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Biochemistry

Purified rat brain calcium- and phospholipid-dependent protein kinase phosphorylates ribosomal protein S6

(phorbol ester tumor promoters/insulin/growth factors/cAMP-dependent protein kinase/protein phosphorylation)

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ABSTRACT **The Ca^{2+}-phospholipid-regulated protein kinase has been purified to homogeneity from a 100,000 × *g* supernatant fluid of rat brain homogenate by a procedure that includes DEAE-cellulose chromatography and successive filtrations on Ultrogel AcA 34 in EGTA and in phosphatidylserine and Ca^{2+}. A more rapid purification consisting of DEAE-cellulose chromatography, Ultrogel AcA 34 gel filtration chromatography, and DEAE-trisacryl chromatography, all in the presence of EGTA, was also developed. Although the enzyme obtained by the latter procedure is not homogeneous, it exhibits properties similar to those of the pure enzyme and is more stable. In addition, the DEAE-trisacryl step permitted resolution of a contaminating Ca^{2+}-inhibitable protein kinase that can interfere with studies of the Ca^{2+}-phospholipid-stimulated enzyme. The homogeneous enzyme, purified about 300-fold, was estimated to have a M_r of 84,000. Its activity was 20- to 30-fold higher in the presence of phospholipid and Ca^{2+} than in the presence of phospholipid and EGTA, EGTA, or Ca^{2+} alone. The specific activity of the activated kinase was 852 nmol of P incorporated into histone per min/mg at 20°C. The pure enzyme underwent autophosphorylation in a Ca^{2+}- and phospholipid-dependent manner. This reaction was inhibited in the presence of histones without affecting the kinetic properties of the enzyme. Under optimal assay conditions, the homogeneous enzyme was activated 10–20% by either 10 μM diolein or 100 nM phorbol 12-myristate 13-acetate. Activation of the purified enzyme by diolein or the phorbol ester was far greater (3- to 4-fold) when aggregated instead of freshly sonicated phospholipids were used, suggesting that these compounds affect the interaction of the enzyme with phospholipids and Ca^{2+}. The purified enzyme catalyzed the phosphorylation of the 40S ribosomal subunit protein S6. The K_m for S6 was ≈1 μM and it was estimated that 2 mol of phosphate were incorporated per mol of S6. The observation that protein S6 can be phosphorylated by the purified Ca^{2+}-phospholipid-dependent protein kinase may link recent reports that phorbol ester tumor promoters activate the Ca^{2+}-phospholipid-dependent protein kinase *in vitro* and stimulate phosphorylation of the ribosomal protein S6 *in vivo*.**

A Ca^{2+}-dependent protein kinase activity that requires phospholipids but not calmodulin was originally described by Takai and colleagues (1, 2) and has been recently purified from bovine heart and from rat brain (3, 4). This new type of protein kinase has been found in a wide variety of tissues and phyla (5, 6). Although protein substrates have been observed in brain (7), heart (8–10), pancreas, liver, vas deferens, adrenal (11), and platelets (12, 13), there is thus far no direct evidence that these phosphorylations are involved in cell regulation and the protein substrates themselves have not been identified. Phenothiazines, anesthetics such as dibucaine, and other phospholipid-interacting drugs inhibit the partially purified Ca^{2+}-phospholipid dependent protein kinase (14–16). Because these drugs also inhibit the functions of Ca^{2+}-calmodulin it has been suggested that the Ca^{2+}-phospholipid-dependent kinase may contain a calmodulin-like domain (17). The recent observation that phorbol ester tumor promoters bind to and activate the Ca^{2+}-phospholipid-dependent protein kinase (18–22) has added a new dimension of interest to this phosphotransferase system. In addition, it has just been reported that the incorporation of phosphate into the 40S ribosomal subunit protein S6 of hepatocytes is increased by the exposure of intact cells to such phorbol esters (23). We now describe a procedure for the complete purification of the Ca^{2+}-phospholipid-dependent protein kinase and demonstrate that this enzyme is activable by the phorbol ester phorbol 12-myristate 13-acetate (PMA) and catalyzes the phosphorylation of the 40S ribosomal subunit protein S6.

