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Effects of Exogenous Phospholipase A_2 and C on Phospholipids in Whole Cells of *Escherichia coli* JElOll and Its NS Mutants

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Whole cells of *Escherichia coli* JE 1011 and its novobiocin-supersensitive (NS) mutants were treated with exogenous phospholipase A_2 and C. The phospholipids in whole cells of JE 1011 were scarcely hydrolyzed by the phospholipases, whereas those of NS mutants including NS6 with complete lipopolysaccharide (LPS) were extensively hydrolyzed. The accessibility of phospholipids in whole cells to exogenous phospholipases bore no relation to the composition of phospholipids in these strains. When the cells of JE 1011 were frozen-thawed or treated with ethylenediaminetetraacetate (EDTA), the phospholipids were hydrolyzed by the phospholipases to the extent similar to those of NS mutants. Parent strain JE 1011 was resistant to hydrophobic substances such as tripropyl- and tributyl-tin. gentian violet and esters of *p*-hydroxybenzoic acid, however, the strain became sensitive to these compounds by EDTA-treatment. On the other hand, all of the NS mutants were sensitive to them. From these results, it was suggested that the accessibility of phospholipids in whole cells to the exogenous phospholipases related to the permeability of hydrophobic substances.

INTRODUCTION

Tamaki *et al.* (1971) previously isolated novobiocin-supersensitive (NS) mutants from *Escherichia coli* K-12 strain JE 1011. Using these strains, we tested the antimicrobial action of organotin compounds and reported that JE 1011 was resistant to tripropyl- and tributyl-tin, whereas all of the NS mutants including NS 6 were sensitive to the compounds (Tatsuguchi *et al., 1979).* From the tentative structure of lipopolysaccharide (LPS) of these strains, it was recognized that the strains from NS 1 to NS5 were "deep rough" mutants with incomplete LPS, whereas NS6 strain had complete LPS found in *Esherichia coli K-12* (Tatsuguchi *et al., 1979).* Many reports have demonstrated that the "deep rough" mutants showed a higher sensitivity to detergents, hydrophobic antibiotics and dyes (Tamaki *et al.,* 1971; Roantree *et al.,* 1969; Nikaido, 1976). The results obtained with the organotin compounds were consistent with the conclusion in these reports, except that NS6 with complete LPS was also sensitive.

The basic structure of biomembrane is the bilayer of phospholipids, and

low molecular hydrophobic substances can readily permeate the membrane (Szabo, 1972). Consequently we speculated that hydrophobic substances were more easily incorporated into the cells of NS mutants than into those of JE 1011, since phospholipids were exposed on the surface of NS mutants including NS6, but not on the surface of JE 1011.

In this paper we describe the effects of exogenous phospholipases on the whole cells of these strains. The results will be discussed in relation to the permeability of hydrophobic substances into the cells.

MATERIALS AND METHODS

Chemicals

Phospholipase A, (EC 3. 1. 1.4, from porcine pancreas) and phospholipase C (EC 3. 1. 4. 3, from *Bacillus cereus*) were purchased from Boehringer Mannhaim Yamanouchi Co. Ltd. Rhodamine 6G (Kanto Chemicals Co. Ltd.), trichloroacetic acid and ammonium molyhdate (Wako Junyaku Co. Ltd.), HEPES (Dojin Chemicals Co. Ltd.), n-hexane, chloroform and methanol (Nakarai Chemicals Co. Ltd.), and palmitic acid (Tokyo Kasei Co. Ltd.) were used. L-α-Phosphatidylcholinc (PC, from egg yolk), L-α-phosphatidyl-DL-glycerol (PG, from egg yolk), L-α-phosphatidylethanolamine (PE, from E. *coli*), L-α-phosphatidyl-L-serine (PS, from bovine brain), and cardiolipin (CL, from bovine heart) were purchased from Sigma Chemicals Co. Ltd. Phosphatidic acid (PA) was prepared from PC using crude phospholipase D from carrot by the method of Long *et al.* (1967). Lysophosphatidylethanolamine (LPE) was prepared from PE using phospholipase A, by the method of Doi and Nojima (1973). Kieselgel H (type 60, Merck Chemicals Co. Ltd.) was used for the adsorbent of thin layer chromatography (TLC).

