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Rapid transmembrane movement of C_6-NBD-labeled phospholipids across the inner membrane of *Escherichia coli*

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Abstract

In this study we have investigated the transmembrane movement of short chain fluorescently labeled phospholipids across the inner membrane of *Escherichia coli*. Exogenously added C_6-NBD-labeled phospholipids rapidly flip across the inner membrane of *E. coli*, as was shown by a dithionite reduction assay applied to inverted inner membrane vesicles (IIMV) isolated from wild type *E. coli* cells. The rate of transmembrane movement of the phospholipid probes incorporated into IIMV is temperature dependent, and shows no phospholipid head group specificity. C_6-NBD-labeled phospholipids translocate across the membrane of IIMV incubated at 37°C with a $t_{1/2}$ of 7 min. After the incorporation into IIMV C_6-NBD-PG is partially converted to CL by CL-synthase. If IIMV are pretreated with proteinase K the conversion of this fluorescent probe to C_6-NBD-CL is not observed anymore, suggesting that the catalytic domain of CL-synthase is at the cytoplasmic site of the plasma membrane of *E. coli*. Newly synthesized C_6-NBD-CL also flips across the inner membrane although at a slower rate than the other phospholipid probes. The transmembrane movement occurs in both directions and is not influenced by treatment of the IIMV with a sulfhydryl reagent or a proteinase, nor by the presence of ATP, or a ΔpH across the membrane of the IIMV. However, the transmembrane movement of the C_6-NBD-labeled phospholipid probes is not observed in LUVETs (large unilamellar vesicles made by extrusion technique) prepared of wild type *E. coli* lipids, indicating that the rapid transmembrane movement of phospholipids across the inner membrane of *E. coli* is a protein-mediated process.

Keywords: C_6-NBD-phospholipid; Inverted inner membrane vesicle; Transmembrane movement; Dithionite; (*E. coli*)

1. Introduction

Phospholipids are the major lipid component in *Escherichia coli*. The most abundant phospholipid in *E. coli* is phosphatidylethanolamine (PE), constituting about 75% of the total phospholipid content in the cell [1]. The two other phospholipids are phosphatidylglycerol (PG) and cardiolipin (CL), which account for about 20% and 5% of the total phospholipid content, respectively. The major part of the phospholipids is localized in the inner membrane in which they are the only lipid component [2,3].

The phospholipids in this membrane structure not only form the barrier between the cytosol and the periplasm, but also have a variety of other functions in the *E. coli* cell. Previous studies showed that specific phospholipid classes are involved in the regulation of enzyme activity, for instance PG affects the enzyme II of the phosphotransferase system [4], and PE the enzyme glycerophosphate acyltransferase [5]. PG serves as precursor for the maturation of the major outer membrane lipoprotein [6] and the periplasmatically localized membrane-derived oligosaccharides [7]. Anionic phospholipids appear to be involved in DNA replication [8], and are involved in the process of protein translocation [9,10]. Furthermore, the polymorphic properties of phospholipids appear to be required for cell viability [11]. These processes are likely to require certain phospholipids at specific times on specific sites of the inner membrane.

Abbreviations: IIMV, inverted inner membrane vesicle(s); LUVET, large unilamellar vesicle made by extrusion technique; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; CL, cardiolipin; PS, phosphatidylserine; TNBS, trinitrobenzenesulfonic acid; NBD, 7-nitrobenz-2-oxa-1,3-diazol; TLC, thin-layer chromatography; DTT, dithiothreitol; PMSF, phenylmethylsulfonyl fluoride; NEM, *N*-ethylmaleimide; Lep, leader peptidase; PhoA, alkaline phosphatase; BSA, bovine serum albumin.

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With respect to the distribution of phospholipids in *E. coli*, it is known that PE is enriched in the outer membrane [3,12]. Surprisingly, knowledge on the topology of the phospholipids in the inner membrane is completely lacking. In the Gram-positive bacterium *Bacillus megaterium* it was shown by Rothman and Kennedy [13], using the amino reactive probe trinitrobenzene sulfonic acid that 31% of the PE in the plasma membrane is situated in the outer leaflet. Furthermore, it was established that newly formed PE is partially transported over the plasma membrane of *Bacillus megaterium* at a rate 30 000-times faster than the rate of spontaneous diffusion of PE across artificial phospholipid membranes [14].

The active sites of the enzymes involved in phospholipid synthesis localized so far, are at the cytosolic side of the inner membrane [15]. To reach their final destination, transport processes of phospholipids are required. Little, if anything, is known about sorting and transport of phospholipids in *E. coli*. Langley et al. [16] showed in pulse chase studies, using a mutant *E. coli* strain lacking functional phosphatidylserine-decarboxylase at the non-permissive temperature, that the accumulating phosphatidylserine (PS) becomes evenly distributed between the inner and outer membrane. Interestingly, the PS present in the outer membrane could be chased back to the inner membrane. These results indicate that there is a bi-directional transport of phospholipids possible between the inner and outer membrane.

To get insight in the transport of phospholipids in *E. coli* we started to investigate the transmembrane movement (flip-flop) of phospholipids across the inner membrane of *E. coli*, in an in vitro system using isolated inverted inner membrane vesicles (IIMV). The transmembrane movement (flip-flop) of fluorescent 7-nitrobenz-2-oxa-1,3-diazol-(NBD)-labeled short chain phospholipid analogues, incorporated into the IIMV, was studied, using the recently developed dithionite assay [17]. Dithionite is a membrane-impermeable reducing agent, which will only reduce the NBD-groups present in the outer leaflet of the membrane, leaving those that have moved to the inner leaflet intact. This assay has been successfully used to determine the time dependent transmembrane distribution of NBD-labeled phospholipids in the plasma membrane of erythrocytes [18], and to investigate the physiological role of the mouse mdr2 gene product expressed in yeast secretory vesicles [19].