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EXPERIMENTAL PROCEDURES

Materials. Histones (III-S), phospholipids, diolein, pepstatin, and aprotinin were from Sigma. [γ-^{32}P]ATP (1 mCi/ml and 30 Ci/mmol or 10 mCi/ml and 3,000 Ci/mmol; 1 Ci = 3.7 × 10^{10} Bq) was purchased from Amersham. Hepes buffer as well as dithiothreitol and leupeptin (acetyl-L-leucyl-L-leucyl-L-arginal) were from Calbiochem. W5, W7, W12, and W13 were a generous gift from H. Hidaka (Mie University, Japan). Ultrogel AcA 34 and DEAE-trisacryl (Industrie Biologique Francaise) were obtained from LKB; diethylaminoethyl-cellulose and P-81 paper were from Whatman. Polyacrylamide gel electrophoresis and protein determination reagents were from Bio-Rad. Calmodulin was isolated from bovine brain (24) according to the method of Dedman *et al.* (25), and the regulatory subunit of the cAMP-dependent protein kinase was purified following the procedure of Erlichman *et al.* (26). The 40S ribosomal subunit was prepared from *Artemia salina* by the procedure described in ref. 27. Scintillant solution "Liquiscint" was from National Diagnostics (Somerville, NJ). X-Omat R and AA films were from Kodak.

Methods. $NaDodSO_4$ (0.1%)/polyacrylamide (10% or 5–15% gradient) gel electrophoreses were performed according to Laemmli (28). Gels were stained by using Coomassie blue or the silver stain method (29). Protein was determined by the Coomassie blue technique (30) using the concentrated protein reagent from Bio-Rad. Phosphorylation of the samples subjected to electrophoresis was achieved under the same conditions as those described below (see assay of the kinase) by using a substrate concentration < 10 μg/ml.

Assay for the Ca^{2+}-Phospholipid-Dependent Protein Kinase. The conditions used were similar to those previously described (1). The standard reaction mixture contained 20 mM Hepes/NaOH buffer, pH 7.5, 10 mM $MgCl_2$, 0.5 mM Ca^{2+} ($CaCl_2$), 60 μg of phosphatidylserine per ml, 6 μg of diolein per ml or 50 nM PMA, 5 mM dithiothreitol, lysine-rich histones at 1 mg/ml, 50 or 100 μM [γ-^{32}P]ATP (specific activity, 500–1,000

Abbreviation: PMA, phorbol 12-myristate 13-acetate.

cpm/pmol), and the enzyme solution to be assayed. Reactions were initiated by the addition of [γ-^{32}P]ATP and were carried out for up to 30 min at 20°C. The reactions were terminated and the ^{32}P-labeled histones were isolated by pipetting 40 μl of the reaction mixture onto Whatman P-81 phosphocellulose papers (1.5 × 1.5 cm) (31). The papers were then washed and assayed for radioactivity in 5 ml of Liquiscint. For electrophoresis, reactions were stopped by adding 0.2 vol of 250 mM Tris·HCl buffer (pH 6.8) containing 5% $NaDodSO_4$, 50% glycerol, 0.01% phenol red, and 0.5% 2-mercaptoethanol and heating at 80°C for 5 min. The following marker proteins were used: β-galactosidase (M_r 116,000), phosphorylase b (M_r 94,000), transferrin (M_r 77,000), bovine serum albumin (M_r 67,000), pyruvate kinase (M_r 57,000), fumarase (M_r 49,000), aldolase (M_r 40,000), carbonic anhydrase (M_r 29,000), and soybean trypsin inhibitor (M_r 21,000). In gel electrophoresis phosphorylation assays, the gels were stained and destained following standard procedures and then dried between two cellophane paper sheets. The dried gels were submitted to autoradiography, after which the radioactive spots were cut out and assayed for radioactivity after overnight incubation in Liquiscint. The Ultrogel AcA 34 columns were calibrated by using apoferritin (61 Å), yeast alcohol dehydrogenase (45 Å), bovine serum albumin (35.5 Å), ovalbumin (27 Å), and α-chymotrypsinogen (21 Å).

