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Evidence That Phosphorylase Kinase Exhibits Phosphatidylinositol Kinase Activity†

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ABSTRACT: Phosphorylase kinase phosphorylates the pure phospholipid phosphatidylinositol. Furthermore, it catalyzes phosphatidylinositol 4-phosphate formation using as substrate phosphatidylinositol that is associated with an isolated trypsin-treated Ca^{2+}-transport adenosinetriphosphatase (ATPase) preparation from skeletal muscle sarcoplasmic reticulum. On this basis a fast and easy assay was developed that allows one to follow the phosphatidylinositol kinase activity during a standard phosphorylase kinase preparation. Both activities are enriched in parallel approximately to the same degree. Neither chromatography on DEAE-cellulose nor that on hydroxyapatite in the presence of 1 M KCl separates phosphatidylinositol kinase from phosphorylase kinase. The presence of a lipid kinase, phosphatidylinositol kinase, in phosphorylase kinase is not a general phenomenon; diacylglycerol kinase can be easily separated from phosphorylase kinase. Polyclonal anti-phosphorylase kinase antibodies as well as a monoclonal antibody directed specifically against the α subunit of phosphorylase kinase immunoprecipitate both phosphorylase kinase and phosphatidylinositol kinase.

During our studies on the sarcoplasmic reticular Ca^{2+}-transport adenosinetriphosphatase (ATPase) we obtained indications that phosphorylase kinase might phosphorylate phosphatidylinositol. This hypothesis is based on the following observations: by immunofluorescence an antigen identical with or related to phosphorylase kinase can be located within the sarcolemma and the sarcoplasmic reticulum (SR) (Heilmeyer, 1975; Gröschel-Stewart et al., 1978; Heilmeyer et al., 1980; Varsanyi et al., 1978). In agreement with these histological observations, isolated rabbit skeletal muscle sarcoplasmic reticulum contains membrane-associated phosphorylase kinase

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activity which represents less than 10% of the total activity measurable in a crude extract. In sucrose gradients this membrane-associated kinase activity sediments with the light and heavy SR fractions (Jennissen et al., 1975; Hörl et al., 1978). Recently, these observations were confirmed and extended: phosphorylase kinase activity was also shown to be present in T tubules (Dombradi et al., 1984). It can be concluded that soluble cytoplasmic phosphorylase kinase is not artificially adsorbed to these membranes. Sarcoplasmic reticulum isolated from I-strain mice muscles that are genetically deficient in cytosolic phosphorylase kinase contains also membrane-associated phosphorylase kinase activity in an amount that is approximately equivalent to that present in SR isolated from wild type mice (Varsanyi et al., 1978). This membrane-associated phosphorylase kinase activity certainly does not function as a glycogen phosphorylase phosphorylating enzyme: it is well established that no phosphorylase *b* to *a* conversion occurs in these I-strain mice muscles (Lyon & Porter, 1963). Thus an alternate function must be envisaged for this membrane-associated kinase.

Enhancement of the Ca^{2+}-transport ATPase activity as well as Ca^{2+} uptake by phosphorylase kinase into SR has been demonstrated by two groups independently (Heilmeyer, 1975; Hörl et al., 1975, 1978; Schwarz et al., 1976; Hörl & Heilmeyer, 1978). Thus, a kinase might play a regulatory role in this ion-transport process; the mechanism by which this is exerted remained unclear.

Recently, we have shown that phosphorylase kinase stimulates the phosphorylation of a membrane component present either in sarcoplasmic reticular vesicles or in isolated SR Ca^{2+}-transport ATPase (Varsanyi & Heilmeyer, 1981). Subsequently, it was shown that phosphate is incorporated into phosphatidylinositol, forming phosphatidylinositol 4-phosphate; additionally, a minor amount (ca. 5%) of phosphatidic acid is produced. In parallel to this phosphatidylinositol 4-phosphate formation the Ca^{2+}-stimulated ATPase activity increases (Varsanyi et al., 1983). The enzyme responsible for such a phosphorylation is phosphatidylinositol kinase. It is generally accepted that phosphatidylinositol kinase is a membrane-bound enzyme [for review, see Irvine (1982)] usually associated with plasma membranes. In liver two forms are described, one associated with plasma membranes and another one with microsomes (Harwood & Hawthorne, 1969). Similarly, it was found that skeletal muscle microsomes contain phosphatidylinositol kinase activity (Harwood & Hawthorne, 1969).

The stimulation of phosphatidylinositol phosphorylation by phosphorylase kinase mentioned above raised the following questions: first, can phosphorylase kinase phosphorylate phosphatidylinositol, and second, if so, can the phosphatidylinositol kinase activity be separated from the phosphorylase kinase activity or are both activities expressed by one enzyme.

In this paper we present enzymological as well as immunological evidence that indeed phosphatidylinositol can be phosphorylated by phosphorylase kinase; another lipid kinase, diacylglycerol kinase, is easily separated from phosphorylase kinase during its isolation.

Materials and Methods

Materials and methods not listed in this paper have been described in a previous paper (Varsanyi et al., 1983). Phosphorylase kinase was purified according to Cohen (1973) and as modified by Jennissen and Heilmeyer (1975). Phosphorylase *b* was isolated according to Fischer and Krebs (1958). The Ca^{2+}-transport ATPase was isolated from the sarcoplasmic reticulum according to De Meis and Hasselbach (1971) and MacLennan (1970). The purity of the isolated material was checked by sodium dodecyl sulfate (SDS) gel electrophoresis [cf. Varsanyi and Heilmeyer (1981)]. Isolated Ca^{2+}-transport ATPase was partially digested with trypsin (freshly dissolved in H_2O, 10 mg/mL, 1:100 w/w) in 0.66 M sucrose, 0.05 M tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl), and 1 mM histidine, pH 8.0, at 27 °C for 5 min. The reaction is stopped with a 10-fold molar excess of soyabean trypsin inhibitor (10 mg/mL); the protein is stored at −20 °C. Phosphatidylinositol was obtained from Sigma.