2. Materials and methods

2.1. Chemicals

1-Palmitoyl-2-6-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)aminocaproyl-*sn*-glycero-3-phosphoethanolamine (C_6-NBD-PE), C_6-NBD-phosphatidylserine (C_6-NBD-PS), C_6-NBD-phosphatidylglycerol (C_6-NBD-PG) and C_6-NBD-phosphatidylcholine (C_6-NBD-PC) were obtained from Avanti Polar Lipids (Birmingham, AL) and were pure as judged by TLC. Sodium dithionite was purchased from Sigma (St. Louis, MO). All other chemicals were analytical grade.

2.2. Growth of bacteria and preparation of inverted inner membrane vesicles (IIMV)

In all experiments *E. coli* wild type strain W3899 [20] was used. Cells were grown at 37°C in LB medium [21].

For the preparation of IIMV, overnight cultures were diluted 1:40 in LB medium and grown to an OD_{660} of 0.7–0.8 (late log phase). The cells were harvested and washed with cold physiological salt solution. Vesicles were isolated essentially as described [22]. Briefly, cells from a 3 l culture were resuspended in 40 ml of buffer K (50 mM triethanolamine-HAc pH 7.5, 250 mM sucrose, 1 mM EDTA). DTT and PMSF were added to 1 mM and 0.375 mM, respectively. The cells were broken by passing the suspension twice through a French press at 8000 psi. After each cycle an extra 1 mM of DTT was added. Cell debris was removed by centrifuging twice at low speed (5 min at $6000 \times g$). The outer membrane fraction was removed by centrifugation in a type 60 Ti rotor (Beckman): after the speed had increased to $165\,000 \times g$ it was maintained for 1 min and then the run was ended. Inner membrane vesicles were pelleted at $165\,000 \times g$ for 90 min in a type 60 Ti rotor. The crude IIMV were resuspended in buffer K and layered on top of a discontinuous sucrose gradient in buffer M (buffer K without sucrose) according to Osborn et al. [2]. The gradients were centrifuged for 14–18 h at $112\,000 \times g$ in an SW 28 rotor (Beckman), after which one broad band, containing the purified IIMV, was collected, diluted in buffer M, and centrifuged at $165\,000 \times g$ for 90 min. Vesicles were resuspended in buffer L (buffer K without EDTA), quickly frozen in liquid nitrogen and stored at −80°C. The concentration of IIMV was determined by their phosphorus content according to Rouser et al. [23]. 45% of the phosphorus content is accounted for by phospholipids as was determined by a total lipid extraction according to Bligh and Dyer [24]. IIMV isolated as above are sealed and 100% inside-out, as was recently shown by van Klompenburg et al. [25].

2.3. Large unilamellar vesicles made by extrusion technique (LUVETs)

E. coli cells in the late log phase (see above) were harvested and lipids were extracted according to Bligh and Dyer. The crude lipid extract was dissolved in a small volume of chloroform and applied to a column packed with Silica 60 (Merck), which was first eluted with chloro-

form to remove traces of neutral lipids. Next the phospholipids were eluted with chloroform/methanol, 1:1 (v/v) [26]. The purified total phospholipid extract was stored in $CHCl_3$ at −20°C. For the preparation of LUVETs, a dry lipid film was hydrated in buffer Z (50 mM triethanolamine-HAc pH 7.5, 1 mM EDTA, 100 mM KCl). The resulting phospholipid suspension with a concentration of 20 mM was extruded 10-times using 400 nm pore size polycarbonate filters [27]. The lipid concentration was determined according to Rouser et al. [23].

2.4. Dithionite assay

C_6-NBD-phospholipids were added from a stock solution of about 1 mM in ethanol to an IIMV or LUVET suspension (0.5 mM phosphorus) in buffer Z on ice. The C_6-NBD-phospholipid added was 0.4 mol% for the IIMV, and 0.2 mol% for the LUVETs with respect to the total of phospholipids. The final concentration of ethanol never exceeded 0.15% (v/v). After the addition of the fluorescent probe the vesicle suspension was put on ice for 15 min in order to facilitate incorporation of the probe. The dithionite assay was performed essentially as described by Meers et al. [28]. After different times of incubation at the temperatures indicated, aliquots of 100 nmol phosphorus of the vesicle suspension were put on ice. To these aliquots dithionite from a 1 M stock solution in 2 M Tris pH 11 was added to a final concentration of 8 mM. After 20 min on ice, the vesicle suspension was centrifuged for 40 min at 315 000 × *g* at 2°C in a TL100 ultracentrifuge (Beckman). The pelleted vesicles were solubilized in 1.2 ml 0.4% (v/v) Triton X-100 in buffer Z, and the C_6-NBD-labeled phospholipids were quantified by fluorescence using an SPF 500C fluorimeter (SLM Aminco) or an LS50B fluorimeter (Perkin Elmer) at 25°C. The excitation wavelength was 470 nm, emission was measured at 540 nm (band widths: 5 nm). Possible hydrolysis of the NBD-labeled phospholipids after incorporation of the probes into IIMV was checked by TLC. No formation of fatty acid was observed during incubations of the IIMV up to 1 h at 37°C.

Upon incorporating C_6-NBD-PG into IIMV, part of the fluorescent label was retrieved as C_6-NBD-cardiolipin (C_6-NBD-CL). In order to quantify the conversion of C_6-NBD-PG to C_6-NBD-CL, and to determine the dithionite-protected pool of C_6-NBD-CL, the phospholipids of the IIMV were analyzed by TLC, before and after treatment with dithionite. The IIMV pellet was resuspended in 100 μl 0.1 M HCl and the lipids were extracted according to Bligh and Dyer. The lipid extracts were applied to Silica 60 plates (Merck) impregnated with 1.2% (w/v) boric acid [29] and the lipids were separated using chloroform/methanol/water/ammonia, 120:75:6:2 (v/v) as eluent. The positions of the fluorescent spots were determined under UV light, the silica was scraped off and collected in a Pasteur pipette on glass wool. The phospholipids were eluted from the silica using 6 ml $CHCl_3$/MeOH, 1:1 (v/v), and dried at 37°C under a stream of nitrogen. Subsequently the lipid film was solubilized in 0.4% (v/v) Triton X-100 and the fluorescence was measured as described above.

