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THE EFFECT OF PHOSPHOLIPASE D ON *ESCHERICHIA COLI* MEMBRANE VESICLES

A RE-EXAMINATION

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SUMMARY

1. The action of commercial cabbage phospholipase D (phosphatidylcholine phosphatidohydrolase, EC 3.1.4.4) on *Escherichia coli* membrane vesicles prepared according to Kaback (1971) Methods in Enzymology (Jakoby, W. B., ed.), Vol. 22, pp 99–120, Academic Press, New York) was re-investigated.

2. With experimental conditions originally described by Milner and Kaback ((1970) Proc. Natl. Acad. Sci. U.S. 65, 683–690), the hydrolytic activity of phospholipase D is absent due to a lack of Ca^{2+} in the incubation medium.

3. Even though the hydrolytic activity of the enzyme is absent, the phospholipase D preparation inhibits uptake of α-methyl-D-glucoside by membrane vesicles.

4. Under incubation conditions where the hydrolytic activity of the phospholipase D is present, the majority of phospholipid hydrolysed in membrane vesicles is phosphatidylethanolamine.

5. The original findings of Milner and Kaback which indicated phospholipase D specifically attacks phosphatidylglycerol in *E. coli* membrane vesicles could not be reproduced. Inhibition of sugar transport in membrane vesicles by phospholipase D is not due to hydrolysis of phospholipids but probably is caused by some other component in the enzyme preparation.

INTRODUCTION

The elucidation of the functional roles of phospholipids is an important goal of membrane biochemistry. At this time one of the better characterized membrane transport systems is the phosphotransferase system found in *Escherichia coli* and two other bacteria [1]. A primary role of the phosphotransferase system is thought to be the transport of certain sugars into the bacterial cell by the process of group translocation [1]. There have been two reports of a specific requirement for phosphatidylglycerol

Abbreviation: α-methyl glucoside, methyl-α-D-glucopyranoside.

for the activation of the phosphotransferase system in *E. coli* [2, 3]. In their work on the characterization of the components of the membrane-bound Enzyme II of the phosphotransferase system, Kundig and Roseman [2] demonstrated that reconstitution of Enzyme II activity (i.e. phosphorylation of sugars), required phosphatidylglycerol for maximum activity. The outcome of this experiment strongly suggests a function for this phospholipid in vivo. However, this extrapolation must be viewed with caution. Milner and Kaback [3] have established that phosphatidylglycerol is necessary for the function of the phosphotransferase system in *E. coli* membrane vesicles. They found that incubation of vesicles with phospholipase D from cabbage, (phosphatidylcholine phosphatidyohydrolase, EC 3.1.4.4) blocked the ability of the vesicles to take up methyl-α-D-glucopyranoside (α-methyl glucoside) from the medium. Upon examination of ^{32}P-labelled phospholipids of phospholipase D-treated vesicles it was found that the levels of phosphatidylethanolamine and diphosphatidylglycerol (cardiolipin), were unaffected by the enzyme, but the amount of phosphatidylglycerol was decreased to approx. 50 % of its value in untreated vesicles. Thus Milner and Kaback concluded that phosphatidylglycerol played an important role in the phosphotransferase system in agreement with the implications from the reconstitution studies of Kundig and Roseman [2].

There is no a priori reason why phospholipase D should hydrolyse only phosphatidylglycerol in membrane vesicles. Recent evidence suggests that membranes prepared by the Kaback method are either an heterogeneous population of inside-out and right-side-out vesicles, or there is an alteration in membrane structure during lysis of the protoplasts [4–7]. With this evidence it seems unlikely that only one phospholipid in the vesicles should be susceptible to attack by phospholipase D.

Observation we have made with intact *E. coli* cells suggest to us that phosphatidylglycerol is not the phospholipid of primary importance in activation of the phosphotransferase system. These findings appear to be in disagreement with the results obtained with vesicles. Therefore we undertook a re-examination of the effect of phospholipase D on the phospholipids in *E. coli* membrane vesicles in order to resolve this apparent conflict.