Bacterial strains and growth conditions

E. *coli* JE 1011 and its NS mutants (NS 1-NS6) were used. The properties of these strains were shown in the report of Tamaki *et al.* (1971). Cultivation was carried out as described in the previous paper (Tatsuguchi *et al.*, 1979).

Contents of phospholipids in whole cells

The cells at the middle exponential phase were used. Phospholipids were extracted with a 2 : 1 chloroform/methanol mixture, according to the method of Bligh and Dyer (1959). The total phosphorus in the lipid fraction was measured by using the procedure of Nakamura (1950).

Treatment of whole cells with phospholipases

The treatment of whole cells with phospholipase A_2 and C, and the determination of hydrolyzed phospholipids were performed as shown in Fig. 1. In the case of phospholipase A_2, free fatty acids liberated from phospholipids were determined colorimetrically at 514 nm by Rhodamine 6G method using

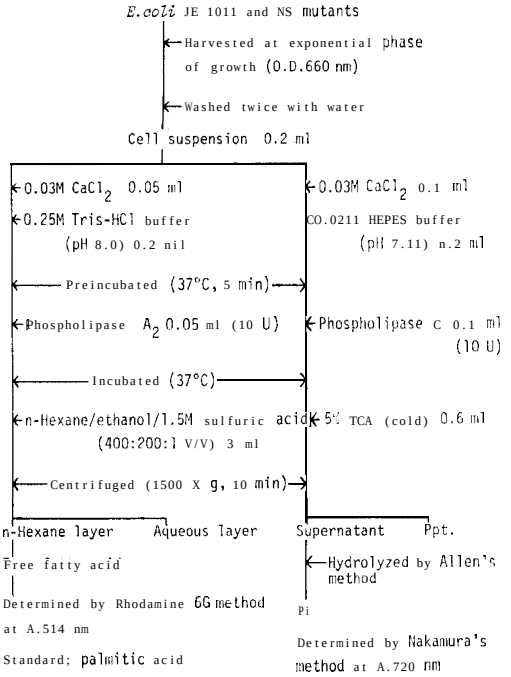

Fig. 1. Schema for treatment of whole cells with phospholipase A, and C, and determination of hydrolyzed phospholipids.

palmitic acid as standard (Hirayama and Matsuda, 1972). After digestion by phospholipase C, organophosphorus in the supernatant was subsequently determined by Nakamura's method at 720 nm (Nakamura, 1950). Controls were simultaneously examined under the same conditions, except for the addition of these enzymes. The amounts of hydrolyzed phospholipids were calculated from the differences in that of free fatty acids or organophosphorus between the cell suspensions incubated with enzymes for 10 min and the controls. The determinants were converted to the values on the basis of 1 gram of cell dry weight (over P_2O_5 in vacuum).

Preparation of frozen-thawed cells and EDTA-treatment

Cells were harvested at the middle exponential phase, then washed twice with water. Thick cell suspensions were frozen in a -20°C freezer overnight, and thawed in an incubator at 30°C.

Treatment of the cells of *E. coli* JE 1011 with EDTA was carried out by the method of Leive (1968).

Composition of phospholipids in whole cells

The lipid fractions were analyzed by TLC. Kieselgel H was applied to a thickness of 0.25 mm on 20×20 cm glass plate and activated at 110°C for 1

hour. The lipid fractions were concentrated to about 10 mg/ml and each 50 μl was applied on the plate. The plate was developed with a solvent of chloroform / methanol / % NH_4OH (65 : 35 : 5, V/V), and the solvent was evaporated under the refluxed N_2 gas. Subsequently the dried plate was developed with a solvent of chloroform / methanol / acetic acid (65 : 25 : 10, V/V) in perpendicular direction. The mixture of standard phospholipids was analyzed in a similar manner as above. Detection of phospholipids was performed by the spraying of Dittmer-Lester reagent (Dittmer and Lester, 1964). Primary amine and choline were characterized by ninhydrin (Skipski *et al., 1962)* and cis-aconite anhydride (Vaskovsky and Suppes, 1971) reagents, respectively. The amounts of phosphorus of each spot were measured by using the method of Yamamoto *et al. (1972).*