The effect of several factors on the transmembrane movement of C_6-NBD-labeled phospholipids was investigated. IIMV were pretreated with 200 μg/ml proteinase K in buffer Z for 15 min at 37°C and put on ice for 10 min prior to the incorporation of the fluorescent probe. Alternatively, IIMV were pretreated with 2 mM *N*-ethylmaleimide (NEM) in buffer Z for 30 min at 37°C, and put on ice for 10 min before the addition of the fluorescent probe. The energy dependence of transmembrane movement was investigated by incubating the IIMV, with a C_6-NBD-labeled phospholipid incorporated, at 37°C in the presence of 10 mM Mg^{2+}-ATP in buffer Z. To investigate the effect of a pH gradient on the transmembrane movement, IIMV with the C_6-NBD-labeled phospholipid incorporated, were incubated at 37°C with 5 mM lactate in buffer Z to induce a ΔpH [30].

2.5. Assay for dithionite permeability in IIMV

To check for dithionite leakage into the IIMV under the conditions used to measure the transmembrane distribution of the NBD-lipids, advantage was taken of the property of a Lep-PhoA fusion protein to form disulfide-bridged dimers. Leader peptidase (Lep) is an integral membrane protein with two transmembrane helices, connected by a small cytoplasmic loop, and a large catalytic domain in the periplasm [31]. Alkaline phosphatase (PhoA) is an enzyme normally present in the periplasm. Wild type *E. coli* strain W3899 was transformed with plasmid pLep-PhoA, which codes for a Lep-PhoA fusion protein under the arabinose promotor. Overnight cultures of W3899/pLep-PhoA were diluted 1:40 in LB media with 50 μg/ml ampicillin. After the culture had reached an OD_{660} of 0.2, every 20 min an aliquot of arabinose was added to a final concentration of 1% (w/v) and growth was continued until an OD_{660} of 0.7–0.8. was obtained. Cells were harvested and IIMV were isolated as described above, except that no DTT was used.

IIMV of W3899/pLep-PhoA were treated as in the dithionite assay, except that instead of a C_6-NBD-phospholipid solution, only ethanol was added to a final concentration of 0.15% (v/v). The dimer/monomer ratio of the Lep-PhoA fusion protein in the IIMV was analyzed by SDS-PAGE under non-reducing conditions (11% gel). The Lep-PhoA fusion protein was visualized by Western blotting and autoradiography with ^{35}S-labeled protein A (Amersham, UK), and identified by its position on the gel, the dependency of its presence on induction, and its reaction with anti-Lep antibody. For quantification the bands of the monomer and dimer Lep-PhoA were scanned on a Phosphor Imager SI (Molecular Dynamics).

2.6. Incorporation of C_6-NBD-labeled phospholipids in E. coli cells with a permeabilized outer membrane

An overnight culture of W3899 was diluted 1:50 in LB media and grown to an OD_{660} of 0.7. Cells from 2 ml of culture were harvested, washed with 1 ml of physiological salt solution (cold), and resuspended in 500 μl of an ice cold physiological salt solution containing 10 mM Tris-HCl pH 8.0, 5 mM EDTA, to permeabilize the outer membrane [32]. 500 pmol of C_6-NBD-PS or C_6-NBD-PG was added to the cell suspension on ice, followed by a 5 min incubation. 200 μl samples were drawn and left on ice or put at 37°C for 30 min. Subsequently the cells were collected by centrifugation and the phospholipids were extracted and analyzed by TLC as already described. Lipids isolated from cells in which C_6-NBD-PG was incorporated were separated as described above. For the isolation of lipids from cells containing C_6-NBD-PS, untreated Silica 60 plates were used, and separation was achieved using $CHCl_3/MeOH/H_2O$, 65:25:4 (v/v) as eluent. Fluorescence was measured as described above.

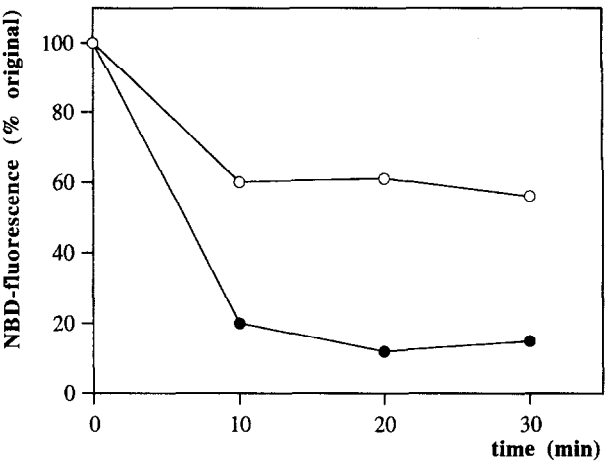

Fig. 1. Time-course of the reduction on ice by dithionite of C_6-NBD-PE present only in the outer leaflet (●) or in both leaflets (○) of LUVETs prepared from an *E. coli* total phospholipid extract. A symmetrical distribution of C_6-NBD-PE was obtained by incorporating the fluorescent probe in the lipid film prior to hydration. Dithionite was added to a final concentration of 8 mM at time zero. For experimental details see Section 2.

3. Results

3.1. Determination of C_6-NBD-PE transmembrane distribution by dithionite

To determine the transmembrane movement of different C_6-NBD-labeled phospholipids, incorporated in the *E. coli* inner membrane from the cytoplasmic side, the dithionite assay was set up and applied to IIMV.

First, the optimal conditions for the selective reduction of C_6-NBD-labeled phospholipids present in the outer leaflet were established using LUVETs prepared from *E. coli* phospholipids. Fig. 1 shows that upon addition of dithionite from a 1 M stock, most of the C_6-NBD-PE is reduced when it is incorporated in LUVETs by external addition. After an incubation for 20 min on ice, 8 mM dithionite has reduced most of the externally added label but only 40% of the label when this is symmetrically distributed in LUVETs, demonstrating that dithionite under these conditions does not penetrate the membrane.

