in the presence of PA was similar to that in the presence of PS or phosphatidylinositol. The inclusion of 1,2-diolein at 10 μg/ml was without effect on the PA-dependent phosphorylation but augmented the phosphorylation due to PS or phosphatidylinositol (data not shown), as expected for protein kinase C. These data are indicative of a specific set of proteins that are phosphorylated by a PA-dependent reaction that is not mediated by protein kinase C.

The effect of PA concentration on the phosphorylation of the 33-kDa protein in the liver soluble fraction and of the

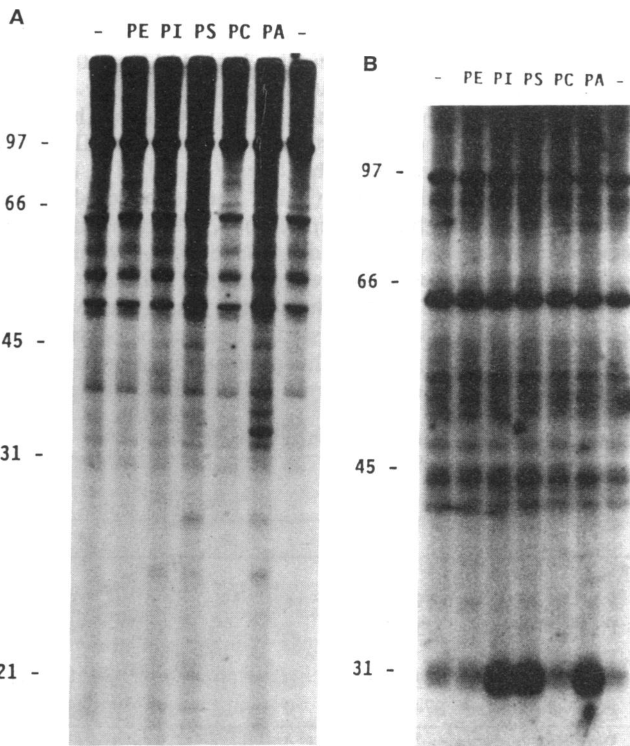

FIG. 2. Phospholipid dependence of protein phosphorylation. Liver (*A*) and heart (*B*) soluble fractions were prepared and 40 μl was assayed in 0.8 mM $CaCl_2$–1 mM EGTA (for liver) or in 0 $CaCl_2$–1 mM EGTA (for heart) plus the indicated phospholipids at 100 μg/ml (both). PE, phosphatidylethanolamine; PI, phosphatidylinositol; PC, phosphatidylcholine.

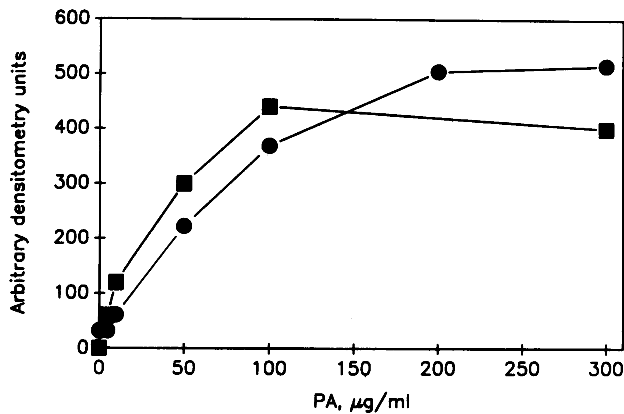

FIG. 3. PA concentration dependence of phosphorylation of the 33-kDa protein from liver and the 30-kDa protein from heart. Liver (squares) and heart (circles) soluble fractions were assayed with the indicated concentration of PA with either 0.85 mM $CaCl_2$–1 mM EGTA (liver) or 0 $CaCl_2$–1 mM EGTA (heart). Results were quantitated by densitometry of the autoradiograms.

30-kDa protein from the heart fraction is shown in Fig. 3. Half-maximal stimulation was obtained at 40–60 μg/ml for both the liver and heart preparations. The liver soluble fraction contained PA at a concentration of 90 ng/ml, as assessed by TLC and Coomassie blue staining; PA was undetectable in the heart fraction.