Phosphatidylinositol kinase activity is assayed in a total volume of 150 μL containing 10 mM [γ-^{32}P]ATP, 10 mM ethylenediaminetetraacetic acid (EDTA), 1 mM ethylene glycol bis(β-aminoethyl ether)*N,N,N',N'*-tetraacetic acid (EGTA), 100 mM KCl, 200 mM Tris-HCl, 6 mg/mL trypsin-treated Ca^{2+}-transport ATPase, and an appropriately diluted aliquot of phosphatidylinositol kinase. A concentration of 10 mM ATP is employed in the assay to avoid depletion even if native ATPase is used in the assay [cf. Figure 2 and Varsanyi et al. (1981)]. The optimum pH for this assay employing phosphorylase kinase as enzyme (cf. Results) was determined with three buffer systems to be 7.6. The mixture is preincubated at 27 °C, and 15-μL aliquots are removed at 0.5, 1, and 1.5 min to determine the radioactivity bound unspecifically. After 2 min the reaction is started by addition of 8 μL of 400 mM $MgCl_2$ (for the standard assay) or 8 μL of 400 mM $MgCl_2$ and 20 mM $CaCl_2$. The calculated free Mg^{2+} is 7.3 mM and free Ca^{2+} 400 μM. Aliquots of 15 μL are removed at appropriate time points and applied to Whatman GF/C filter paper disks, which are washed 2 times with 10% trichloroacetic acid, 1% K_2HPO_4, and 1% $Na_4P_2O_7$, followed by a third wash with 10% trichloroacetic acid. The filter disks are washed with water and acetone and dried at 100 °C. Radioactivity is determined as described previously. As controls, radioactivity incorporation in the absence of the substrate (i.e., the trypsin-treated ATPase) was carried out to determine the autophosphorylation of phosphorylase kinase as well as in the absence of phosphorylase kinase to determine autoincorporation into the ATPase. Under assay conditions protein phosphorylation was measured as follows: After the radioactivity was counted following trichloroacetic acid precipitation as described above, the filter disks were extracted with chloroform/methanol/concentrated HCl (100:100:0.6 v/v). This organic solvent extracts lipid-bound radioactivity but not protein-bound radioactivity. The filter disks were further washed consecutively with acetone, water, and acetone. The remaining radioactivity was determined as described above. For calculation of phosphatidylinositol kinase activity this background value was subtracted.

Diacylglycerol kinase activity is assayed in the same mixture as described for the phosphatidylinositol kinase except that the Tris buffer is replaced by 200 mM KH_2PO_4 and 25 mM NaF. The optimum pH, 7.2, for this activity was determined by employing three different buffer systems. One unit of both kinase activities is defined as 1 nmol of phosphate transferred per minute.

Phosphorylase kinase activities are determined on an Autoanalyzer according to Jennissen and Heilmeyer (1974) and as modified by Hessova et al. (1985a). For determination of the A_2 activity, the free Ca^{2+} concentration in the pH 8.2 assay is adjusted to 100 μM, according to Kilimann and Heilmeyer (1982a,b). One unit of kinase activity corresponds to 1 μmol of inorganic phosphate (P_i) transferred to phosphorylase *b* subunit (M_r 100 000) per minute.

Thin-layer chromatography was carried out as described by Varsanyi et al. (1983) with the modification that perchloric

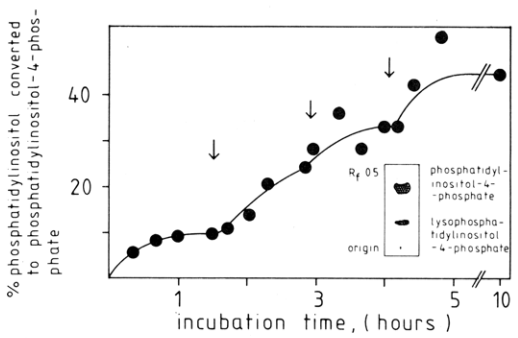

FIGURE 1: Phosphatidylinositol 4-phosphate formation was assayed as described under Materials and Methods. Arrows indicate the time points at which 0.72–0.80 mg/mL phosphorylase kinase (DEAE pool) was readded to the mixture. The ordinate shows the percentage of phosphatidylinositol converted to the 4-phosphate derivative. The inset shows product identification by thin-layer chromatography followed by autoradiography.

acid instead of trichloroacetic acid together with 25 mg/mL albumin was employed for precipitation of incorporated radioactivity.

Phosphatidylinositol Phosphorylation Catalyzed by Phosphorylase Kinase. Phosphatidylinositol (2.5 mg) is suspended in 500 μL of 100 mM KCl, 10 mM Tris-HCl, and 2.4% Triton X-100, pH 7.0. The suspension is sonicated for 1 h at 40–50 °C. An aliquot of this suspension (final concentration, ca. 1 mM phosphatidylinositol) is preincubated at 4 °C for 15 min in a total volume of 240 μL containing 100 mM KCl, 100 mM Tris-HCl (pH 7.6), 10 mM EDTA, 1 mM EGTA, 1 mM dithioerythritol (DTE), 0.4% Triton X-100, and 0.75 mg/mL phosphorylase kinase. After the temperature was raised to 36 °C and after addition of 10 mM [γ-^{32}P]ATP, two aliquots of 15 μL are added each to 300 μL of chloroform/methanol/concentrated HCl (100:100:0.6 v/v) to determine the nonlipid radioactivity extracted into the organic phase. Phosphorylation is initiated by addition of 20 μL of 400 mM $MgCl_2$. During incubation, samples of 15 μL are removed from the incubation mixture, stirred with 300 μL of the above acidic organic solvent mixture, and stored in ice. To these samples is added 40 μL of 1 M HCl, and the resulting suspension is centrifuged at 2000*g*. The upper and middle phases are eliminated, and the lower chloroform phase is further washed with 500 μL of chloroform/methanol/0.2 M HCl (3:47:50 v/v). To obtain a clear phase separation, sometimes 500 μL of chloroform is additionally employed. The sample then is cleared by centrifugation at 2000*g*. The amount of phosphatidylinositol 4-phosphate is determined by counting the radioactivity present in the chloroform phase. An aliquot is used for thin-layer chromatography, and the product is identified by autoradiography as described previously.