Purification of the Ca^{2+}-Phospholipid-Dependent Protein Kinase. A summary of the purification procedure is outlined in Table 1.

Extraction. Fresh brains from four rats were removed and washed in ice-cold buffer A (20 mM Hepes/NaOH buffer, pH 7.5, containing 0.3 M sucrose, 2 mM EDTA, 10 mM EGTA, 2 mM dithiothreitol, 2 mM phenylmethylsulfonyl fluoride, 10 μg of pepstatin per ml, 10 μg of soybean trypsin inhibitor per ml, 10 μg of leupeptin per ml, and 25 μg of aprotinin per ml), which had been degassed under vacuum and saturated with nitrogen prior to the addition of dithiothreitol and proteinase inhibitors. The brain was homogenized in 8 vol of buffer A by using a glass-Teflon pestle homogenizer and the homogenate was centrifuged at 100,000 × *g* for 1 hr. The pellet was discarded and the supernatant fluid that contained >90% of the Ca^{2+}-phospholipid-dependent protein kinase activity was adjusted to an ionic strength of 0.7–0.9 mmho.

DEAE-cellulose chromatography. The 100,000 × *g* supernatant fluid was applied to a DEAE-cellulose column (2 × 5 cm; flow rate, 30 ml/hr) equilibrated with 20 mM Hepes/NaOH buffer, pH 7.5, containing 2 mM dithiothreitol, 2 mM EDTA, and 2 mM EGTA (buffer B). The column was washed with 5 vol of buffer B, followed by washing with 2 vol of buffer B containing 0.1% Triton X-100 and finally 1 vol of buffer B. The enzyme was eluted with a linear gradient of NaCl (0–0.3 M) in buffer B (twice, 250 ml each time). Fractions containing enzyme activity (106–110 ml) were pooled, made 0.2 mg/ml with respect to soybean trypsin inhibitor, and adjusted to 70% saturation in ammonium sulfate. The proteins were allowed to precipitate for 10 min at 4°C and then were centrifuged at 3,000 rpm in a Sorvall HL-8 rotor for 30 min at 2°C. The pellets were dissolved in a minimal volume of 20 mM Hepes/NaOH buffer, pH 7.5, containing 0.5 mM EDTA, 0.5 mM EGTA, and 2 mM dithiothreitol (buffer C).

Ultrogel AcA 34 filtration chromatographies. The concentrated solution (4 ml) was applied to an Ultrogel AcA 34 filtration column (1.6 × 95 cm; flow rate, 12 ml/hr) equilibrated and run in buffer C. Fractions (2 ml) containing Ca^{2+}-phospholipid-dependent protein kinase activity (Fig. 1) were pooled and concentrated by ammonium sulfate precipitation, after addition of 0.2 mg of soybean trypsin inhibitor per ml. The concentrate (2 ml) was dialyzed 6 hr against buffer C, made 60 μg/ml in phosphatidylserine, 6 μg/ml in diolein, and 2 mM in Ca^{2+}, and applied to a second Ultrogel AcA 34 filtration column (1.6 × 95 cm) equilibrated and run in 20 mM Hepes/NaOH buffer, pH 7.5, containing 0.5 mM $CaCl_2$, 2 mM dithiothreitol, 20 μg of phosphatidylserine per ml, 2 μg of diolein per ml, and 50 μg of soybean trypsin inhibitor per ml (buffer D) at 12 ml/hr (Fig. 1). The homogeneous enzyme obtained by this procedure retained full activity for only 1 day at 4°C, despite attempts to stabilize it in either glycerol (10–20%), polyethylene glycol (0.5–1.0%), sucrose (25%), or dithiothreitol (5 mM).