MATERIALS AND METHODS

Preparation of cells, membrane vesicles and phospholipids

E. coli strain K12X15 (λ^-, str-s), a prototrophic strain, obtained here from R. Middleton was used for this work. The bacteria were grown in flasks with gyratory action (200 rev./min) at 37 °C. The growth medium was 112 mM Tris–HCl (pH 7.2) containing per litre the following compounds: 1.6 g glucose, 0.25 g NH_4Cl, 0.025 g $(NH_4)_2SO_4$, 0.05 g NaCl, 0.05 g $MgCl_2 \cdot 6H_2O$, 0.3 g Na_2HPO_4 and 0.15 g KH_2PO_4. For experiments requiring ^{32}P-labelled phospholipids the growth medium was supplemented with $H_3{}^{32}PO_4$ at a concentration of 2.5 μCi/ml.

Membrane vesicles were prepared from mid-log-phase cells according to the procedure of Kaback [8] and stored at −95 °C. Electron microscopy of negatively stained preparations indicated the absence of intact cells. In our vesicle preparations the ratio of protein (mg/ml) to total lipid phosphorus (μmoles/ml) was typically in the range 2.5–2.8.

Unless otherwise specified phospholipids were extracted by suspending cells or vesicles in no less than 10 ml of chloroform–methanol (1 : 1, v/v), for each g wet weight of material. A second extract of the same volume of chloroform–methanol (2 : 1, v/v) was combined with the first and washed once with aq 0.9 % NaCl and twice with methanol–water (1 : 1, v/v). The extracted phospholipids were stored in chloroform–methanol (1 : 1, v/v) at −15 °C.

Egg lecithin, isolated from fresh egg yolks, was purified by alumina and silicic acid chromatography according to Wells and Hanahan [9]. The egg lecithin was stored as a stock solution in chloroform–methanol (1 : 1, v/v) at −15 °C. When the purified lipid was chromatographed on a silica gel G plate with the solvent system: chloroform–methanol (4 : 1, v/v), 2 % water, 2 % pyridine, and stained with molybdate reagent [10] only a single spot was observed. However, analysis of the deacylation products of the purified lipid by anion-exchange chromatography [11], indicated the preparation was contaminated with 4.8 % phosphatidylgycerol and 0.42 % phosphatidic acid.

Enzyme Assay

Phospholipase D, Type 1, from cabbage, was purchased from Sigma Chemical Co. Phospholipase D stock solutions were prepared immediately before use in 50 mM potassium phosphate buffer (pH 6.6). Activity of phospholipase D towards egg lecithin and *E. coli* mid-log-phase phospholipids was determined essentially by the procedure described by Yang [12]. All experiments were done in duplicate with Ca^{2+} omitted from one sample which acted as a control. To the dried lipid in a 10-ml tube, fitted with a teflon-lined screw-cap, was added 0.2 ml 0.4 M acetate buffer (pH 5.6), 0.2 ml 0.2 M $CaCl_2$ in 0.4 M acetate buffer (pH 5.6) and 0.5 ml double-distilled water. For the control the $CaCl_2$ was replaced by 0.2 ml of acetate buffer (pH 5.6). The mixture was incubated with shaking for 5 min at the reaction temperature. The enzyme, 27 μg of protein in 0.1 ml of 50 mM potassium phosphate buffer (pH 6.6) was then added, and the incubation continued for a further 1 min. The reaction was initiated by the addition of 0.5 ml of washed ethyl ether and the mixture was incubated with shaking for 10 min. The reaction was terminated by extraction of the mixture with 3 ml of chloroform–methanol (2 : 1, v/v). A second extract of 3 ml of chloroform–methanol (2 : 1, v/v) was combined with the first and the combined extract was washed once with aq. 0.9 % NaCl and twice with methanol–water (1 : 1, v/v). The phospholipids were analysed as described below.