Results

Figure 1 shows that the pure phospholipid, phosphatidylinositol, can be phosphorylated by isolated phosphorylase kinase. Phosphatidylinositol 4-phosphate formation ceases after a ca. 1-h incubation. It is due to inactivation of the kinase. Readdition of phosphorylase kinase leads to a further formation of an approximately equivalent amount of phosphatidylinositol 4-phosphate. This procedure can be repeated several times until essentially all phosphatidylinositol is converted to phosphatidylinositol 4-phosphate. Product identification (Figure 1, inset) shows that only phosphatidylinositol 4-phosphate is formed; the lysophosphatidylinositol 4-phosphate is a breakdown product obtained due to the acidic extraction conditions (Varsanyi et al., 1983). Auto-

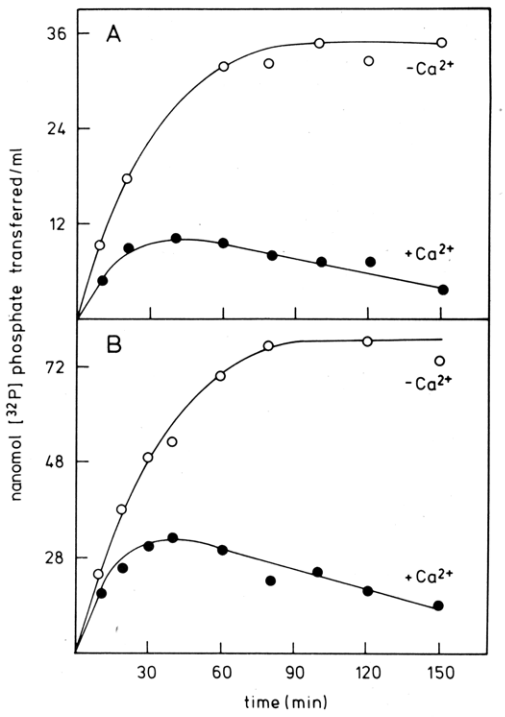

FIGURE 2: Phosphorylation employing as substrate purified Ca^{2+}-transport ATPase was carried out in the absence (A) or presence (B) of 300 μg/mL purified phosphorylase kinase (DEAE pool) at nanomolar (○) and micromolar (●) free Ca^{2+} as described under Materials and Methods.

phosphorylation of phosphorylase kinase that occurs during this incubation does not interfere with the determination of the lipid kinase activity since the phosphorylated protein is not extracted into the organic solvent. The specific phosphatidylinositol kinase activity, calculated from the initial phospholipid phosphorylation rate, is approximately 0.9 nmol of phosphate transferred min^{-1} mg^{-1}.

This extraction procedure is cumbersome and not well suited for a routine assay of phosphatidylinositol kinase activity. Therefore, a new assay was developed on the basis of the following observation: endogenous phosphatidylinositol kinase in the isolated Ca^{2+}-transport ATPase catalyzes incorporation of trichloroacetic acid precipitable radioactivity mainly as phosphatidylinositol 4-phosphate when this ATPase preparation is incubated with [γ-^{32}P]ATP in the absence of added Ca^{2+}, i.e., at ca. 2 nM free Ca^{2+} (Figure 2; for product identification, see below). In the presence of micromolar free Ca^{2+} the phosphatidylinositol 4-phosphate formed decomposes rapidly due to the presence of phosphomonoesterase (Schäfer, Varsanyi, and Heilmeyer, unpublished results) (Figure 2A). Addition of purified phosphorylase kinase enhances ca. 2-fold both the incorporation rate and the amount of incorporated phosphate (Figure 2B). The observed increase in phosphatidylinositol 4-phosphate formation is too small to assay an exogenous added phosphatidylinositol kinase in this system. For this reason, the endogenous phosphatidylinositol kinase activity was eliminated by a short trypsin treatment of isolated Ca^{2+}-transport ATPase (see Materials and Methods). Thereupon, neither in the presence of nanomolar free Ca^{2+} nor at micromolar free Ca^{2+} does phosphatidylinositol 4-phosphate accumulate (Figure 3A). However, this phosphorylated phospholipid is formed again upon exogenous phosphorylase kinase addition (Figure 3B). At 2 nM free Ca^{2+} a linear relationship is obtained between phosphatidylinositol 4-phosphate formation and incubation time up to approximately 40 min (Figure 3B). The initial rate increases linearly

Table I: Comparison of Parallel Enrichment of Phosphorylase Kinase and Phosphatidylinositol Kinase to That of Diacylglycerol Kinase[a]

fraction	phosphorylase kinase			phosphatidylinositol kinase		
	units	units/mg	purification (x-fold)	units	units/mg	purification (x-fold)
part A						
crude extract	3243	0.09	1	438	0.01	1
pH 6.1 precipitate	2330	1.2	14	90	0.04	4
100000g supernatant	1495	2.4	27	390	0.47	47
Sepharose 4B	1166	10.6	121	11	1.5	150
DEAE-cellulose	516	11.5	131	1.4	1.1	110

fraction	diacylglycerol kinase		
	units	units/mg	purification (x-fold)
part B			
crude extract	4434	0.03	1
supernatant after pH 6.1 precipitation	5701	0.04	1.3
DEAE pool	1051	2.5	82
1.82 M $(NH_4)_2SO_4$, precipitate	1087	14	459
Sepharose pool	608	17	557

[a] Phosphatidylinositol kinase and diacylglycerol kinase assays were carried out as described under Materials and Methods; the activity A_2 of phosphorylase kinase was determined only.