DEAE-trisacryl ion-exchange chromatography. In some experiments, the active fractions from the first AcA 34 gel filtration were loaded directly on a 1 × 1.5 cm column of DEAE-trisacryl previously equilibrated with buffer C. The column was washed sequentially with 25 ml of buffer C, 25 ml of the same buffer containing 0.1% Triton X-100, and 10 ml of buffer C. Enzyme was eluted with a linear gradient of NaCl (0–0.3 M) in buffer C (twice, 75 ml each time) (Fig. 2). Fractions from any step of this procedure could be pooled and made 30% (wt/vol) in sucrose and 2 mM in EGTA. Under these conditions, 80% of the Ca^{2+}-phospholipid-dependent activity is retained after storage for 1 wk at 4°C.

Unless otherwise indicated, all of the experiments reported here were performed with homogeneous enzyme within 24 hr of its complete purification.

RESULTS

Comments on the Purification. The protein kinase activity after the first gel filtration step was activated 15- to 20-fold by the addition of phospholipids in the presence of Ca^{2+}. However, in the absence of phospholipid, activity was higher in the presence of EGTA than in the presence of Ca^{2+}. After the second gel filtration, enzyme activity was eluted as a high molecular weight species (M_r > 300,000). This enzyme was not inhibited by Ca^{2+} in the absence of phospholipid and was activated by both Ca^{2+} and phospholipids. The difference in the sensitivity of the enzyme preparation to activation by phospholipid and Ca^{2+} may be due to the presence of a contaminating Ca^{2+}-

Table 1. Purification of the Ca^{2+}-phospholipid-dependent protein kinase from rat brain

Fraction	Volume, ml	Activation			Specific activity, nmol/min per mg	Protein, mg	Activity, nmol/min	Purification, fold	Yield, %
		PL-Ca^{2+}/Ca^{2+}	PL-Ca^{2+}/EGTA	+PMA/−PMA					
Supernatant fluid	60	10–15	10–15	—	2.6	650.0	1,696.5	1	100
DEAE eluate	108	12–18	12–18	1.33	31.3	35.0	1,102.7	12	65
Filtration I	24	15–20	2	1.16	14.2	30.0	425.0	—	—
Filtration II	12	15–25	15–25	1.10	739.7	0.115	84.8	283	5

The enzyme activity is the difference between the activities in the presence and absence of Ca^{2+} and phospholipids (PL). Activation PL-Ca^{2+}/Ca^{2+} or PL-Ca^{2+}/EGTA represents the activation of the enzyme upon addition of Ca^{2+} and phospholipids; the activities in the presence of Ca^{2+} alone or in the presence of EGTA alone are taken as reference. Activation +PMA/−PMA represents the activation of the enzyme upon addition of PMA (or diolein) in the presence of Ca^{2+} and phospholipids. All other conditions were as described in the text. Filtration I and filtration II refer to the first and the second Ultrogel AcA 34 gel filtrations, respectively.

