PHOSPHOLIPASE A ACTIVITY WITH INTEGRATED PHOSPHOLIPID VESICLES IN INTACT CELLS OF AN ENVELOPE MUTANT OF *ESCHERICHIA COLI*

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1. Introduction

The outer membrane (OM) of *E. coli* has been found to be the site of a phospholipid (PL) deacylation–reacylation cycle [1] the perturbation of which can modify the physico–chemical properties of the external layer of the envelope. Cells treated with physical [2,3] or chemical factors [4] or infected with phage [5] show an increase in PL deacylation products, particularly lysophosphatidylethanolamine (LPE). We reported earlier that envelope alterations resulting from the *envC* mutation in *E. coli* are expressed as aberrant morphology and division, antibiotic and detergent hypersensitivity, release of periplasmic enzymes and modified OM composition [6–8]. These anomalies are paralleled by alterations in PL metabolism during growth and particularly by the production of a large proportion of LPE [9]. Since it has been suggested that phospholipase A activity is triggered by factors affecting the structure of the cell envelope [10] we compared the activity of this enzyme in homogenized $envC^+$ and $envC^-$ cells and in intact cells incubated with PL vesicles containing [^{3}H]phosphatidylethanolamine (PE). Sonicated cells of both strains actively hydrolysed exogenous PE. In contrast, intact wild-type cells exhibited only traces of phospholipase A activity whereas intact $envC^-$ cells had a high activity.

2. Materials and methods

2.1. *Bacteria and growth*

The origin, genotype, properties and culture of *E. coli* K-12 P678 and of the isogenic *envC* mutant PM 61 have been described [11,12].

2.2. *Isolation of purified phospholipids*

envC cells were grown on a complete medium containing 5 g tryptose (Difco), 5 g yeast extract (Difco), 8 g NaCl, 2 g glucose, and 2 mCi 2(*n*)-[^{3}H]glycerol per liter and were harvested in stationary growth phase. The lipids were extracted as in [7]. Neutral lipids and free fatty acids were removed by passage through a silicic acid column in $CHCl_3$, and the PL eluted with CH_3OH. PL extracts were banded on preparative pre-coated silicagel plates (2 mm thickness, Merck) and separated successively in three solvent systems: 1, $CHCl_3$–CH_3OH–NH_4OH–H_2O (70/30/4/2, by vol.); 2, $CHCl_3$–CH_3OH–H_2O (65/25/4, by vol.); 3, $CHCl_3$–CH_3OH–CH_3COOH (65/25/8, by vol.). After each migration, vertical strip of the silicagel plate is developed with appropriate reagents (ammonium molybdate spray and iodine vapours for total PL, ninhydrin for aminophospholipids) and the individual PL bands scraped off and eluted batchwise with successively $CHCl_3$–CH_3OH (2:1) $CHCl_3$–CH_3OH (1:1) and CH_3OH (twice). Finally, the [^{3}H]PL were concentrated and their purity verified on pre-coated silicagel plates (0.25 mm thickness, Merck) in the acidic solvent. The specific radioactivity of each was also determined from these plates after elution. Lipid phosphate was measured according to [13].

2.3. *Vesicle preparation*

PL vesicles were prepared by extensive sonication (60 min, 4°C) of total [^{3}H]PL or purified [^{3}H]PE

supplemented with unlabelled total PL (1:1) in 10 mM Hepes buffer (pH 7.5).

2.4. *Vesicle–cell interactions*

Intact cells in 10 mM Hepes buffer (pH 7.5) were incubated with vesicles (400 nmol [^{3}H]PL, spec. radioact. 400 cpm/nmol) in the presence of $CaCl_2$ at 37°C in conditions similar to those used in [14]. The cells were separated from the unintegrated vesicles by centrifugation through 10% Ficoll in 10 mM Hepes buffer–5 mM EDTA (pH 7.5) at 10 000 × *g*, 10 min, 4°C. The pellets were washed twice with cold 10 mM Hepes buffer (pH 7.5), suspended in 0.4 ml of the same buffer, and 100 μl resulting cell suspension were withdrawn, transferred to scintillation vials containing 10 ml of a scintillation cocktail for aqueous samples (Ready Solv GP Beckman) and the radioactivity determined.