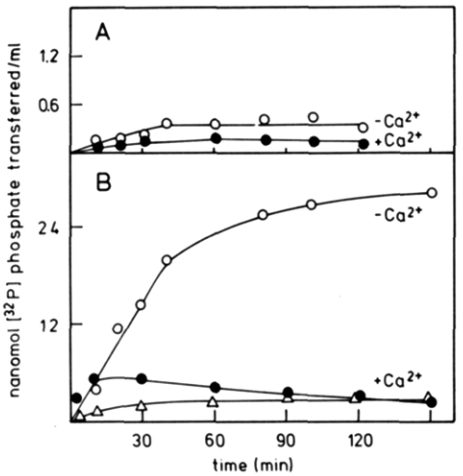

FIGURE 3: Phosphorylation employing as substrate trypsin-treated Ca^{2+}-transport ATPase was carried out in the absence (A) and presence (B) of 300 μg/mL purified phosphorylase kinase (DEAE pool) at nanomolar (○) and micromolar (●) free Ca^{2+} as described under Materials and Methods. Autophosphorylation of phosphorylase kinase was determined under the same assay conditions at nanomolar free Ca^{2+} (△).

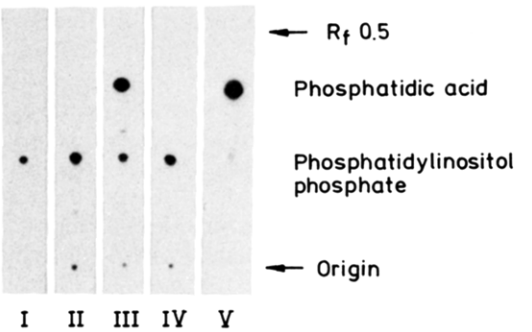

FIGURE 4: Phosphorylation was carried out at nanomolar free Ca^{2+} as described under Materials and Methods. The formed products were extracted and identified by thin-layer chromatography as described previously. (I) Phosphatidylinositol 4-phosphate formation catalyzed by the endogenous phosphatidylinositol kinase as described in Figure 1A. (II) Additionally, 300 μg/mL phosphorylase kinase (DEAE pool) was added as described in Figure 1B. (III) Diacylglycerol kinase (100 μg/mL; final product as shown in Table IB) was added. Trypsin-treated Ca^{2+}-transport ATPase was employed as substrate with added purified phosphorylase kinase (IV) or diacylglycerol kinase (V).

with the amount of kinase added up to 600 μg/mL (not shown); at micromolar free Ca^{2+} fast decomposition is observed again. Autophosphorylation of phosphorylase kinase was measured in parallel: it represents ca. 10% of the total incorporated radioactivity as determined following extraction of the lipid with the organic acidic solvent (see Materials and Methods). For calculation of phosphatidylinositol phosphorylation this amount was subtracted from the total amount of phosphate incorporated (see Materials and Methods).

Purified phosphorylase kinase catalyzes phosphatidylinositol 4-phosphate formation, as does the endogenous kinase present in isolated Ca^{2+}-transport ATPase (Figure 4, lanes I and II). There is no qualitative difference between native and trypsin-treated Ca^{2+}-transport ATPase (Figure 4, lanes II and IV). Approximately 86% of the incorporated radioactivity is extractable with chloroform/methanol/concentrated HCl; i.e., most of the radioactivity is bound as an acidic phospholipid. Autoradiography following thin-layer chromatography of this extractable material shows that more than 95% of the radioactivity is present as phosphatidylinositol 4-phosphate, employing either the isolated or the trypsin-treated Ca^{2+}-transport ATPase together with phosphorylase kinase (Figure 4, lanes II and IV). Diacylglycerol kinase produces additionally phosphatidic acid when native Ca^{2+}-transport ATPase is used (Figure 4, lane III); however, it produces exclusively phosphatidic acid when the trypsin-treated ATPase is employed (Figure 4, lane V). Again, a linear relationship is obtained between the concentration of diacylglycerol kinase and initial rate of phosphatidic acid production (not shown). The initial rate of phosphatidic acid but not that of phosphatidylinositol 4-phosphate formation is enhanced by 25 mM NaF; however, this difference varies with the enzyme preparation employed. Phosphatidic acid is synthesized at the same rate in the presence of nanomolar or micromolar free Ca^{2+} (not shown).

On the basis of these observations an assay was designed that determines the incorporation rate of trichloroacetic acid precipitable radioactivity (Materials and Methods). This assay procedure is easier to handle than the usual extraction of the formed phosphorylated phospholipids with acidic organic solvents (see above).