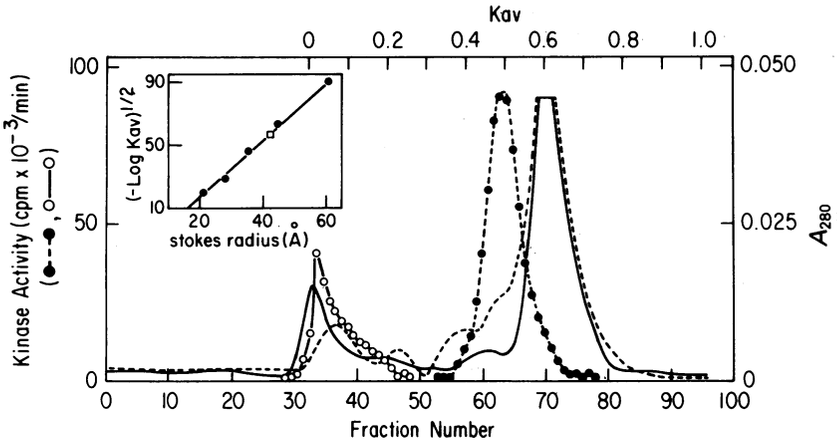


FIG. 1. Ultrogel AcA 34 chromatography of Ca^{2+}-phospholipid-dependent protein kinase. The concentrated DEAE-cellulose eluate was filtered in the presence of EGTA. ---, Absorbancy at 280 nm; ●——●, enzyme activity assayed in the presence of Ca^{2+} and phospholipids. The enzyme collected from the first Ultrogel AcA 34 filtration was concentrated and filtered in the presence of Ca^{2+} and phospholipids. ——, Absorbancy at 280 nm; ○——○, activity of the enzyme assayed in the presence of Ca^{2+} and phospholipids. (*Inset*) Determination of the Stokes radius of the Ca^{2+}-phospholipid-dependent protein kinase. All other conditions were as described in *Results*.

inhibitable, phospholipid-insensitive protein kinase that is removed in the final step of the purification. This is supported by the observation that two peaks of protein kinase activity are eluted from DEAE-trisacryl when this step is performed with the enzyme derived from the first gel filtration (see *Methods*). Enzyme in the first peak was stimulated as much as 30-fold by the addition of Ca^{2+} and phospholipid. Although this enzyme is not homogeneous and has a specific activity that is only 10–20% of that of the pure protein, its properties are the same as those of the fully purified Ca^{2+}-phospholipid-dependent protein kinase. The protein kinase in the second peak was inhibited by Ca^{2+} (Fig. 2). Experiments in which the first peak was isolated and rechromatographed on DEAE-trisacryl suggest that the second peak may be a degraded or modified form of the Ca^{2+}-phospholipid-activated protein kinase in the first peak.

Properties of the Purified Enzyme. From its behavior upon gel filtration in the presence of EGTA, it was possible to calculate a Stokes radius (mean ± SD) of 42.5 ± 0.5 Å and a M_r of 94,639 ± 2,214 or 103,917 ± 2,432, by using for these calculations the published sedimentation coefficient for the rat brain enzyme (2) or the bovine heart enzyme (3), respectively. In $NaDodSO_4$/polyacrylamide gel electrophoresis, the purified enzyme exhibited one protein band corresponding to a M_r of 84,000. Activation by phospholipids was observed only in the presence of Ca^{2+}. In the presence of optimal concentrations of Ca^{2+} and freshly sonicated phospholipids there was a small additional activation of the enzyme (10–20%) (Table 1) by either 10 μM diolein or 100 nM PMA. However, when the phospholipids were allowed to aggregate, the activation by either diolein or PMA increased 3- to 4-fold. The activation observed by a combination of optimal concentrations of diolein and PMA was not greater than either alone. A K_m of 3 μM and a V_{max} of 50 nmol of P incorporated into histone per min/mg were found in the presence of 0.5 mM Ca^{2+}. The addition of phospholipids (K_a, 10 μM for phosphatidylserine) in the presence of Ca^{2+} resulted in an increase in the K_m for ATP to 8–10 μM and an increase of the V_{max} to 772 nmol of P incorporated per min/mg of enzyme. The K_a of the pure enzyme for Ca^{2+} was 3–5 μM in the presence of phosphatidylserine, in the presence or absence of diolein. The enzyme was maximally active between pH 7 and pH 8.5. Activation by Ca^{2+} and phospholipids was constant between pH 6 and pH 9.