3.2. Transmembrane redistribution of C_6-NBD-PE in IIMV, but not in LUVETs

C_6-NBD-PE was incorporated into IIMV and LUVETs, the vesicles were incubated for 1 h at 0°C or 37°C, and subsequently treated with dithionite on ice. After the incubation the label is still present on PE as analyzed by TLC (data not shown). In the case of the IIMV, the protected pool of fluorescent label is about 4-fold higher at 37°C than at 0°C, as shown in Fig. 2. This protected pool becomes accessible to dithionite when the vesicles are solubilized with Triton X-100. No significant change in accessibility of C_6-NBD-PE to dithionite is observed in LUVETs after incubation at 0°C or 37°C, with the protected pool of C_6-NBD-PE being similar to that found for IIMV incubated at 0°C. From the temperature dependent occurrence of protected C_6-NBD-PE in IIMV, and from the fact that this protected pool can be reduced after solubilizing the vesicles with detergent, it is concluded that the probe has the ability to redistribute (flip) across the membrane. 5% of the NBD-label incorporated into IIMV can not be reduced even if the IIMV are solubilized with Triton X-100. This is due to the experimental conditions selected for the topology assay. Higher concentrations of dithionite are able to reduce all the label present both in

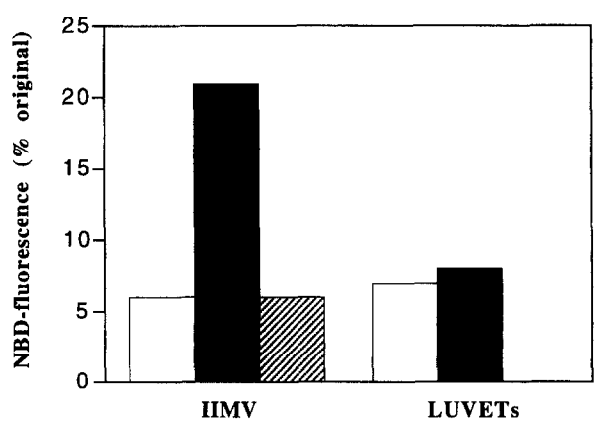

Fig. 2. Comparison of the C_6-NBD-PE fluorescence, protected against reduction with dithionite in IIMV and LUVETs after incubating at 37°C (black bars) or 0°C (open bars). After incubating for 1 h at the temperature indicated, vesicles were treated with dithionite, pelleted, and the fluorescence remaining was measured as described in Section 2. In a control experiment IIMV were incubated for 1 h at 37°C and solubilized with Triton X-100 prior to the dithionite treatment (hatched bar).

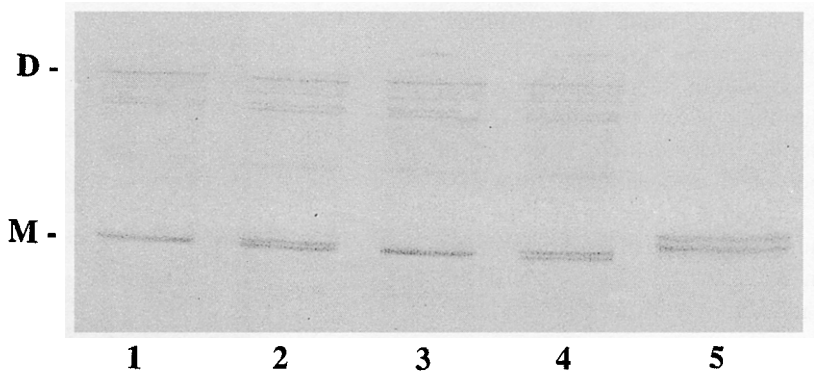

Fig. 3. Treatment with dithionite does not reduce the disulfide bond of a Lep-PhoA fusion protein in IIMV. IIMV of W3899/pLep-PhoA were incubated for 1 h at 0°C (lanes 1 and 2), or 37°C (lanes 3 and 4), treated with dithionite (lanes 2 and 4) under identical conditions as used in the assay for transmembrane movement, and analyzed by SDS-PAGE under non-reducing conditions, followed by Western blotting using an anti-Lep antibody. An autoradiogram of the Western blot is shown with D denoting the dimer of Lep-PhoA, and M the monomer. In lane 5 the IIMV were solubilized with 0.4% (v/v) Triton X-100 prior to treatment with dithionite. In the presence of dithionite for unknown reasons the monomer band shows a double appearance.

intact and solubilized IIMV (on ice), but at these concentrations dithionite permeates the membrane, as was detected in LUVETs symmetrically labeled with C_6-NBD-PE (data not shown). Under the conditions used, dithionite does not permeate the membrane of LUVETs. However, this is not a guarantee that this is also the case for IIMV. If dithionite permeates the membrane of the IIMV the amount of protected C_6-NBD-PE found may be an underestimate of the actual value. To check for this, a dithionite permeability assay was set up.

3.3. Dithionite does not permeate the membrane of the IIMV

IIMV isolated from cells of strain W3899/pLep-PhoA grown in the presence of arabinose, contain a Lep-PhoA fusion protein which can form disulfide-bridged homodimers. This disulfide bridge is situated in the lumen of the IIMV. If dithionite penetrates the membrane it should be able to reduce the dimer. The loss of dimer is a measure of the extent of dithionite permeation. Fig. 3 shows that no loss of dimer is observed in the IIMV after treatment with dithionite. The amounts of dimer present were quantified and the values are presented in Table 1. Lane 5 of Fig. 3 shows that most of the dimer disappears if the vesicles are solubilized prior to the dithionite treatment, proving that dithionite reduces the disulfide bridge if it has access to it. It is concluded that no significant permeation of dithionite in IIMV occurs during the dithionite treatment, irrespective of the temperature of the incubation preceding the treatment with dithionite.