The enrichment of phosphatidylinositol kinase throughout the purification of cytosolic phosphorylase kinase is summarized in Table IA. Phosphatidylinositol kinase activity is enriched approximately to the same degree as phosphorylase kinase. It has to be taken into account that in crude extracts the assay of phosphatidylinositol kinase determines simultaneously diacylglycerol kinase; the latter, however, requires fluoride in the assay (see above). The diacylglycerol kinase

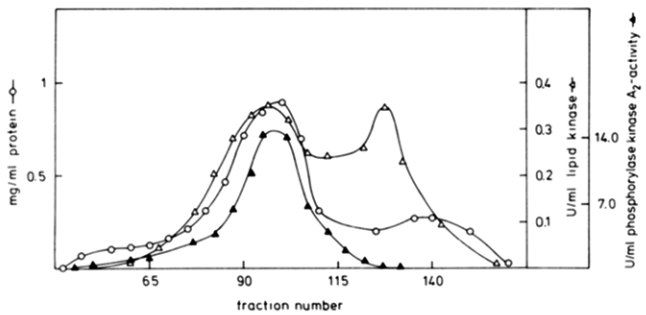

FIGURE 5: Sepharose 4B (10 × 30 cm) was equilibrated in buffer containing 50 mM sodium glycerol 2-phosphate, 2 mM EDTA, 1 mM dithioerythritol, and 0.02% NaN_3, pH 7.0. The material from the 100000*g* supernatant (cf. Table I) was concentrated by precipitation with 1.9 M $(NH_4)_2SO_4$. The precipitated material was solubilized in 130 mL of the equilibration buffer and applied to the column. Fractions of 13 mL were collected, and enzyme activities as well as protein were determined as described under Materials and Methods. Fractions 85–100 were pooled.

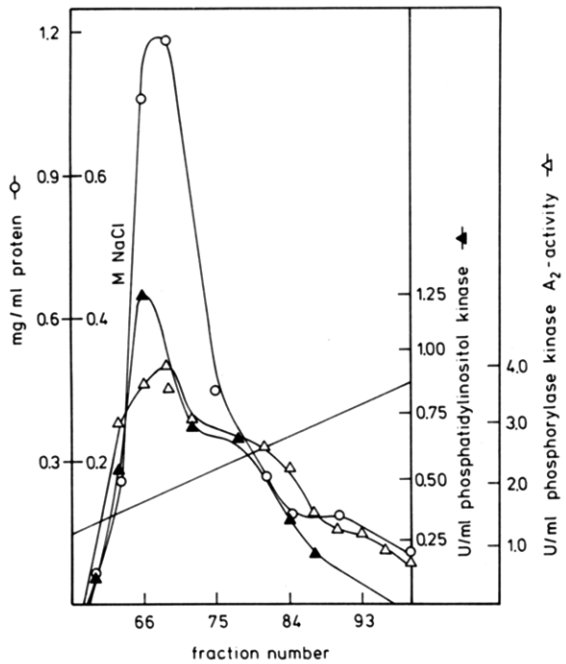

FIGURE 6: Pooled fractions from the Sepharose 4B column (see legend, Figure 5) were applied to DEAE-cellulose (DE 52, Whatman, 5 × 25 cm) preequilibrated in the same buffer as employed for gel filtration (see Figure 5). The enzyme was eluted with a linear gradient of 0–0.5 M NaCl. Fractions of 5 mL were collected and assayed for protein and enzymatic activities as described under Materials and Methods. Fractions 64–82 were pooled, and the protein was concentrated by precipitation with 1.9 M $(NH_4)_2SO_4$.

and the phosphatidylinositol kinase are mostly separated during the acid precipitation step (compare parts A and B of Table I) and finally resolved by gel filtration on Sepharose 4B. The elution profile shows two kinase peaks, designated as lipid kinase activities, that catalyze such a phosphorylation (Figure 5). Product identification shows that the first one, which parallels the phosphorylase kinase activity, contains phosphatidylinositol kinase whereas the second one contains diacylglycerol kinase activity (not shown). This separation of these two lipid kinases is, at least in part, responsible for the change in the ratio of phosphorylase kinase to phosphatidylinositol kinase activity in the collected pools from approximately 5:1 in the crude extract to 100:1 following gel filtration over Sepharose 4B (see Table I). Following Sepharose 4B chromatography the phosphatidylinositol and phosphorylase kinase containing fractions were applied to DEAE-cellulose.

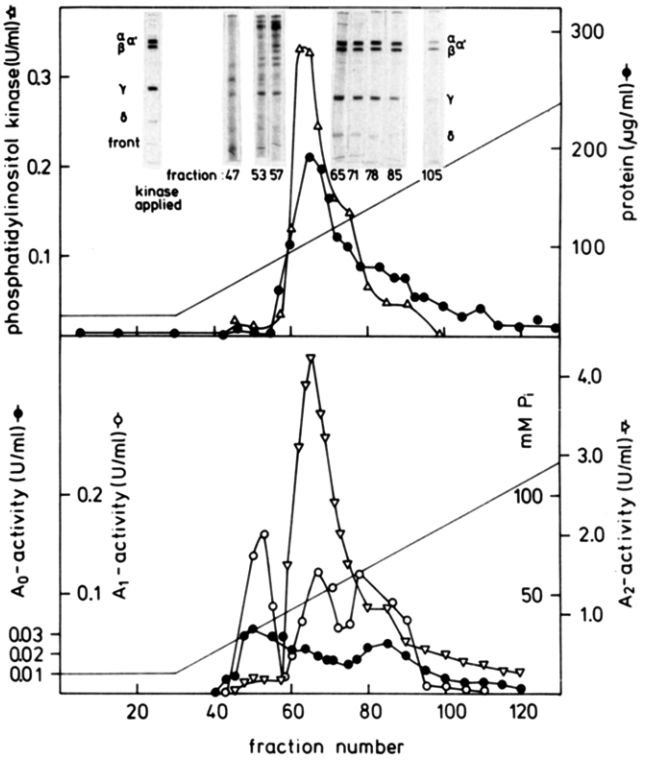

FIGURE 7: Hydroxyapatite (0.6 × 10 cm) was equilibrated with 50 mM sodium glycerol 2-phosphate, 2 mM EGTA, 2 mM dithioerythritol, 10 mM KH_2PO_4, and 1 M KCl, pH 6.8. The precipitated material from the DEAE chromatography step (see legend, Figure 6) was dialyzed against the same buffer for 5 h at 0 °C. The protein was eluted with a linear gradient (200 mL) from 10 to 200 mM KH_2PO_4 at 4.5 mL/h. Fractions of 1.2 mL were collected and assayed for enzymatic activities and phosphate as described under Materials and Methods. From the fractions indicated (insets), 10–20 µg of protein was precipitated with 10% trichloroacetic acid and applied to gel electrophoresis in the presence of sodium dodecyl sulfate as described under Materials and Methods.