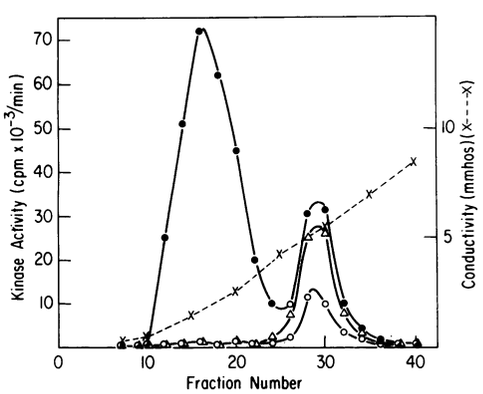


FIG. 2. DEAE-trisacryl ion-exchange chromatography of the active fractions of the first Ultrogel AcA 34 gel filtration. Proteins were eluted by a linear gradient (0–0.3 M NaCl) (x---x), and histone kinase activity was measured in the presence of EGTA (△——△), in the presence of Ca^{2+} (○——○), and in the presence of Ca^{2+} and phospholipid (●——●).

The Ca^{2+}-phospholipid-dependent activity was strongly inhibited by trifluoperazine, a drug known to inhibit many of the Ca^{2+}-calmodulin-dependent enzymes (32) (Fig. 3). The apparent K_i of this drug was about 15 μM. W7 and W13, the chlorinated naphthalenesulfonamide derivatives (33–35), inhibited with apparent K_is of 32 and 60 μM, respectively, whereas W5 and W12, the corresponding nonhalogenated derivatives, did not affect the enzyme activity at the same concentrations.

Like other protein kinases, the Ca^{2+}-phospholipid-dependent protein kinase undergoes autophosphorylation (Fig. 4). This reaction is Ca^{2+}- and phospholipid-dependent. The same kinetic properties were observed for the kinase by using the enzyme itself or exogenous substrates, such as histones or 40S ribosomal subunit protein S6 (see below). Autophosphorylation of the kinase was inhibited in the presence of exogenous substrates. There was complete inhibition in the presence of 0.1 mg of histone per ml and 50% inhibition in the presence of the same amount of 40S ribosomal subunit protein. The state of phosphorylation of the enzyme did not affect its activation by Ca^{2+} and phospholipid or the initial rates of phosphorylation of exogenous substrates. The enzyme was neither phosphorylated nor its enzymic properties altered by incubation for 60 min with 6×10^3 units of the cAMP-dependent protein kinase.

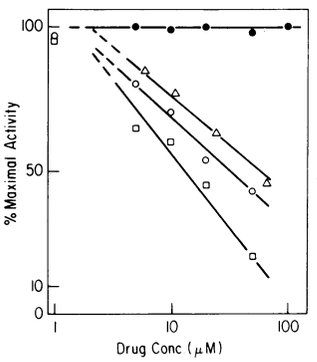


FIG. 3. Effects of drugs on the enzyme activity. Activity of the Ca^{2+}-phospholipid-dependent protein kinase (final concentration, 1 μg/ml) was measured as a function of increasing concentrations of trifluoperazine (□), chlorinated naphthalenesulfonamide derivatives W7 (△) and W13 (○), and the corresponding nonchlorinated derivatives (●) W5 and W12.

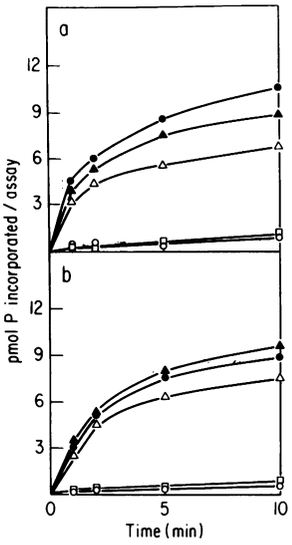
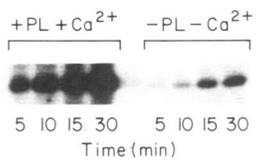


FIG. 4. (*a*) Time course of the autophosphorylation of the Ca^{2+}-phospholipid-dependent protein kinase or (*b*) time course of phosphorylation of ribosomal protein S6 by the Ca^{2+}-phospholipid-dependent protein kinase. Assays were performed by using a final concentration of 1 μg of enzyme protein per ml, in the presence of EGTA (○), in the presence of Ca^{2+} (□), in the presence of Ca^{2+} and phospholipid (△), or in the presence of Ca^{2+}, phospholipid, and either diolein (●) or PMA (▲).