Phosphorylation of Exogenous Proteins. A variety of exogenous proteins were tested as substrates for PA-dependent phosphorylation by the liver and heart soluble preparations by using SDS/PAGE and measuring radioactivity by scintillation counting in the excised radioactive bands. The heart preparation phosphorylated protamine in a PA-dependent manner but not myelin basic protein, casein, protamine sulfate, filamin, troponin, or histones III-S, V-S, VI-S, and VII-S (Table 1 and data not shown). It should be noted that this method is not as sensitive as autoradiography and is suitable only for highly phosphorylated substrates. The liver preparation phosphorylated protamine and histone III-S in a PA-dependent manner but not histones V-S and VI-S, casein, or phenylalanine hydroxylase (Table 1 and data not shown). In both liver and heart preparations, PS- plus diolein-dependent phosphorylation and PA-dependent phosphorylation exhibited markedly different patterns (Table 1).

Phosphorylation of Peptide Substrates. Using the liver and heart soluble fractions, we tested a number of peptides as substrates for PA-dependent phosphorylation: Arg-Arg-Leu-Ser-Ser-Leu-Arg-Ala (a substrate for S6 kinase and protein kinase C), Arg-Lys-Arg-Ser-Arg-Ala-Glu (cGMP-dependent kinase), Pro-Leu-Ser-Arg-Thr-Leu-Ser-Val-Ser-SerNH_2 (calmodulin-dependent protein kinase), Arg-Arg-Arg-Glu-Glu-Glu-Thr-Glu-Glu-Glu (casein kinase II), Arg-Arg-Lys-Ala-Ser-Gly-Pro-Pro-Val (protein kinase C), Leu-Arg-Arg-Ala-Ser-Leu-Gly (cAMP-dependent kinase), and Arg-Arg-Leu-Ile-Glu-Asp-Ala-Glu-Tyr-Ala-Ala-Arg-Gly and Asp-Arg-Val-Tyr-Ile-His-Pro-Phe (epidermal growth factor receptor kinase and other tyrosine kinases) (21–27). Of these peptides, the peptide substrates for S6 kinase, cAMP-dependent protein kinase, and cGMP-dependent protein kinase were phosphorylated in that order of efficacy by both heart and liver preparations (Table 1). PS plus diolein supported the phosphorylation of the S6 and cAMP-dependent kinase substrates by heart and liver preparations and additionally supported the phosphorylation of the substrate for the cGMP-dependent kinase in the heart preparation (Table 1). The phosphorylation of the S6 peptide required calcium in the liver but did not in the heart, as observed for the endogenous substrates (Fig. 1). These experiments apparently exclude a number of well-characterized protein kinases as candidates for the putative PA-dependent kinase.

Phosphorylation of the 30-kDa Heart Protein. When the heart supernatant fraction was heated to 50°C for 10 min, ≈90% of the kinase activity assayed with either PA or PS plus diolein for the 30-kDa protein was lost. Using this heated heart preparation as a substrate, we assayed for Ca^{2+}-independent PA-dependent phosphorylation in cytosolic extracts that did not have the 30-kDa phosphoprotein (Table 2). Spleen, lung, testis, liver, and brain extracts (in that order of efficacy) phosphorylated the 30-kDa heart protein in a PA-dependent manner. When PS plus diolein were substituted

Table 2. Phosphorylation of the 30-kDa heart protein by soluble fractions of various tissues

	^{32}P incorporated, pmol per 4 min per mg of protein				
	Brain	Liver	Testis	Spleen	Lung
PA	11.9	18.0	22.4	22.9	25.7
PS + diolein	44.3	25.7	25.3	40.0	40.0

Soluble fractions of the tissues were prepared. The heart fraction was heated at 50°C for 10 min to inactivate heart kinases and 20 μl of the heated heart fraction and 20 μl of the tissue soluble fractions were incubated with the phosphorylation mixture [1 mM EGTA in the absence of Ca^{2+} and H_2O, PA (100 μg/ml), or PS (100 μg/ml) plus 1,2-diolein (10 μg/ml)] for 4 min at 37°C. The 30-kDa protein from the heated heart fraction was localized by autoradiography and excised, and radioactivity was analyzed by liquid scintillation chromatography. Values are the average of duplicates corrected for the residual kinase activity in the heated heart fraction.