As shown in Figure 6, both activities were found to coelute. By this ion-exchange chromatography an enzyme is obtained that consists of approximately 95% of the α, β, γ, and δ subunits of phosphorylase kinase (Figure 7, inset); contaminating polypeptides are clearly visible. To purify phosphorylase kinase to apparent homogeneity, the enzyme was chromatographed on hydroxyapatite equilibrated with buffer containing 1 M KCl (Figure 7). Most of the contaminating proteins are eluted at a very low phosphate concentration (ca. 30 mM) together with some material of the same molecular weight as the γ (43 000) and δ (16 700) subunits. The peak phosphatidylinositol kinase activity elutes at ca. 50 mM phosphate and coincides with the elution of the main A_2 activity peak of phosphorylase kinase. Two shoulders of phosphatidylinositol kinase activity elute at ca. 60 and 75 mM phosphate, respectively, which again correspond to phosphorylase kinase A_2, A_1, and A_0 activities. The peak fraction (65) shows the same specific phosphatidylinositol kinase activity (1.5 units/mg) as the applied material; the specific A_2 activity remains also approximately constant. Figure 7 clearly demonstrates that hydroxyapatite chromatography removes most of the contaminating polypeptides from the phosphorylase kinase but the phosphatidylinositol kinase activity still coelutes with it. The amount of these contaminants is below 1% in fraction 65 and below 0.1% in fraction 85; the elution profile of the phosphatidylinositol kinase activity does not follow these contaminating polypeptides but that of phosphorylase kinase. However, in different preparations a varying ratio of phosphorylase kinase to phosphatidylinositol kinase (compare

Table II: Immunoprecipitation of Phosphorylase Kinase and Phosphatidylinositol Kinase[a]

incubation mixture	phosphorylase kinase units/mL	%	phosphatidylinositol kinase units/mL	%
part A				
enzyme	3789	100	0.87	100
enzyme, *S. aureus*	4036	100	0.85	100
enzyme, polyclonal antibody	3181	79	0.46	53
enzyme, polyclonal antibody, *S. aureus*	1757	45	0.15	18
part B				
enzyme	1811	100	0.39	100
enzyme, *S. aureus*	1811	100	0.35	91
enzyme, antibody KIN 666/IIIC6	738	42	0.50	130
enzyme, antibody KIN 666/IIIC6, *S. aureus*	92	5	0.14	37

[a] The polyclonal antibody was a purified IgG fraction; the monoclonal antibody was purified by protein A–Sepharose. Enzyme (150 μL) (purified phosphorylase kinase, 1.5 mg/mL) was mixed with 150 μL of 10-fold-diluted polyclonal antibody (40 mg/mL) and preincubated 30 min at 4 °C with gentle shaking. Then 150 μL of 10% suspension *S. aureus* cowan I-strain cells (Calbiochem) in phosphate-buffered saline was added. After 15 min the mixture was centrifuged for 1 min in an Eppendorf microfuge. In the case of the monoclonal antibody 250 μL of (0.84 mg/mL) enzyme, 125 μL of antibody (0.42 mg/mL), and 60 μL of *S. aureus* suspension were used. In the supernatants phosphorylase kinase and phosphatidylinositol kinase activities were determined as described under Materials and Methods.

Tables I and II) is obtained in the purified enzyme. It could be interpreted as a separation of phosphorylase kinase from phosphatidylinositol kinase; alternatively, differential stability of different species of phosphorylase kinase exhibiting different phosphatidylinositol kinase activities could be responsible for this phenomenon.

Several effectors that influence the Ca^{2+}-dependent activities of phosphorylase kinase like phosphorylation by the catalytic subunit of the cAMP-dependent protein kinase or calmodulin did not affect the phosphatidylinositol kinase activity. Autophosphorylation as well did not exert any effect on the phosphatidylinositol kinase activity.

Polyclonal anti-phosphorylase kinase antibodies inhibit the A_2 activity of phosphorylase kinase [for characterization of the antibody and the preimmune serum, see Gröschel-Stewart et al. (1978) and Hörl et al. (1978)]. Similarly, phosphatidylinositol kinase is inhibited by these antibodies; it has been tested that the preimmune serum has no effect (not shown). To minimize coprecipitation, a low antibody concentration was chosen which inhibits the phosphorylase kinase and phosphatidylinositol kinase activity partially (Table IIA). Absorption of the antigen–antibody complex onto *Staphylococcus aureus* removes 45% and 66% of each activity, respectively (Table IIA).

From a series of monoclonal antibodies against phosphorylase kinase (Hessova et al., 1985b) the most specific which exhibits high affinity toward the α subunit of phosphorylase kinase (K_D = 10^{-9} M) was purified from ascites fluid [for details, see Plenker (1985)]. This antibody inhibits the A_2 activity and stimulates the A_0 activity (Hessova et al., 1985b). When a ratio of one antibody binding site to one α subunit is employed in the binding assay, the A_2 activity is inhibited to 58% whereas the phosphatidylinositol kinase activity is weakly stimulated (ca. 1.3-fold; see Table IIB). Taking these two activites in the presence of the antibody as 100%, absorption of the antigen–antibody complex to *S. aureus* removes 88% of the phosphorylase kinase activity and 72% of the phosphatidylinositol kinase activity.