RESULTS

Treatment of whole cells with phospholipases

The contents of total phospholipids of whole cells are shown in Table 1. In the whole cells of NS2, NS 4 and NS 6, the contents of phospholipids were extremely low. These values did not increase by using the "hot methanol" method which was used in the extraction of lipids from the cells with rigid cell wall such as *Chlorella* (Paula and Heath, 1975), and even under the conditions added standard PE (0.5 mg) as the carrier (data not shown). It was suggested that the low values did not result from the loss in the partition process.

Table 1. The contents of phospholipids in the whole cells and the amounts of phospholipids hydrolyzed by exogenous phospholipase A, and C. Results are presented as the mean±S.D. of 12-15 (total phospholipids) or 5-7 (phospholipids hydrolyzed by A, and C) specimens. Each value is calculated on the basis of cell dry weight (μmoles/g dry weight). Percentages of hydrolyzed phospholipids to total phospholipids are listed in parentheses.

Strain	Total phospholipids μ moles/g	Phospholipids hydrolyzed by phospholipase A, μ moles/g (%)	Phospholipids hydrolyzed by phospholpiase C μ moles/g (%)
JE1011	113.9± 8.1	6.3±1.5 (5)	2.5±0.8 (2)
NS1	152.8± 16.0	20.8±3. 1 (14)	1.7. 8-tl.5 (12)
NS2	29.5± G.2	14.8±2.6 (50)	6.7±0.4 (23)
NS3	170.1±15.0	22.6i4.3 (13)	17.3±1.2 (10)
NS4	29.0± 5.2	17.5±3.5 (60)	4. 6-1-O. 5 (16)
NS5	144.5±15.0	22.8t4.8 (16)	16.4±1. 4 (11)
NS6	48.4± 0.3	34.8 F3.4 (72)	31.0f2.5 (64)

When whole cells were treated with phospholipase A,, the phospholipids of NS6 with complete LPS were most remarkably hydrolyzed, and those of the other NS mutants were hydrolyzed more extensively than those of JE 1011 (Table 1). Similar results were obtained by the treatment of the whole cells with phospholipase C except for the case of NS 2 and NS4 (Table 1).

The amounts of phospholipids hydrolyzed by phospholipase A, were much more extensive than those hydrolyzed by phospholipase C in the all strains,

especially it was remarkable in the whole cells of NS2 and NS4. The digestion of phospholipids in the whole cells of JE 1011 was scarcely observed by the treatment with phospholipase C, but observed to some extent by A,. The results may be related to the fact that phospholipase A produced lysophosphatides which acted as detergent and disaggregated the membrane structure (Kamio and Nikaido, 1976).

Treatment of frozen-thawed cells with phospholipase C

By freeze-thawing, a considerable amount of membrane components including protein, phospholipids and LPS was released from the cells of *E. coli* (*Souzu*, 1980). The effects of phospholipase C on frozen-thawed cells were examined. The phospholipids of JE 1011 were hydrolyzed in a similar manner to those of NS mutants (Table 2), although the exogenous phospholipase little hydrolyzed the phospholipids in the intact cells of JE 1011 (Table 1). Phospholipids in the frozen-thawed cells of NS6 were hydrolyzed to lesser extent than those in the intact cells (Tables 1 and 2). The results were apparent reduction caused by the fact that the cellular components had almost exhaustively released from the cells of NS6 only by freeze-thawing. In the other NS mutants, the phospholipids were hydrolyzed more extensively than those in the intact cells (Table 2).

Table 2. The effects of phospholipase C on the phospholipids of frozen-thawed cells.