Table 1
Dithionite treatment of W3899/pLep-PhoA IIMV does not result in a loss of Lep-PhoA dimer [a]

Temperature (°C)	Dithionite (8 mM)	Dimer
0	−	100 ± 21
0	+	103 ± 18
37	−	100 ± 24
37	+	100 ± 24
0 [b]	+	15 ± 9

[a] IIMV of W3899/pLep-PhoA were treated as described in the legend of Fig. 3. Dimer and monomer bands were quantified with a Phosphor Imager, and the relative contribution of the dimer band to the total of the Lep-PhoA fusion protein was determined. The data are related to the intensity of the dimer band obtained after incubating for 1 h at 0°C, without dithionite treatment. Values are presented ± S.D. ($n = 4$).
[b] Sample treated with Triton X-100.

3.4. Temperature dependence of transmembrane movement of C_6-NBD-PE

The effect of temperature on the rate of transmembrane movement of C_6-NBD-PE was investigated. After the incorporation of C_6-NBD-PE into IIMV, the vesicles were incubated at 0°C, 25°C, or 37°C for several time intervals,

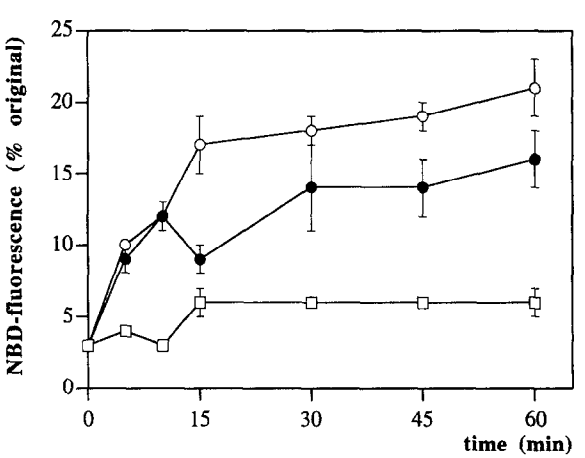

Fig. 4. The rate of transmembrane movement of C_6-NBD-PE in IIMV is temperature dependent. After incubation of the IIMV with C_6-NBD-PE incorporated at 0°C (□), 25°C (■), and 37°C (○) for the indicated periods of time, IIMV were treated with dithionite, and the residual fluorescence was related to the original fluorescence intensity (i.e., the intensity before dithionite treatment). The error bars depict the standard deviation ($n \geq 3$).

and subsequently treated with dithionite on ice. Fig. 4 shows that the rate of transmembrane movement is temperature dependent. At 0°C the pool of fluorescence protected against reduction with dithionite is low (6%) and does not change during a 1 h period of incubation. However, at 25°C and 37°C the pool of fluorescent label protected against reduction with dithionite increases in time and levels off at a maximum value which is higher after incubation at 37°C than at 25°C. At 37°C, C_6-NBD-PE moves across the inner membrane with an estimated $t_{1/2}$ of 7 min.

3.5. Phospholipid specificity of the transmembrane movement

To investigate whether transmembrane movement depends on the nature of the phospholipid head group, different C_6-NBD-labeled phospholipids were incorporated into the IIMV after which the vesicles were incubated at 37°C for several periods of time. Upon incorporation into IIMV, C_6-NBD-PS is quickly ($t_{1/2} < 7$ min) decarboxylated to C_6-NBD-PE by the enzyme PS-decarboxylase, even at low temperatures (data not shown). Using this biosynthetic route C_6-NBD-PE is directed into the membrane in a similar way as is the case for PE in the in vivo situation. Fig. 5 shows that the phospholipid analogues tested, even the one resembling PC, which is not an endogenous lipid of *E. coli*, rapidly flip across the membrane of the IIMV. Furthermore, the rate of transmembrane movement is similar for these phospholipid analogues, and a similar maximum level of fluorescence protected against reduction is observed, which amounts to 20% of the original fluorescence incorporated into the IIMV. The rates of transmembrane movement of C_6-NBD-PE, directly incorporated into IIMV or generated out of incorporated C_6-NBD-PS are similar, suggesting that the transmembrane movement of PE is not directly coupled to PS-decarboxylase activity.

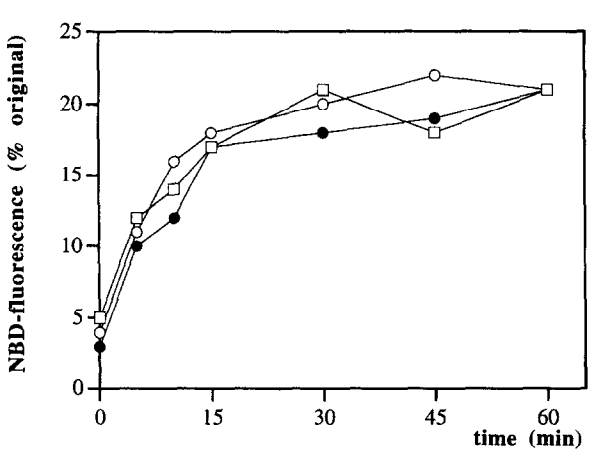

Fig. 5. The rate of transmembrane movement at 37°C of C_6-NBD-labeled phospholipids in IIMV is not dependent on the nature of the phospholipid head group. The experiments were performed as described in the legend of Fig. 4. The following fluorescent phospholipid analogues were added to IIMV: C_6-NBD-PE (●), C_6-NBD-PS (○), and C_6-NBD-PC (□). Error bars are left away for clarity. Standard deviations never exceeded 3% ($n \geq 3$).