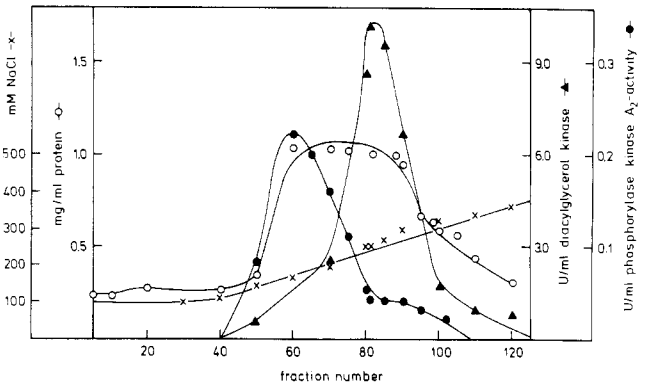

FIGURE 8: The supernatant of the pH 6.1 acid precipitation step during the phosphorylase kinase preparation (see Table IA) was readjusted to pH 6.8 with triethanolamine. The conductivity was 2.48 mS. DE 52 cellulose (600 mL), equilibrated in 10 mM sodium glycerol 2-phosphate, 2 mM EDTA, 1 mM dithioerythritol, and 0.02% NaN_3, pH 6.8, was added per 9 L of supernatant, and the mixture was poured into a column (5 × 24 cm). Protein was eluted with a linear gradient up to 0.5 M NaCl. Enzymatic activities and protein were determined as described under Materials and Methods. Fractions 70–98 were pooled, and the protein was precipitated by 1.82 M $(NH_4)_2SO_4$.

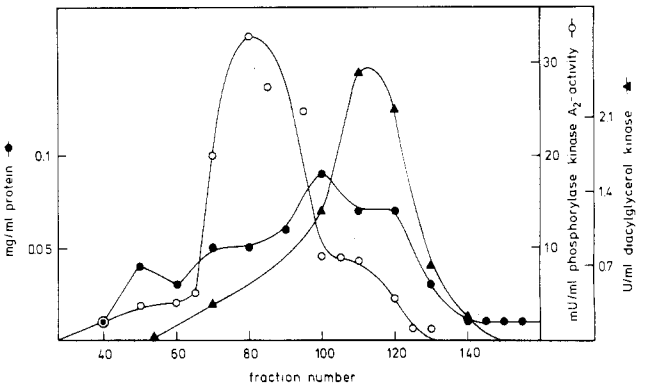

FIGURE 9: The same Sepharose 4B column as described in the legend of Figure 5 was employed. The pooled and concentrated fractions from the DEAE column (Figure 8) were applied. Fractions were analyzed for protein and enzymatic activities as described under Materials and Methods.

DEAE-cellulose chromatography (Figure 8) and gel filtration over Sepharose 4B (Figure 9) clearly separate diacylglycerol kinase activity from residual phosphorylase kinase activity; this demonstrates that this lipid kinase is not associated with phosphatidylinositol kinase. When these two steps and acid precipitation are included, the diacylglycerol kinase activity was enriched ca. 400-fold from a crude extract (Table IB). This preparation does not catalyze phosphatidylinositol phosphorylation (Figure 4, lane V).

Discussion

As demonstrated in Figure 2, phosphatidylinositol kinase activity is present in isolated Ca^{2+}-transport ATPase and catalyzes the endogenous phosphatidylinositol 4-phosphate formation. This finding confirms earlier results that skeletal muscle phosphatidylinositol kinase is a membrane-bound enzyme (Harwood & Hawthorne, 1969). The fact that the endogenous phosphatidylinositol kinase activity disappears upon trypsin treatment opens the possibility to use as a substrate the trypsin-treated Ca^{2+}-transport ATPase for assaying phosphatidylinositol kinase activity (see below).

On the other hand, the results shown in Figure 1 demonstrate that soluble phosphorylase kinase is also able to catalyze phosphatidylinositol phosphorylation. It explains that phos-

phorylase kinase can reconstitute the trypsin-treated Ca^{2+}-transport ATPase in respect to phosphatidylinositol phosphorylation (Figure 3).

Isolated trypsin-treated Ca^{2+}-transport ATPase can be used as a convenient substrate for a phosphatidylinositol kinase assay. The tight association of the formed phosphatidylinositol 4-phosphate with the protein allows one to precipitate the incorporated radioactivity with trichloroacetic acid. It yields a much easier procedure than using the pure phospholipid as substrate because it avoids the extraction procedures. Interferences by protein phosphorylation including autophosphorylation of phosphorylase kinase in respect to trichloroacetic acid precipitable radioactivity are small and are always subtracted (see Materials and Methods). The main drawback of this method is the decomposition of phosphatidylinositol 4-phosphate in the presence of micromolar Ca^{2+}, which is not yet overcome. Studies on this degrading enzyme are in process.

Two phosphatidylinositol kinase activites have been demonstrated here: one associated with SR membranes and another one that is soluble, i.e., phosphorylase kinase. Obviously then one must ask if phosphorylase kinase is contaminated with phosphatidylinositol kinase or if phosphorylase kinase itself expresses an intrinsic phosphatidylinositol kinase activity. The latter hypothesis is supported by copurification of both enzymatic activities from a crude extract, similarity in size as judged by gel filtration, and similarity in charge as judged by elution from DEAE-cellulose. The remaining 5% contaminating polypeptides in the DEAE-cellulose-purified phosphorylase kinase can be excluded to carry the phosphatidylinositol kinase activity; they are separated by chromatography on hydroxyapatite in the presence of 1 M KCl. As shown in Figure 7, the main phosphatidylinositol kinase activity appears to be separable from the small amount of contaminants; phosphatidylinositol kinase activity coelutes with the main A_2 activity of phosphorylase kinase and the main protein. Moreover, the specific activity remains essentially unchanged in comparison to the applied material. Furthermore, the SR-associated phosphorylase kinase activity is—as the cytosolic phosphorylase kinase—associated with phosphatidylinositol kinase activity and can be extracted from the membranes with Triton X-114 (Behle and Varsanyi, unpublished results). It is in agreement with the fact that phosphatidylinositol kinase activity cannot be dissociated from phosphorylase kinase activity even in the presence of detergents.