Table 1. Phosphorylation of exogenous substrates

	Peptide								Protein				
	1	2	3	4	5	6	7	8	Protamine	Histone III-S	Histone V-S	Histone VI-S	Histone VII-S
Heart													
PA	79	27	4	4	0	59	0	0	1.8	0	0	0	0
PS + diolein	138	45	5	5	15	84	0	11	1.7	0	1.3	1.3	0.7
Liver													
PA	542	22	0	0	0	129	0	0	30.1	1.2	0	0	ND
PS + diolein	674	2	0	17	11	201	0	0	23.1	7.0	42	3.2	ND

Peptides were tested at 200 μM. Peptides: 1, Arg-Arg-Leu-Ser-Ser-Leu-Arg-Ala (a substrate for the S6 kinase and protein kinase C); 2, Arg-Lys-Arg-Ser-Arg-Ala-Glu (cGMP-dependent kinase); 3, Pro-Leu-Ser-Arg-Thr-Leu-Ser-Val-Ser-SerNH_2 (calmodulin-dependent protein kinase); 4, Arg-Arg-Arg-Glu-Glu-Glu-Thr-Glu-Glu-Glu (casein kinase II); 5, Arg-Arg-Lys-Ala-Ser-Gly-Pro-Pro-Val (protein kinase C); 6, Arg-Arg-Ala-Ser-Leu-Gly (cAMP-dependent kinase); 7, Arg-Arg-Leu-Ile-Glu-Asp-Ala-Glu-Tyr-Ala-Ala-Arg-Gly; 8, Asp-Arg-Val-Tyr-Ile-His-Pro-Phe (peptides 7 and 8 are substrates for epidermal growth factor receptor kinase and other tyrosine kinases) (21–27). Proteins were tested at 100 μg/ml. Liver incubations were conducted at 0.85 mM $CaCl_2$–1 mM EGTA. Heart incubations were conducted at 1 mM EGTA. Activities in the absence of lipid were subtracted from lipid-dependent activities. ND, not determined. Phosphorylation is expressed as pmol of phosphate incorporated per min per ml.

for PA, the order of efficacy was brain > lung, spleen > testis > liver (Table 2). In a parallel experiment, without the heated heart fraction, the specific activities of the unheated heart fraction were 13.2 and 16.6 pmol/4 min·mg for the PA- and PS- plus diolein-dependent activities, respectively.

DISCUSSION

The data here are suggestive of the existence of a PA-dependent protein kinase(s). Multiple endogenous substrates were phosphorylated in soluble fractions from liver, brain, testis, and lung in a PA- and Ca^{2+}-dependent manner. Ca^{2+} activated at 360–800 nM, which is within the physiological range of cytosolic Ca^{2+} in stimulated cells. The liver preparation also phosphorylated exogenous proteins and peptide substrates in the presence of Ca^{2+}. Soluble heart substrates were phosphorylated in a PA-dependent manner that did not require Ca^{2+}, and a 30-kDa substrate from heart was phosphorylated by soluble fractions from liver, brain, spleen, testis, and lung in a Ca^{2+}-independent PA-dependent fashion.

PA stimulated phosphorylation over the range of 50–200 μg/ml. PA levels are ≈250 pmol/mg (wet weight) in unstimulated hepatocytes (28) and double on treatment with 10 nM vasopressin (29). By assuming a volume of 0.5 μl/mg (wet weight) and a molecular weight of 720 for PA, it can be calculated that PA levels of 360–720 μg/ml are present in the hepatocyte. Thus, the estimated intracellular PA concentration is within the range supporting PA-dependent phosphorylation. The physical state and subcellular localization of PA in stimulated and unstimulated hepatocytes are unknown and would obviously be important in the regulation of PA-dependent phosphorylation.

Although we have not yet been able to exclude substrate and phosphatase effects, the wide range of exogenous substrates and the observation of PA-dependent phosphorylation at 30 sec of incubation argue against these explanations. We believe that these findings are strong evidence for the widespread occurrence of both Ca^{2+}-dependent and Ca^{2+}-independent forms of PA-dependent protein kinase.

Phosphorylase kinase (30, 31) and protein kinase C (32–34) have been reported to be activated by PA under certain conditions. We have not observed PA-dependent phosphorylation of phosphorylase in the liver preparation, but this protein is highly phosphorylated in incubation mixtures without lipid additives, and further phosphorylation may be impossible. PA has been reported to support little protein kinase C activity at physiological levels of Ca^{2+} (32). Additionally, in the liver and other preparations, the pattern of protein phosphorylation observed with PA was very different from that seen with PS plus diolein (Fig. 1*A*). It is evident that the PA-dependent phosphorylation cannot be explained by the usual PS- plus diolein-dependent protein kinase C. The possibility remains that a certain isoform of protein kinase C with a different lipid specificity may mediate PA-dependent phosphorylation. Regardless of the exact mechanism, PA stimulates the phosphorylation of certain proteins. It is possible that the resulting phosphoproteins mediate the effects of PA, whether elicited by agonists or added exogenously. PA-dependent protein kinases may provide the link between hormonal activation of phospholipase D and the cellular actions of PA, establishing PA as a genuine second messenger.

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