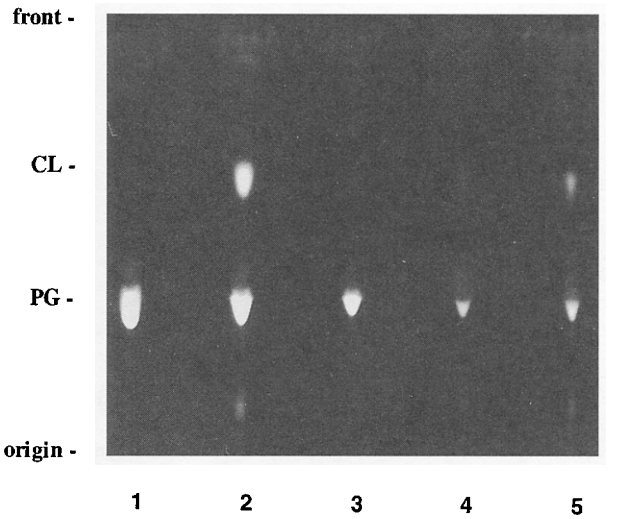

Fig. 6. After the incorporation of C_6-NBD-PG into IIMV it serves as a substrate for CL synthase when the IIMV are incubated at 37°C. After incubating the IIMV, with C_6-NBD-PG incorporated, for 15 min at 0°C (lane 1), or for 15 min (lane 2–4) or 60 min (lane 5) at 37°C, the phospholipids of IIMV were analyzed by TLC, and spots of NBD-labeled phospholipids were visualized by UV. IIMV were treated with proteinase K (200 μg/ml) for 15 min at 37°C, and put on ice for 10 min prior to the incorporation of C_6-NBD-PG (lane 3). Furthermore IIMV were treated with dithionite after the incubation at 37°C (lanes 4, 5).

3.6. C_6-NBD-PG: conversion into C_6-NBD-CL and transmembrane movement

After the incorporation into IIMV, C_6-NBD-PG was partially converted to NBD-labeled CL upon incubation at 37°C (Fig. 6, lane 2). Incubating IIMV on ice did not result in the synthesis of C_6-NBD-CL (Fig. 6, lane 1), and upon proteinase K pretreatment (Fig. 6, lane 3) the conversion at 37°C was completely blocked, suggesting that the active site of CL-synthase is at the cytoplasmic leaflet of the inner membrane. Fig. 7 shows that the conversion is rapid and levels off at 40% of the fluorescent label being converted into C_6-NBD-CL. This conversion provides an opportunity to study the transmembrane transport of both the PG and CL analogues and in addition it allows to compare the kinetics of CL-synthesis with the kinetics of its subsequent transmembrane movement. In Fig. 6, lanes 4 and 5 illustrate that part of both phospholipids flip across the membrane of IIMV, as they are not reduced by dithionite. This effect has been quantified and Fig. 8 shows that C_6-NBD-PG moves across the membrane with a half-time of about 7 min, a value which is in the same order as the rates observed for the other phospholipid analogues in Fig. 5. C_6-NBD-CL moves across the membrane approx. 2.4-fold slower than C_6-NBD-PG (Fig. 8). The rate of trans-

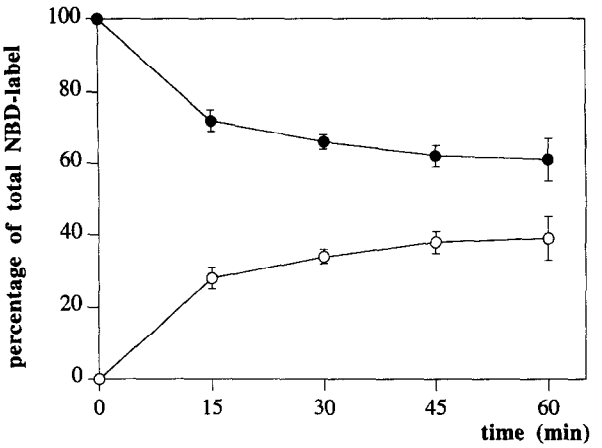

Fig. 7. Conversion of C_6-NBD-PG (●) into C_6-NBD-CL (○) by IIMV at 37°C. IIMV with C_6-NBD-PG-incorporated were incubated at 37°C for several time periods. IIMV were pelleted, after which the phospholipids were extracted and applied to TLC. CL and PG spots were scraped off, the lipids eluted, and the associated fluorescence measured. The error bars depict the standard deviation ($n = 3$).

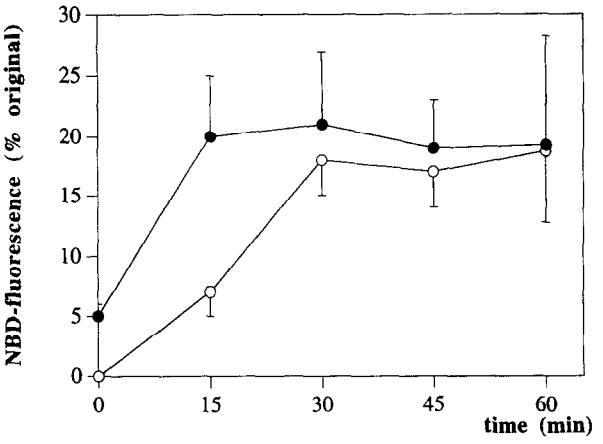

Fig. 8. Time dependence of the protection from reduction by dithionite of C_6-NBD-PG (●), and C_6-NBD-CL (○) in IIMV. The data are expressed as a percentage of the total amount of the corresponding NBD-lipid present at each time point. After the incubation at 37°C vesicles were treated with dithionite and collected. IIMV were further treated as described in the legend of Fig. 7. The error bars depict the standard deviation ($n = 3$).

membrane transport of C_6-NBD-CL is approx. 1.7-fold slower than its rate of synthesis (compare Figs. 7 and 8).

3.7. Which factors affect the transmembrane movement of C_6-NBD-labeled phospholipids

To investigate whether proteins are involved in the transmembrane movement of C_6-NBD-labeled phospholipids, IIMV were either pretreated with proteinase K or NEM. Table 2 reveals that the process of transmembrane movement of C_6-NBD-labeled PE, PS (decarboxylated to PE), and PG, is not influenced by the proteinase treatment and that the transmembrane movement of C_6-NBD-PE is not affected by the pretreatment with NEM. Furthermore, it was checked whether the transmembrane movement of C_6-NBD-labeled phospholipids is energy-dependent. The presence of a pH gradient (generated by 5 mM lactate) during the incubation at 37°C, has no effect on the process of transmembrane movement (see Table 2). A decrease in the transmembrane movement of C_6-NBD-labeled lipids is detected when IIMV are incubated for 15 min at 37°C in the presence of 10 mM Mg^{2+}-ATP. However, this decrease was also found when only Mg^{2+} was present during the incubation at 37°C. Therefore, the inhibitory effect on transmembrane movement observed for Mg^{2+}-ATP most likely is caused by Mg^{2+}.