The procedure used for duplication of the experiment reported by Milner and Kaback [3], was as follows. The reaction medium containing membrane vesicles (4.2 mg membrane protein, 1.46 μmoles lipid phosphorus), LiCl (final concentration 0.3 M), $MgSO_4$ (final concentration 0.01 M), phosphoenolpyruvate (final concentration 0.1 M) and 50 mM potassium phosphate buffer (pH 6.6) in a total volume of 1.6 ml was incubated with shaking at 46 °C for 5 min. Then phospholipase D (2.35 mg protein) in 0.4 ml of 50 mM potassium phosphate buffer (pH 6.6) was added and the reaction mixture incubated with shaking for 20 min at 46 °C. The reaction was terminated by extracting with chloroform–methanol as described above. As a control the incubation was repeated without the addition of phospholipase D.

To measure the activity of the enzyme in the presence of Ca^{2+}, vesicles (2.12 mg membrane protein, 0.85 μmole lipid phosphorus) were incubated with 0.1 M

imidazole–HCl buffer (pH 6.6) and 40 mM $CaCl_2$ final concentration in a total volume of 1.9 ml for 5 min at the assay temperature. Then phospholipase D (0.625 mg protein) in 0.1 ml 50 mM potassium phosphate buffer (pH 6.6) was added and the mixture incubated for 10 min before terminating the reaction by chloroform–methanol extraction. As a control the assay was repeated but Ca^{2+} was omitted. The hydrolytic activity of phospholipase D was determined from the production of phosphatidic acid. One enzyme unit is defined as the amount of enzyme which forms 1 μmole of product per min.

Phospholipid analysis

Phospholipids were analyzed in terms of their deacylation products obtained by mild alkaline hydrolysis. The deacylation products were chromatographed on AG1-X2 anion exchange resin columns (0.4 cm × 40 cm), and eluted with a gradient prepared in a three chamber gradient mixer. The first two chambers contained aq. 0.1 M ammonium formate – 0.02 M borate (pH 8.5), and the third chamber contained aq. 0.625 M ammonium formate–0.02 M borate (pH 8.5). The flow rate of the gradient through the columns was 0.42 ml/min. Approx. 44 2.3-ml fractions were collected. The fractions were analyzed for total phosphate using the Bartlett method as described previously [11]. In all experiments described in this work there was a minimum recovery of 90 % of the total lipid phosphorus originally present. For a determination of ^{32}P radioactivity, 0.1-ml samples from the fractions eluted from the anion-exchange columns were counted in 15 ml of water. Radioactivity was determined in a Nuclear-Chicago I liquid scintillation spectrometer. The counting efficiency was 32.9 %.

Transport studies

The uptake of α-methyl glucoside by membrane vesicles and the effect of phospholipase D on transport were assayed as described by Kaback [8] and Milner and Kaback [3]. Millipore filters were counted in 15 ml of scintillation fluid (toluene–ethanol (12 : 7, v/v) containing 5.5 g/l of Permablend (Packard)) with an efficiency of 56 %.

Chemicals

Methyl-[α-D-*gluco*-^{14}C]-pyranoside, specific radioactivity 52 Ci/mole, was purchased from Calatomic. $H_3{}^{32}PO_4$, carrier free, was purchased from New England Nuclear. Phosphoenolpyruvate, trisodium salt, was obtained from Sigma Chemical Co. All other chemicals used were of reagent grade.

Determination of Protein

Protein was determined by the method of Lowry et al. [13] using bovine serum albumin as a standard.

RESULTS

The effect of phospholipase D on the uptake of α-methyl glucoside by *E. coli* K12X15 membrane vesicles is shown in Fig. 1. There is a precipitous decrease in uptake of sugar after incubation of vesicles with phospholipase D, in agreement with