Strain	Hydrolyzed phospholipids (μmoles/g cell dry weight)
JE1011	29.3
NS1	39.0
NS2	17. 5
NS3	31.0
NS4	13.0
NS5	32.0
NS6	24. 0

Digestion of phospholipids in EDTA-treated cells by phospholipases

It is known that the complex of LPS-protein-lipids leaks from the cells of gram-negative bacteria by brief treatment with EDTA (Leive and Shovlin, 1968), and that membrane permeability of hydrophobic substances increases (Leive, 1968). Further, we pointed out that LPS, protein and phospholipids were released from the cells of JE 1011 by EDTA-treatment, and that the treated cells became sensitive to tripropyl- and tributyl-tin compounds (Tatsuguchi *et al.*, 1979).

The effects of phospholipase A_2 and C on EDTA-treated cells are shown in Fig. 2. The phospholipids of EDTA-treated cells were remarkably hydrolyzed by each of phospholipase. Free fatty acids were released even from the EDTA-treated cells without added enzyme during the incubation (Fig. 2(a)). The results were in good agreement with the experiment of Hardaway

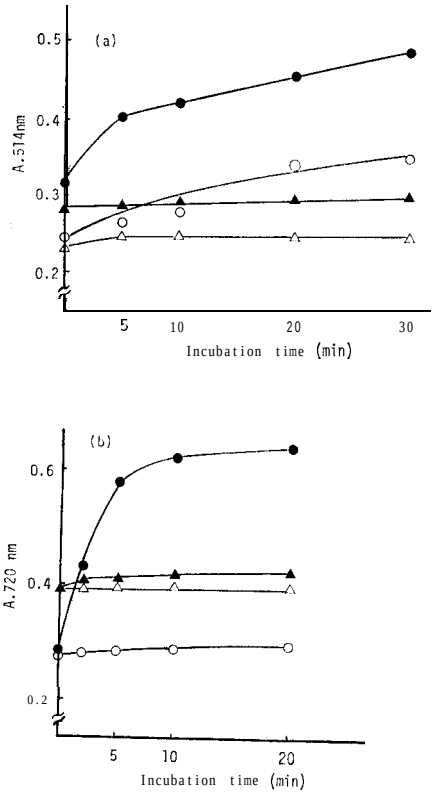

Fig. 2. The digestion of phospholipids of EDTA-treated cells of JE1011 by phospholipases. (a) phospholipase A,, (b) phospholipase C. o-o: EDTA-treated cells without enzyme, ●—●: EDTA-treated cells with enzyme, h--n : intact cells without enzyme, ▲--▲: intact cells with enzyme.

using *E. coli* K-12 strain *S-15* in which the endogenous phospholipase A was activated by EDTA-treatment (Hardaway and Buller, 1979).

Composition of phospholipids in whole cells

In order to examine whether the variation of the accessibility of phospholipids in whole cells to exogenous phospholipases was attributed to the differences in the composition of phospholipids, phospholipids of these strains were analyzed by TLC. Chromatogram of standard phospholipids was shown in Fig. 3(a). Being compared with Rf of standard phospholipids and characterizing the polar head groups, each spot of lipid fractions from these strains was identified except for 3 spots in those from NS2, NS4 and NS6 (Fig. 3(b) and (c)). PC and PS were not detected in all lipid fractions. The phospholipids of NS 2 and NS4 were unusual (Fig. 3(b)). These strains contained 2 unknown phospholipids (UK-I'L(1) and UK-PL(2) in Fig. 3(b)), whereas they did not contain PE which occupied 60–80 % in the phospholipids of *E.*