3.8. Transmembrane movement of C_6-NBD-labeled phospholipids in E. coli cells with a permeabilized outer membrane

In the in vitro assay described above phospholipids analogues are inserted from the cytoplasmic side of the membrane. The ability of C_6-NBD-labeled phospholipids to move from the outside to the inside of the inner

Table 2
The effect of a pretreatment of IIMV with proteinase K or NEM, or the presence of a ΔpH or Mg^{2+}-ATP during the incubation at 37°C on the transmembrane movement of C_6-NBD-labeled phospholipids in IIMV [a]

	Proteinase K		NEM		ΔpH		Energy source		
	+	−	+	−	+	−	−	Mg^{2+}-ATP	Mg^{2+}
C_6-NBD-PE	17	17	23	21	18	18	19	12	16
C_6-NBD-PS [b]	18	17	ND		ND		20	14	15
C_6-NBD-PG [c]	16	16	ND		18	18	15	8	11
C_6-NBD-PC	ND		ND		ND		16	11	11

[a] IIMV were pretreated as described in Section 2, and incubated for 15 min at 37°C, after the addition of the C_6-NBD-labeled phospholipid. The data are presented as the NBD-fluorescence remaining after dithionite reduction and expressed as percentage of the original fluorescence present. ND: not determined.

[b] After C_6-NBD-PS is added to IIMV it is rapidly decarboxylated to C_6-NBD-PE. Proteinase K treatment had no effect on the decarboxylation.

[c] C_6-NBD-PG is a substrate for CL-synthase, so the protected pool of fluorescent label depicted in the table in the absence of proteinase K contains both C_6-NBD-PG and C_6-NBD-CL. Proteinase K pretreatment blocks the conversion of PG to CL. The protected pool of fluorescent label now only contains C_6-NBD-PG.

Table 3
EDTA treated *E. coli* cells of wild type strain W3899 synthesize C_6-NBD-PE and C_6-NBD-CL from incorporated C_6-NBD-PS and C_6-NBD-PG, respectively [a]

Incorporated NBD-lipid	Temperature of incubation (°C) [b]	C_6-NBD-PE (% of total fluorescence)	C_6-NBD-CL (% of total fluorescence)
C_6-NBD-PS	0	41±5	–
C_6-NBD-PS	37	82±4	–
C_6-NBD-PG	0	–	0
C_6-NBD-PG	37	–	50±2

[a] The amounts of newly formed NBD-labeled phospholipids are shown as a percentage of the total fluorescence present on the TLC plate ± S.D. ($n = 3$).
[b] After incorporation of the NBD-labeled lipids, the cells were incubated for 30 min at the temperature indicated.

membrane of *E. coli* cells was also investigated. For this purpose C_6-NBD-labeled phospholipids were incorporated in cells of *E. coli*, of which the outer membrane was permeabilized by EDTA [32]. No major disintegration of the cells was observed, and the cells were homogeneously labeled with C_6-NBD-phospholipid, as checked by fluorescence microscopy (data not shown). Under these conditions 50–60% (data not shown) of the NBD-labeled phospholipid added becomes incorporated in the *E. coli* cells. The transmembrane movement was determined by the ability of the phospholipids to serve as substrates for the enzymes of the biosynthetic routes, which have their active sites at the cytoplasmic side of the inner membrane. The results in Table 3 indicate that both C_6-NBD-PS and C_6-NBD-PG after incorporation into EDTA-treated *E. coli* cells, flip across the inner membrane. A large fraction of C_6-NBD-PS is decarboxylated to C_6-NBD-PE even when the cells are incubated at 0°C, indicating that inward transmembrane movement of C_6-NBD-PS already occurs at this temperature. The transmembrane movement of C_6-NBD-PG at 0°C could not be determined because the enzyme CL-synthase is not active at this temperature (as shown in Fig. 6). However, at 37°C 50% of the C_6-NBD-PG incorporated in the cells is converted to C_6-NBD-CL (Table 3), demonstrating rapid inward transport of the PG analogue.

4. Discussion

In the present study we have investigated the transmembrane movement of C_6-NBD-labeled phospholipids across the inner membrane of *E. coli* in in vitro systems. Using a dithionite reduction assay, it was shown that C_6-NBD-labeled lipids are able to rapidly flip across the membrane of *E. coli* inverted inner membrane vesicles (IIMV) in a temperature dependent way. At 37°C C_6-NBD-PE equilibrates over the membrane of IIMV with a $t_{1/2}$ of 7 min. In contrast, in LUVETs prepared from wild type *E. coli* lipids, no transmembrane movement of C_6-NBD-PE was detected, consistent with the reported slow flip-flop rates of natural phospholipids ($t_{1/2}$ of 80 days [33]) in artificial phospholipid bilayers.

In the dithionite assay IIMV are incubated with 8 mM dithionite for 20 min on ice to reduce the fluorescent label present in the outer leaflet of the vesicles. Under these conditions dithionite is able to reduce 95% of the fluorescent label when this is incorporated into IIMV on ice and therefore present in the outer leaflet of the IIMV only. The assay was set up in this way because other protocols did not work. The dithionite assay as described by McIntyre and Sleight [17], and Ruetz and Gros [19], where dithionite is added to vesicle suspensions at room temperature, did not work in the case of IIMV. On-line fluorescence measurements showed that no stable level of fluorescence is reached during the incubation with dithionite at room temperature (data not shown). Furthermore, flip-flop is likely to continue during the incubation at these higher temperatures, complicating the interpretation of the values obtained. The conditions of the dithionite assay were validated in experiments with LUVETs, and with the use of a Lep-PhoA marker in the IIMV. In LUVETs not more than 40% of the C_6-NBD-PE is reduced when this is symmetrically distributed over the membrane, demonstrating that the label is selectively reduced in the outer leaflet. Dithionite does not permeate the membrane of IIMV as evidenced by the absence of any disulfide bridge reduction in the Lep-PhoA dimer.