2.5. *Phospholipase A assay*

2.5.1. Using disrupted cells

Cells harvested in early stationary growth phase were sonicated in 8 periods of 30 s at 4°C. The unbroken cells were removed at 1500 × *g*, 4°C and the supernatant taken as crude extract. [^{3}H]PE, 15 μl (1500 nmol, spec. radioact. 114 cpm/nmol) were deposited at the bottom of a tube, evaporated under a stream of nitrogen, dissolved in 0.5 ml diethylether and sonicated for 15 s. To this were added 0.5 ml of the crude cell extract in 130 mM Hepes buffer pH 8.0 (6–8 mg protein) and 0.1 ml of 40 mM $CaCl_2$. The mixture was incubated at 37°C for 30 or 60 min with frequent energetic agitation, the PL extracted according to [15] and separated on pre-coated silicagel plates. Spots corresponding to PE and LPE were scraped off and put into Eppendorf tubes, the PL eluted successively with $CHCl_3$–CH_3OH (1:1), CH_3OH and CH_3OH–H_2O (5:1), and the silicagel separated by centrifugation. The combined eluates were transferred directly into scintillation vials, the solvent evaporated by infrared heat under a stream of air and the radioactivity determined [7].

2.5.2. Using intact cells

Cells were suspended in 10 mM Hepes buffer (pH 7.5) at ~10 mg protein/ml. The incubation mixture contained 0.2 ml vesicle suspension prepared with [^{3}H]PE (400 nmol, spec. radioact. 217 cpm/nmol) and unlabelled total PL (1:1), 0.2 ml cell suspension, 0.1 ml 100 mM $CaCl_2$ in 10 mM Hepes buffer (pH 7.5) and the reaction carried out for 60 min at 37°C with agitation. Cold 100 mM EDTA in 10 mM Hepes buffer (pH 7.5) 0.125 ml, was then added and the samples kept 15 min in ice. The cells were separated from the unintegrated vesicles by centrifugation through 10% Ficoll–5 mM EDTA in 10 mM Hepes buffer (pH 7.5) at 10 000 × *g*, 10 min, 4°C and washed twice with cold Hepes buffer. The PL were extracted directly from the cells by the procedure in [15], separated by thin-layer chromatography, eluted and counted as described.

3. Results

3.1. *Phospholipase A activity in disrupted cells*

The results summarized in table 1 show that phospholipase activity in crude extracts of sonicated *envC*$^-$ cells was approximately twice that in wild-type (*envC*$^+$) preparations. This observation supports the idea that the high proportion of LPE found in *envC*$^-$ cells is the consequence of higher phospholipase A activity [9]. However, the enzyme activities in mutant and parent envelope fragments may be

Table 1
Phospholipase A assay using disrupted cells

	30 min	60 min
envC$^+$	0.286	0.49
envC$^-$	0.65	0.93
$\frac{envC^-}{envC^+}$	2.27	1.9

Cells harvested in the early stationary growth phase were disrupted by sonication (8 periods of 30 s at 4°C). Intact cells were pelleted at 1500 × *g* for 15 min at 4°C, and the supernatant used for the detection of the phospholipase activity. Protein (6–8 mg) from the crude extract in 130 mM Hepes buffer (pH 8.0) was incubated with 1500 nmol [^{3}H]PE in diethylether in the presence of Ca^{2+} and the reaction carried out for 30 or 60 min at 37°C. The reaction was stopped by PL extraction as in [15]. Further details are in section 2.5.1. The enzyme activity is expressed in nmol LPE synthesized/mg cell protein and the results calculated for 100 nmol [^{3}H]PE/sample

affected differently by sonication and by the presence of diethylether in phospholipase A assay. Therefore, the values shown in table 1 cannot be considered as actual activities in intact cells.

3.2. *Transfer of PL vesicles*

To study phospholipase A activity in whole cells we applied the PL transfer technique [14] using PL vesicles and intact recipient bacteria. Figure 1 shows the kinetics of PL transfer into *envC*$^+$ and *envC*$^-$ cells. Exogenous PL was incorporated to a greater extent into *envC* than into wild-type cells regardless of their age.

As fig.2 shows, the rate of PL transfer is affected by the ratio vesicular PL:cell protein. Although exponential and early stationary *envC* cells incorporated exogenous PL at the same rate, exponential wild-type cells incorporated them more rapidly than early stationary cells.