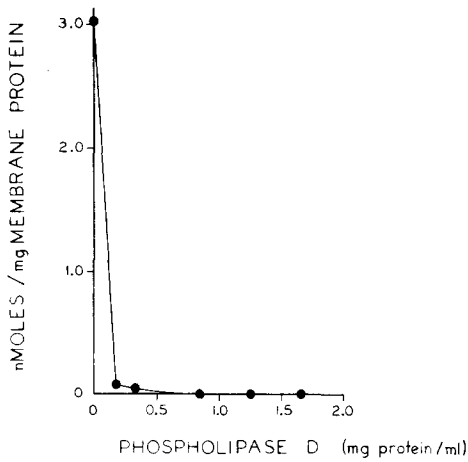

Fig. 1. Effect of phospholipase D on uptake of α-methyl glucoside by *E. coli* K12X15 membrane vesicles. Assay mixtures (0.1 ml) containing final concentrations of 50 mM potassium phosphate buffer (pH 6.6), 0.3 M LiCl, 10 mM $MgSO_4$, 0.1 M *P*-enolpyruvate, 0.5 mg membrane protein and phospholipase D at the concentrations shown were incubated at 46 °C for 15 min. α-Methyl [^{14}C]glucoside (52 Ci/mole) was then added to give a final concentration of $3.3 \cdot 10^{-5}$ M and a total reaction volume of 0.15 ml. The incubation was continued for 5 min at 46 °C. The reactions were terminated as described previously [8], and the Millipore filters analysed for radioactivity as described in Materials and Methods.

the findings of Milner and Kaback [3]. It is important to note that no Ca^{2+} is present in the assay.

The hydrolytic activity of the cabbage phospholipase D was characterized with purified egg phosphatidylcholine, and the phospholipids extracted from *E. coli* K12X15 mid-log-phase cells. The results are displayed in Table I. The activity of the enzyme was measured by the production of phosphatidic acid. At pH 5.6 the specific activity of the enzyme was 1.17 units/mg protein at 26 °C and 0.75 unit/mg protein at 46 °C determined with egg phosphatidylcholine, and 1.46 units/mg protein at 26 °C and 0.88 unit/mg protein at 46 °C using the *E. coli* phospholipids. There was no detectable hydrolysis of phospholipids when Ca^{2+} was omitted from the reaction medium, in agreement with previous reports [14, 15]. The experiments with the *E. coli* phospholipids demonstrate that the majority of phospholipid hydrolysed is phosphatidylethanolamine with a maximum of 10 % of phosphatidylglycerol being degraded. By increasing the level of enzyme or lengthening the incubation time all of the phosphatidylglycerol could be hydrolysed but not without extensive hydrolysis of phosphatidylethanolamine. No hydrolysis of diphosphatidylglycerol was detected.

E. coli K12X15 membrane vesicles from cells grown in the presence of $^{32}P_i$ were used to examine the effects of phospholipase D on the vesicle phospholipids. The conditions for incubation of the vesicles with phospholipase D were the same as described previously [3] except that a larger reaction volume (2 ml) was used. Fig. 2 shows the elution profiles of the deacylated products of phospholipids extracted from vesicles after incubation with phospholipase D. As a control the incubation was repeated but phospholipase D was omitted from the reaction medium. The

TABLE I

ACTIVITY OF PHOSPHOLIPASE D WITH EGG LECITHIN OR *E. COLI* PHOSPHOLIPIDS

Assay mixtures contained egg lecithin (1.19 μmoles phosphorus) or *E. coli* mid-log-phase phospholipids (0.94 μmole phosphorus), 160 mM acetate buffer (pH 5.6), phospholipase D, (27 μg protein) with (+) or without (−) 40 mM $CaCl_2$ in a total volume of 1 ml plus 0.5 ml washed ethyl ether. Procedure is described in Materials and Methods.

Sample	Temp. (°C)	Phospholipid	Total phosphorus (μmoles)		Specific activity of phospholipase D (units/mg protein)
			$+Ca^{2+}$	$-Ca^{2+}$	
Egg lecithin	26	Phosphatidylcholine	0.86	1.12	1.17
	26	Phosphatidylglycerol	0.05	0.07	
	26	Phosphatidic acid	0.33	0.01	
	46	Phosphatidylcholine	0.94	1.11	0.75
	46	Phosphatidylglycerol	0.05	0.06	
	46	Phosphatidic acid	0.22	0.02	
E. coli phospholipids	26	Phosphatidyl-ethanolamine	0.31	0.67	1.46
	26	Phosphatidylglycerol	0.14	0.17	
	26	Phosphatidic acid	0.42	0.02	
	26	Cardiolipin	0.04	0.04	
	46	Phosphatidyl-ethanolamine	0.45	0.69	0.88
	46	Phosphatidylglycerol	0.16	0.18	
	46	Phosphatidic acid	0.25	0.02	
	46	Cardiolipin	0.04	0.05	