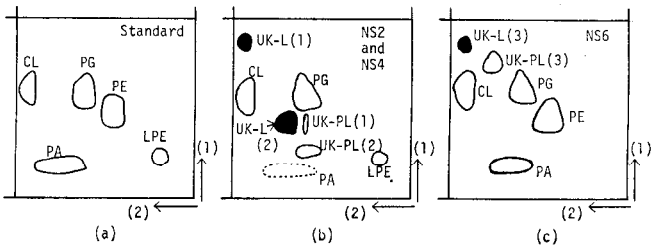

Fig. 3. Thin layer chromatograms of total lipid fraction. Developing solvent: (1) Chroloform : methanol : 28 %NH_4OH=65 : 35 : 5. (2) Chloroform : methanol : acetic acid=65 : 25 : 10. (a) standard phospholipids, (b) total lipids fraction from NS2 and NS4, (c) total lipids fraction from NS6. Open circular spots were detected by Dittmer-Lester reagent, closed spots were revealed by carbonization after spraying of Dittmer-Lester reagent.

coli (Nagata et *al.*, 1967; Kaback, 1971). New spots (UK-L(l) and UK-L(2) in Fig. 3(b)) revealed by carbonization after spraying of Dittmer-Lester reagent. The spot of UK-L(2) was most dense of all spots. This lipid has not been identified by far. Unknown phospholipids occurred in the lipid fraction from NS6 (UK-PL(3) in Fig. 3(c)) and nonphosphorus lipids revealed by carbonization (UK-L(3) in Fig. 3(c)).

The amounts of phosphorus from each spot were determined and represented as molar percentages (Table 3). The composition of phospholipids in strain NS 1 was similar to that in NS3 and NS5, and NS2 and NS 4 were similar too. The composition in NS6 was discriminated from these groups. And the lipid fractions of NS2, NS4 and NS 6 contained nonphosphorus lipids. We will identify these lipids and investigated whether these lipids effect on the accessibility of phospholipids to exogenous phospholipases. However, the differences in the composition of phospholipids did not directly participate in the accessibility of phospholipids to exogenous phospholipases in the whole cells of *E. coli* JE 1011 and its NS mutants.

Table 3. The composition of phospholipids in whole cells of *E. coli* JE 1011 and its NS mutants. Results are represented as percentages of phosphorus molar. UD: undetected.

Strain	LPE	PE	PG	CL	PA	UK-PL(1)	UK-PL(2)	UK-PL(3)
JE1011	UD	78	10	3	Trace	UD	UD	UD
NS1	4	UD	55	8	Trace	UD	UD	UD
		78		6			9	UD
NS2	16	UD	10	22	Trace	UD	UD	UD
								UD
NS3	12	72	4	8	Trace	UD	UD	UD
NS6	Trace	60	8	26	4	UD	UD	2

DISCUSSION

Gram-negative bacteria are enclosed by an envelope consisting of three portion, inner or cytoplasmic membrane, a peptidoglycan layer and the outer

membrane. The outer membrane, which is exposed to the environment, provides the cell with a passive barrier (Nikaido, 1976). It was conceivable that the variation in the barrier function of the outer membrane influenced on the sensitivities of gram-negative bacteria to hydrophobic substances.

Kamio and Nikaido (1976) proposed that the outer surface of the outer membrane of wild type gram-negative bacteria was occupied with protein and LPS, and did not expose phospholipids, whereas most of the outer membrane of "deep rough* mutants were composed of lipid bilayer (Kamio and Nikaido, 1976). From the hypothesis, they explained that hydrophobic substances could easily permeate the outer membrane of "deep rough" mutants. On the other hand, Verkleiji *et al.* (1977) suggested that a part of the phospho-Iipids was organized as a bilayer in the outer membrane of some wild types of gram-negative bacteria, and that the phospholipids on the outer surface of the membrane were concealed behind LPS and protein (Alphen *et al.,* 1977). As described above, the organization of phospholipids in the outer membrane has been argued by many investigators.

Concerning with antimicrobial action of organotin compounds, we reported that ***E.*** *coli* JE 1011 was resistant to the hydrophobic compounds, whereas its NS mutants including NS 6 were sensitive to them(Tatsuguchi *et al.,* 1979). Hydrophobic dye such as gentian violet and highly hydrophobic esters of *p*-hydroxybenzoic acid had strong antimicrobial action to all NS mutants (data not shown). These results suggested that the surfaces of NS mutants including NS6 were able to permeate hydrophobic substances easily.