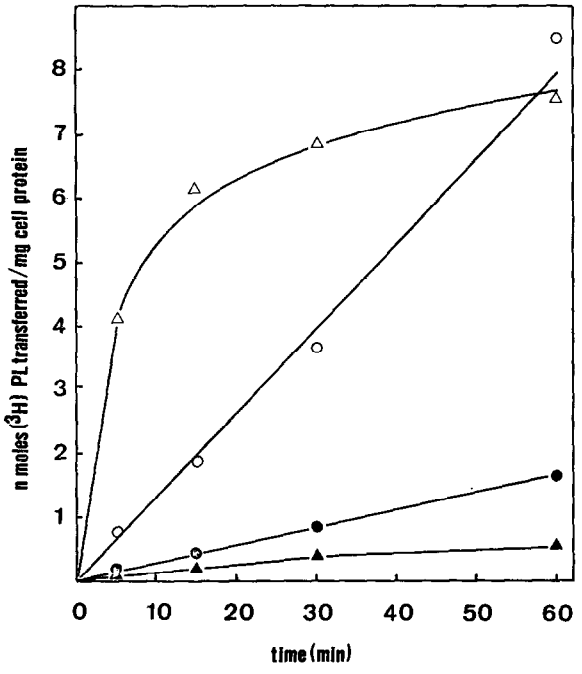

Fig.1. Time course of PL transfer into *envC*$^+$ and *envC*$^-$ cells. Cells harvested in exponential or early stationary growth phase were suspended in 10 mM Hepes buffer (pH 7.5) at 10 mg cell protein/ml, and incubated with PL vesicles (400 nmol [^{3}H]PL) at 37°C in the presence of 20 mM $CaCl_2$. At the indicated times PL transfer was stopped by addition of 20 mM EDTA and the samples treated as detailed in section 2.4. The results are calculated for 100 nmol vesicular [^{3}H]PL/sample. Exponential, *envC*$^+$ (●), *envC*$^-$ (○); early stationary, *envC*$^+$ (▲), *envC*$^-$ (△).

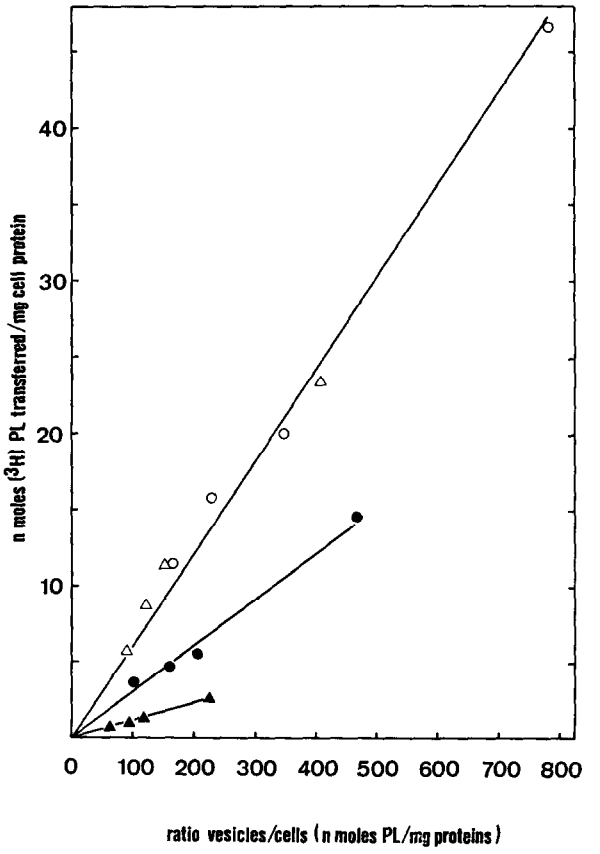

Fig.2. Influence of the ratio vesicular PL/cell protein on the PL transfer into *envC*$^+$ and *envC*$^-$ cells. Exponential and early stationary cells were harvested, suspended at different cell protein concentrations in 10 mM Hepes buffer (pH 7.5) and incubated with PL vesicles (400 nmol [^{3}H]PL) in the presence of 20 mM $CaCl_2$ for 60 min at 37°C. The PL transfer was stopped by addition of 20 mM EDTA and the samples treated as detailed in section 2.4. Exponential, *envC*$^+$ (●), *envC*$^-$ (○); early stationary, *envC*$^+$ (▲), *envC*$^-$ (△). The results are calculated for 100 nmol vesicular [^{3}H]PL/sample.

We tested the effect of [Ca^{2+}] on PL transfer in both types of cells (fig.3). At all concentrations of Ca^{2+} the incorporation of PL into *envC* was more efficient than into the wild-type cells. The peak at 10 mM Ca^{2+} with exponential *envC* cells was reproducible but its interpretation is not clear.