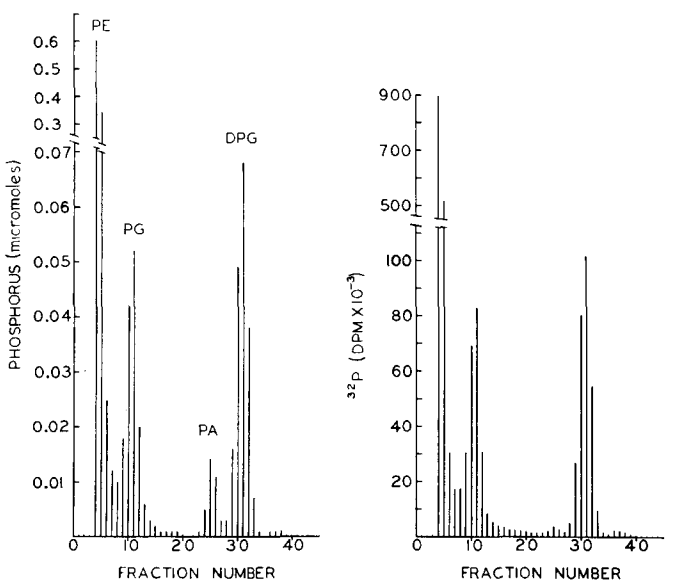

Fig. 2. Chromatography of deacylation products of phospholipids extracted from *E. coli* K12X15 membrane vesicles incubated with phospholipase D. The deacylation products were fractionated on an AG1-X2 anion-exchange column (0.4 cm × 40 cm) with a pH 8.5 gradient of ammonium formate at a flow rate of 0.42 ml/min. The elution profile is displayed in terms of μmoles of phosphorus and $^{32}P_i$ radioactivity.

TABLE II

EFFECT OF PHOSPHOLIPASE D ON PHOSPHOLIPIDS IN *E. COLI* MEMBRANE VESICLES

Assay contained membrane vesicles (4.2 mg membrane protein, 1.46 μmoles lipid phosphorus), 0.3 M LiCl, 0.01 M $MgSO_4$, 0.1 M *P*-enolpyruvate, 50 mM potassium phosphate buffer (pH 6.6) with (+) or without (−) phospholipase D (2.35 mg protein) in a total volume of 2 ml incubated for 20 min at 46 °C. Procedure is described in Materials and Methods.

Sample	Phospholipid	Total phosphorus (μmoles)	Total activity (dpm × 10^{-6})	Specific radioactivity (Ci/mole lipid phosphorus)	Specific activity of phospholipase D (units/mg protein)
+Enzyme	Phosphatidyl-ethanolamine	0.98	1.458	0.668	$2.3 \cdot 10^{-4}$
	Phosphatidyl-glycerol	0.15	0.249	0.734	
	Phosphatidic acid	0.03	0.010	0.141	
	Cardiolipin	0.18	0.280	0.699	
−Enzyme	Phosphatidyl-ethanolamine	0.94	1.423	0.679	
	Phosphatidyl-glycerol	0.17	0.262	0.687	
	Phosphatidic acid	0.02	0.010	0.223	
	Cardiolipin	0.18	0.288	0.710	