Phosphorylase kinase could also not be separated from phosphatidylinositol kinase on affinity matrices like Blue Sepharose or ATP–Sepharose. Since these ligands bind to kinases in general, this result might be expected. A high-affinity inhibitor for phosphatidylinositol kinase is, to our knowledge, not known at the moment that could be employed to synthesize an affinity matrix.

It seems to be a discrepancy that a protein kinase, phosphorylase kinase, could be a phospholipid kinase too, namely, phosphatidylinositol kinase. At nanomolar free Ca^{2+}, at which phosphatidylinositol kinase activity is measured, phosphorylase kinase expresses the A_0 activity (Kilimann & Heilmeyer, 1982a,b). A_0 is a low activity in comparison to the A_2 activity but has been demonstrated recently to be an intrinsic activity of phosphorylase kinase (Hessova et al., 1985a,b). This A_0 activity is still ca. 100-fold higher than the phosphatidylinositol kinase activity. No measurements of the phosphatidylinositol kinase activity in the presence of micromolar Ca^{2+} are possible due to product decomposition. Thus no comparison of the phosphatidylinositol kinase activity to the Ca^{2+}-dependent A_1 and A_2 activities of phosphorylase kinase is possible at the moment [for definition of activity, see Kilimann and Heilmeyer (1982a,b)]. Ca^{2+} inhibition of phosphatidylinositol kinase has been described in other systems, too (Irvine, 1982; Harwood & Hawthorne, 1969).

A hypothesis that resolves the discrepancy mentioned above would be that phosphorylase kinase is or contains a phosphatidylinositol kinase kinase and hence activates phosphatidylinositol kinase by phosphorylation. It would be in line with the property of phosphorylase kinase being a protein kinase rather than a phospholipid kinase. However, this interpretation seems not to be probable since the pure phospholipid, phosphatidylinositol, can be phosphorylated by phosphorylase kinase without the presence of other membrane components (see Figure 1).

Phosphatidylinositol kinase activity is measured below substrate saturation in the assay described, and the influence of the association of the substrate, phosphatidylinositol, with the ATPase is unknown. The specific phosphatidylinositol kinase activity of the isolated phosphorylase kinase (ca. 1.5 units/mg) is ca. 60-fold higher than that of the enzyme present in skeletal muscle microsomes (Harwood & Hawthorne, 1969). However, in these membranes it is impossible to estimate the degree of enrichment for this enzyme. In other membrane systems the specific phosphatidylinositol kinase activity that was also assayed by employing membrane-associated phosphatidylinositol as substrate was found to be of the same magnitude (1–2 units/mg; Cooper & Hawthorne, 1976) as reported here for phosphorylase kinase. The parallel inhibition and immunoprecipitation of phosphorylase kinase together with phosphatidylinositol kinase (cf. Table II) with the polyclonal antibody support the hypothesis that both activities are expressed by the same protein. However, purified phosphorylase kinase was used as antigen; a contamination with a separate phosphatidylinositol kinase could have induced anti-phosphatidylinositol kinase antibodies, which would explain the observed inhibition pattern and coprecipitation of both activities. This explanation, however, seems not to be plausible since by employing similar conditions, a monoclonal antibody directed specifically against the α subunit of phosphorylase kinase leads also to coprecipitation of both phosphorylase kinase and phosphatidylinositol kinase. Furthermore, the small activation of phosphatidylinositol kinase activity by this monoclonal antibody is qualitatively similar to the A_0 activation [see Hessova et al. (1985b)]. Thus the observations with the monoclonal antibody are also in agreement with the interpretation that both activities are expressed by the same protein. However, one can calculate that coprecipitation would occur under the conditions employed if this monoclonal antibody would cross-react with approximately the same affinity with phosphatidylinositol kinase and phosphorylase kinase. This assumption seems to be again not plausible since this antibody was selected on the basis of its specificity. Other monoclonal antibodies against phosphorylase kinase showed a much higher cross-reactivity with the other subunits, β and γ, of phosphorylase kinase and other nonrelated proteins (Hessova et al., 1985b).

It seems not to be a general phenomenon of a lipid kinase to associate with phosphorylase kinase. Phosphorylase kinase was shown to express phosphatidylinositol kinase but not diacylglycerol kinase activity. The latter kinase can be easily separated from phosphatidylinositol kinase and phosphorylase kinase. A smaller molecular weight as demonstrated by gel filtration and a different charge as shown by salt elution from DEAE-cellulose differentiates diacylglycerol kinase from

phosphorylase kinase. The stimulation by fluoride could be due to inhibition of a phosphatase that might be present at variable concentrations in different fractions obtained during purification.

In summary, the presented evidence favors the idea that phosphorylase kinase expresses phosphatidylinositol kinase activity. However, the physiologically relevant phosphatidylinositol kinase is probably only the membrane-associated kinase, which might express as a side activity phosphorylase kinase activity inasmuch as phosphorylase kinase expresses phosphatidylinositol kinase as a side activity. The results shown here might help to elucidate the structure of this membrane-bound enzyme, which one might envisage to show similarity to phosphorylase kinase.

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Registry No. Phosphorylase kinase, 9001-88-1; phosphatidylinositol kinase, 37205-54-2.

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