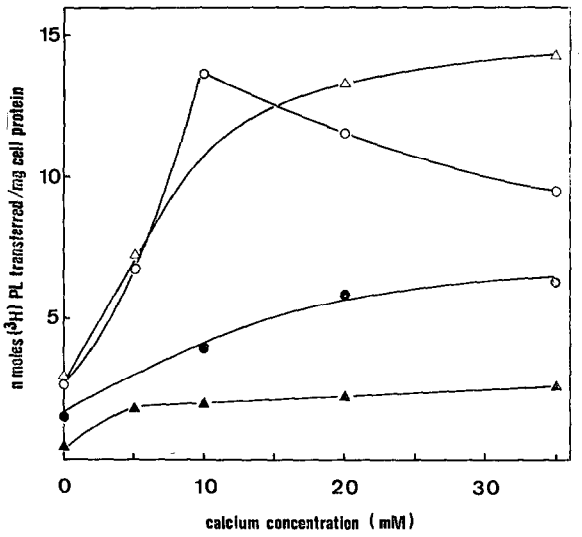

Fig.3. Calcium dependence of PL transfer into *envC*$^+$ and *envC*$^-$ cells. Cells harvested in exponential or early stationary growth phase were suspended in 10 mM Hepes buffer (pH 7.5) at 10 mg cell protein/ml, and incubated with PL vesicles (400 nmol [^{3}H]PL) in the presence of 0, 5, 10, 20, 35 mM $CaCl_2$ for 60 min at 37°C. The PL transfer was stopped by addition of 0, 5, 10, 20, 35 mM EDTA and the samples treated as detailed in section 2.4. The results are calculated for 100 nmol vesicular [^{3}H]PL/sample. Exponential, *envC*$^+$ (●), *envC*$^-$ (○); early stationary, *envC*$^+$ (▲), *envC*$^-$ (△).

Table 2
Phospholipase A assay using intact cells

	Exponential	Stationary
envC$^+$	0.4	0.8
envC$^-$	4.4	12.0
$\frac{envC^-}{envC^+}$	11	15

Cells harvested in exponential or early stationary growth phase were suspended in 10 mM Hepes buffer (pH 7.5) at 10 mg cell protein/ml, incubated with PL vesicles containing [^{3}H]PE (400 nmol) and unlabelled total PL (1:1) in the presence of 20 mM $CaCl_2$ at 37°C for 60 min. Further details are in section 2.5.2. The enzyme activity is expressed in nmol/LPE synthesized . h^{-1} . mg cell protein^{-1} and the results calculated for 100 nmol radioactive PE transferred/sample

3.3. *Phospholipase A activity in whole cells*

The results obtained with whole bacteria and described in table 2 were very different from those observed with crude extracts of sonicated cells. Only traces of phospholipase A activity were found in whole wild-type cells but *envC* cells showed significant hydrolysis of PE to LPE. The ratios of mutant to parent activity were 11 and 15 for exponential and stationary cells, respectively. It is noteworthy that early stationary cells of both strains were more active than log cells.

4. Discussion

Little is known about the physiological role of phospholipase A in PL metabolism. It has been suggested that the deacylation–reacylation cycle could be involved in the remodelling of the PL layer as a reaction of the cell to environmental changes. Indeed, it has been demonstrated that the increased sensitivity of bacteria to actinomycin D following treatment with EDTA is accompanied by an activation of the phospholipase A activity [16].

The results presented here show conclusively that the activity of phospholipase A in intact cells of *E. coli* with normal envelope is very low, although the same cells disrupted by sonication hydrolyse PE actively. On the other hand, the envelope mutant *envC* which is characterized by an alteration of the permeability barrier displayed significant phospholipase A activity in intact cells.

These findings together with evidence of a relatively high content of LPE in growing *envC* lend support to the concept that an alteration of the control mechanism of phospholipase A activity could be responsible at least in part for the alteration of the permeability barrier observed in this strain.

Determination of phospholipase A activity using labelled substrate introduced into membranes of intact cells of *E. coli* as described here is evidently a more physiological method than that using sonicated cells. It should be noted that the optimal conditions for transfer of PL into *E. coli* cells such as incubation time, the ratio vesicular PL:cellular protein, and [Ca^{2+}] were similar to those found [14] in *Salmonella typhimurium*.

Of particular interest is the observation that the transfer of PL vesicles into *envC*$^-$ cells was more efficient than into the isogenic *envC*$^+$ strain. It should be

noted that the sugar compositions of *envC*$^+$ and *envC*$^-$ lipopolysaccharides are identical [17]. In contrast, in *S. typhimurium* the transfer of PL into the cells was dependent upon the nature of the lipopolysaccharide in the OM and maximal rates of transfer were observed with a heptoseless (*Re*) mutant [14]. However, since deep rough (*Rd* and *Re*) mutants of *S. typhimurium* as well as *envC* mutant of *E. coli* are sensitive to phospholipase C [18,19] this implies that the OM of these types of strains contains PL exposed on the cell surface. Thus it seems reasonable to conclude that the rate of transfer of PL from vesicles into cells is the function of accessibility of cellular PL.

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