It seems reasonable to consider that phospholipids among three components of the outer membrane participate in the permeability of hydrophobic substances. The results in this report showed that the exogenous phospholipases hydrolyzed phospholipids in the whole cells of NS mutants more extensively than those of JE 1011 (Table 1). It was suggested that the surface phospholipids of NS mutants including NS6 were easy of access to exogenous phospholipases. The phospholipids of JE 1011 were remarkably hydrolyzed after freeze-thawing or EDTA-treatment (Table 2 and Fig. 2). The results suggested that the inaccessibility of phospholipids in the whole cells of JE 1011 to exogenous phospholipases results from the absence of phospholipids on the outer surface or the shielding of phospholipids by the other outer membrane components.

The phospholipids in the whole cells of NS2 and NS 4 were slightly hydrolyzed by phospholipase C (Table 1). The time course of treatment with the enzyme was shown in Fig. 4. The successive release of phosphorus was observed in the cells without added enzyme, but not observed in the other strains (data not shown). Consequently, the amounts of phospholipids hydrolyzed by phospholipase C were relatively small in the cells of NS2 and NS4. If the initial values were subtracted from the values at 10 min incubation of NS 2 and NS 4 with added enzyme, 14.3 mg/g cell dry weight and 15.9 mg/g cell dry weight were obtained, respectively. These values were 62 % and 71% of the total phospholipids.

The percentages of the hydrolyzed phospholipids in whole cells by phos-

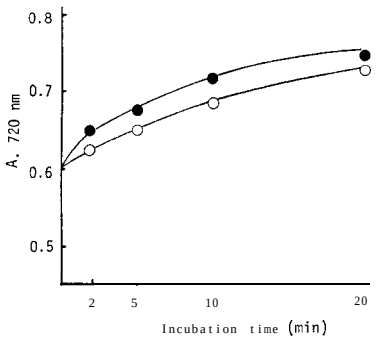

Fig. 4. Time course of the digestion in the whole cells of NS2 by phospholipase C. o-o: added no enzyme, •—•: added 10 U enzyme. NS4 was not shown, but was similar to the results in NS2.

pholipase A, to the total phospholipids were calculated, and 50, 60 and 72 % were obtained for NS2, NS 4 and NS 6, respectively (Table 1). Considering the above facts (Fig. 4), it seemed that the values by phospholipase C were also similar to those by A,. In these NS mutants, exogenous phospholipases might penetrate to the inner portion and hydrolyzed not only the phospholipids in the outer memberne but also those in the cytoplasmic membrane. In the whole cells of NS 1, NS3 and NS5, the digestion of phospholipids was much more extensive than JE 1011, but this was about 10–16 % of the total phospholipids. Therefore it seemed that the exogenous phospholipases only hydrolyzed the surface phospholipids in the whole cells of NS 1. NS3 and NS5.

NS mutants were divided into 2 groups accoring to their accessibility to exogenous phospholipases. Those were the lower group of NS 1, NS3 and NS 5, and the higher group of NS 2, NS4 and NS 6. The analyses of the proteins in the cell envelopes by polyacrylamide gel electrophoresis showed that NS 1, NS 3 and NS5 did not lack "major proteins" in the outer membrane, but NS2 and NS4 lacked one species of them and NS6 lacked them all (Suda, 1981). The protein of the outer membrane is composed from several tens of species and the so-called "major proteins" account for 70 % of the total protein (Schnaitman, 1970). It was suggested that the "major proteins" in the outer membrane might influenced on the accessibility of phospholipids to exogenous phospholipases. Further study using other outer membrane protein mutants is necessary to reveal the relationship.

From these results, it was found that the whole cells of JE 1011 were resistant to exogenous phospholipases, comparing with those of all NS mutants which were sensitive to them, although the accessibility of all NS mutants was variable. And it was suggested that the accessibility to exogenous phospholipases related to the permeability of hydrophobic substances, and that the organization of phospholipids, protein and LPS in the outer membrane influenced on the accessibility.

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