Phosphorylation of Ribosomal Protein S6. Protein S6, a component of the 40S ribosomal subunit, was found to be a substrate for the Ca^{2+}-phospholipid-dependent protein kinase (Fig. 4). The behavior of the enzyme with lysine-rich histones or 40S ribosomal subunit protein S6 as substrate is summarized in Fig. 5. Phosphorylation of S6 is markedly enhanced by phospholipid and Ca^{2+} and, like the phosphorylation of histone, can be stimulated further by diolein or PMA (Fig. 4). Under the conditions employed, the cAMP-dependent protein kinase catalyzed the incorporation of ≈1 mol of P and the Ca^{2+}-phospholipid-dependent protein kinase catalyzed the incorporation of 2 mol of P per mol of S6 (see Fig. 5 legend and Fig. 6*a*). As indicated in Fig. 6*b*, the phosphorylation of ribosomal protein S6 by the Ca^{2+}-phospholipid-dependent protein kinase and by the cAMP-dependent protein kinase appears additive. It should be noted that, at the time indicated by the arrow in Fig. 6*a*, an additional aliquot of the original kinase did not further increase the amount of ^{32}P incorporated into S6.

DISCUSSION

A procedure has been developed that permits a three-step, 280-fold purification of the Ca^{2+}-phospholipid-dependent protein kinase to homogeneity. Ribosomal 40S subunit protein S6 was found to be a substrate for this purified enzyme. The general



Substrate	Km (μM)	Vmax (nmol/min/mg)
Histones (III-S)	4.74	772
S6	0.8	283

FIG. 5. (*Upper*) Autoradiogram showing the time course of phosphorylation of the ribosomal protein S6 in the presence or absence of Ca^{2+} and phospholipids (PL). (*Lower*) Comparison of the kinetic properties of the enzyme with lysine-rich histones or ribosomal S6 protein as substrate. In both experiments the final enzyme protein concentration was 1 μg/ml. Calculation of the molarity of ribosomal protein S6 was based upon the assumption that (*i*) there is one molecule of S6 in each 40S particle, (*ii*) the extinction coefficient of the 40S particle at A_{260} is 11.4 mg^{-1}, and (*iii*) the 40S particle M_r is 1.3×10^6 (36).

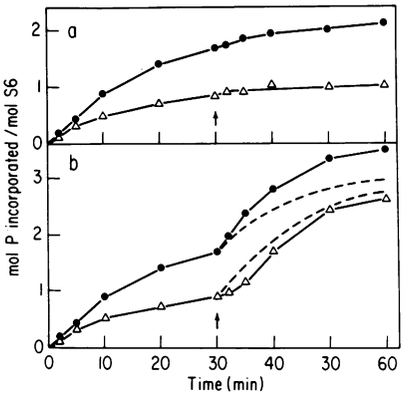


FIG. 6. (*a*) Time course of the phosphorylation of the ribosomal 40S subunit protein S6 by the catalytic subunit of the cAMP-dependent protein kinase (△) (6×10^3 units/ml) and by the Ca^{2+}-phospholipid-dependent protein kinase (●) (1 μg of enzyme protein per ml). At the time indicated by the arrow, an additional aliquot of the same protein kinase was added (see text). (*b*) Time course of the phosphorylation of S6 by the catalytic subunit of the cAMP-dependent protein kinase first and then by the Ca^{2+}-phospholipid-dependent protein kinase (△) (arrow) or by the Ca^{2+}-phospholipid-dependent protein kinase first followed by the catalytic subunit of the cAMP-dependent protein kinase (●) (arrow). Dashed lines indicate theoretical curves predicted from two independent phosphorylations.