results of analysis of phospholipids from vesicles incubated in the presence and absence of phospholipase D are presented in Table II. We find there is no difference between phospholipid levels with or without phospholipase D. The same results were obtained when the experiments were repeated with vesicles made from cells of *E. coli* strain B. Therefore it is evident that phospholipase D is inactive with these assay conditions (specific activity $\leqslant 2.3 \cdot 10^{-4}$ unit/mg protein). This is not surprising since it is well established that phospholipase D requires Ca^{2+} for activation [14, 15]. It is apparent from Fig. 2 and Table II that the specific radioactivity of phosphatidic acid from the vesicles is approximately one-sixth of that of the three other phospholipids. Phosphatidic acid isolated from vesicles suspended in 0.1 M potassium phosphate buffer (pH 6.6), which were extracted immediately after warming to room temperature, had a specific radioactivity of 0.96 Ci/mole phosphorus. Also the level of phosphatidic acid in the vesicles is much lower than found after incubation in the reaction medium at 46 °C for 20 min. Therefore, it would seem that phosphatidic acid (or perhaps lysophosphatidic acid) is a de novo metabolic product under conditions of incubation for the phospholipase D assay.

It was of interest to see if phospholipase D exhibited any specificity when incubated with vesicles under conditions where the enzyme is active (i.e. with 40 mM Ca^{2+}). It was necessary to replace the potassium phosphate buffer used previously in the incubation medium with imidazole–HCl buffer. The results of these experiments are shown in Table III. The specific activity of phospholipase D with the new assay conditions was $4.3 \cdot 10^{-2}$ unit/mg protein at 26 °C and $4.0 \cdot 10^{-2}$ unit/mg protein at

TABLE III

EFFECT OF PHOSPHOLIPASE D ON PHOSPHOLIPIDS IN *E. COLI* MEMBRANE VESICLES IN THE PRESENCE OF Ca^{2+}

Assay contained membrane vesicles (2.1 mg membrane protein, 0.85 μmole lipid phosphorus), 95 mM imidazole–HCl buffer (pH 6.6), 2.5 mM potassium phosphate buffer (pH 6.6) and phospholipase D (0.625 mg protein) in a total volume of 2 ml, with (+) or without (−) 40 mM $CaCl_2$. The procedure is described in the text.

Temp.	Phospholipid	Total phosphorus (μmoles)		Specific activity of phospholipase D (units/mg protein)
		$-Ca^{2+}$	$+Ca^{2+}$	
26	Phosphatidylethanolamine	0.35	0.56	$4.3 \cdot 10^{-2}$
	Phosphatidylglycerol	0.09	0.11	
	Phosphatidic acid	0.27	0.01	
	Cardiolipin	0.11	0.12	
46	Phosphatidylethanolamine	0.36	0.57	$4.0 \cdot 10^{-2}$
	Phosphatidylglycerol	0.10	0.11	
	Phosphatidic acid	0.26	0.01	
	Cardiolipin	0.10	0.11	

46 °C. There is no indication of specificity of phospholipase D for phosphatidylglycerol. At either 26 or 46 °C the majority of the phospholipid hydrolysed is phosphatidylethanolamine, with at most 10 % of the phosphatidylglycerol being degraded.

CONCLUSIONS

The present work demonstrates that phospholipase D inhibits transport of α-methyl glucoside by *E. coli* membrane vesicles under conditions where the hydrolytic activity of the enzyme is absent, due to a lack of Ca^{2+}. When vesicles are incubated with phospholipase D in the presence of 40 mM Ca^{2+} the majority of phospholipid hydrolysed is phosphatidylethanolamine. Evidence presented by Milner and Kaback [3] for specific hydrolysis of phosphatidylglycerol in vesicles by phospholipase D in the absence of Ca^{2+} and the implied requirement of phosphatidylglycerol for α-methyl glucoside transport is not supported by our findings.

How does one account for the inhibition of sugar transport when the vesicles are incubated with inactive phospholipase D? Commercial phospholipase D is a very crude preparation [12, 16]. Therefore, it is not unreasonable to conclude that inhibition of transport by the vesicles is due to the action of some component of the phospholipase D preparation unrelated to the phospholipase D activity.

Finally, we would suggest that further work is necessary to delineate the requirements and functional roles of phospholipids in the phosphotransferase system of *E. coli*, and the mechanism of inhibition of sugar transport in membrane vesicles by phospholipase D.

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