properties described for the Ca^{2+}-phospholipid-dependent histone kinase activity are also true for its S6 kinase activity and for the autophosphorylation activity of the enzyme. Phosphorylation of S6 catalyzed by the Ca^{2+}-phospholipid-dependent protein kinase and the cAMP-dependent protein kinase is approximately additive, indicating that these kinases may catalyze phosphorylation at distinct sites. Under the conditions employed, ≈1 P was incorporated into S6 by the cAMP-dependent protein kinase. The Ca^{2+}-phospholipid-dependent protein kinase catalyzed the incorporation of 2 mol of P per mol of S6. Interestingly, the extent of phosphorylation by the cAMP-dependent protein kinase is somewhat greater when S6 is first phosphorylated by the Ca^{2+}-phospholipid-dependent protein kinase. This may explain in part why incorporation of phosphate into S6 by the cAMP-dependent protein kinase has been reported to vary between 1 and 2 mol of P per mol of S6 (36–40). In intact cells, two sites are phosphorylated in response to cAMP (38).

The observation that the Ca^{2+}-phospholipid-dependent protein kinase can catalyze the phosphorylation of S6 is of considerable interest because in intact cells insulin, growth factors such as epidermal growth factor, and other conditions that promote cell growth rapidly induce the phosphorylation of S6 (41–51). Phosphorylation precedes the enhanced synthesis of macromolecules and may be important in ribosome function and protein synthesis (51–53). The first step in the action of these growth-promoting peptides, specific binding to cell-surface receptors, is well documented. Little is known about the subsequent steps or enzymes responsible for the multiple non-cAMP-dependent phosphorylations of S6. The evidence presented here that S6 is a substrate for the purified Ca^{2+}-phospholipid-dependent protein kinase and that this kinase is activated by phorbol ester tumor promoters suggests a role for this enzyme in growth regulation. However, it will be important to compare the sites phosphorylated *in vivo* with those phosphorylated by the Ca^{2+}-phospholipid-dependent protein kinase *in vitro*.*

* Experiments in collaboration with J. Martin-Pérez and G. Thomas indicate that the Ca^{2+}-phospholipid-dependent protein kinase phosphorylates one pair of peptides (10a and 10b) *in vitro* that are also phosphorylated *in vivo* in response to serum or growth factors (ref. 41; unpublished data).

Another observation linking S6 phosphorylation by the Ca^{2+}-phospholipid-dependent protein kinase *in vitro* to physiologically relevant events is that the phorbol ester tumor promoter PMA increases phosphate incorporation into S6 in intact hepatoma cells (23). Our results and those published (19, 20, 54, 55) suggest that the effects of the phorbol esters on the Ca^{2+}-phospholipid-dependent protein kinase may be complex and include facilitation of the interaction of the enzyme with phospholipids as well as its binding to membrane-bound PMA with resultant activation by membrane phospholipids. In the presence of PMA, substrates associated with the plasma membrane or with intracellular membranes such as the endoplasmic reticulum may then become accessible to the active translocated kinase. The observation reported here that the purified enzyme is only slightly activated by phorbol esters (or diolein) in the presence of optimal concentrations of phospholipids and Ca^{2+} but is substantially activated when assayed with suboptimal concentrations of phospholipid is consonant with the suggestion that these compounds act by facilitating interaction of the enzyme with phospholipid. Consistent also is the recent report that PMA induces phosphorylation of serine residues in the epidermal growth factor receptor of intact cells (56). It is possible that the Ca^{2+}-phospholipid-dependent protein kinase is also involved in the phosphorylation of serine residues in the insulin receptor. Thus, the potentially important connection between phorbol ester tumor promoters and growth-promoting hormones may be mediated by Ca^{2+}-phospholipid-dependent phosphorylation of cellular proteins such as S6 and plasma membrane receptors for growth regulatory peptides.

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