BSA extraction, which is a widely used method in determining the distribution of fluorescently labeled short chain phospholipid analogues, and spin-labeled phospholipid analogues over the plasma membrane of eukaryotes (for a review see [34]), did not work in the case of IIMV. After the incorporation of C_6-NBD-PE into IIMV on ice, not more than about 70% of the probe could be re-extracted after incubating for 30 min with 5% (w/v) BSA on ice. This number could not be increased by longer incubation times or higher concentrations of BSA (data not shown).

When the data are corrected for the fraction non-reducible label it can be calculated that at equilibrium 15% of all C_6-NBD-labeled phospholipids have moved across the inverted inner membrane. The fact that for all C_6-NBD-labeled lipids the rate of transmembrane movement is the same at 37°C, indicates that this process in *E. coli* is not head group specific. Even C_6-NBD-PC, an analogue of PC, a lipid class which is normally not present in *E. coli* cells, shows the same rate of transmembrane movement as the other lipid probes. This is in agreement with an earlier study of Langley et al. [16], which showed that in a *psd* mutant PS was transported from its site of synthesis to the outer membrane, thereby flipping across the inner membrane. PS is normally rapidly decarboxylated to PE and is therefore not present in the membranes of an *E. coli* cell. The fact that PS was flipped across the inner membrane

indicates that the machinery involved in this process is not specific for the endogenous *E. coli* phospholipids.

The equilibrium distribution of ~ 6:1, cytoplasmic/periplasmic side obtained for the C_6-NBD-labeled phospholipids is very unlikely to correspond to the distribution of the endogenous phospholipids across the *E. coli* inner membrane. Possibly, the transmembrane movement of C_6-NBD-labeled lipids in the IIMV is restricted in the in vitro system. When C_6-NBD-labeled phospholipids are added to the IIMV at a ten-fold lower concentration, the same distribution pattern is observed after a 1 h incubation at 37°C (data not shown), suggesting that it is not the amount of fluorescent probe incorporated into the IIMV that determines its transmembrane distribution obtained at equilibrium. It can not be excluded that the kinetics of transmembrane movement are different in the case of a lower amount of NBD-phospholipid. In the in vivo situation phospholipids are transported to the outer membrane or can be used in the periplasm for protein modification [6], and for the formation of periplasmatically localized membrane-derived oligosaccharides [7]. This could provide a sink for the phospholipid transport process which is most likely not present in the in vitro system. Therefore, transmembrane transport towards the periplasmic side of the inverted inner membrane vesicle might stop at an early stage.

The fact that C_6-NBD-PS and C_6-NBD-PG are used by the enzymes of the biosynthetic routes is an argument for the use of these artificial phospholipids as model for the endogenous phospholipids. C_6-NBD-PS is, even at low temperatures, quickly converted into C_6-NBD-PE by the PS-decarboxylase. By using this biosynthetic pathway C_6-NBD-PE was introduced into the inner membrane via a route mimicking the in vivo situation. The rate of transmembrane movement of the resulting C_6-NBD-PE was similar to that obtained when this lipid analogue was directly incorporated into IIMV, indicating that transmembrane movement of PE and decarboxylation of PS are processes that are not directly coupled. C_6-NBD-PG was shown to be a substrate for the synthesis of C_6-NBD-CL by CL-synthase. If IIMV were pretreated with proteinase K no C_6-NBD-CL was synthesized from C_6-NBD-PG, indicating that the catalytic domain of CL-synthase is situated at the cytosolic side of the membrane, as was found for the other phospholipid biosynthetic enzymes [15].

The conversion of C_6-NBD-PG to C_6-NBD-CL only occurs at higher temperatures and is less rapid than the decarboxylation of C_6-NBD-PS. This makes it a nice tool to investigate the transmembrane movement of a phospholipid generated in situ. C_6-NBD-CL is rapidly synthesized at 37°C. After synthesis it is partially flipped across the membrane of IIMV, at a rate lower than the rate of synthesis, indicating that also the synthesis and transmembrane movement of C_6-NBD-CL are not directly coupled. The slower rate of the transmembrane movement of CL could have several causes. Possibly the slower rate of transmembrane movement of CL is simply due to its molecular structure with two negative charges in the head group and four acyl chains. Alternatively, the acyl chain composition may affect the flipping rate of the NBD-labeled phospholipids. It is assumed that each fluorescent labeled CL contains one NBD group. This assumption is based on the consideration that the amount of C_6-NBD-PG is much less than the amount of endogenous PG. As a result C_6-NBD-CL contains more long-chain fatty acids than the other fluorescent probes. The length of the fatty acids might cause the C_6-NBD-CL to be more tightly packed in the membrane and consequently it will not flip as easily as the other probes. This reasoning suggests that the rate of transmembrane movement of the endogenous *E. coli* phospholipids is slower than that of the short chain NBD-labeled phospholipids reported here. In this context it should be added that Colleau et al. [35] have reported that the NBD label slows down the rate of phospholipid translocation in the plasma membrane of mammalian cells.

As described in the literature (for review see [34]) several flip-flop processes of phospholipids in eukaryotic cells are sensitive to sulfhydryl reagents and/or dependent on a source of energy, indicative for a protein-mediated process. The rate and extent of the transmembrane movement in *E. coli* IIMV could not be altered by a pretreatment with proteinase K, NEM, or the presence of Mg^{2+}-ATP or a ΔpH during the temperature incubation. The effect observed with Mg^{2+}-ATP was accounted for by Mg^{2+} (see Section 3). Possibly, Mg^{2+} might cause a tighter packing of the phospholipids, by diminishing electrostatic head group repulsion. This would make it more difficult for the fluorescent probe to flip across the membrane. Although no specificity was seen between the different C_6-NBD-labeled phospholipids, transmembrane movement did not occur in LUVETs at 37°C, indicating that proteins are involved in this process.

The ability of the fluorescent probe to serve as a substrate for the enzymes of the biosynthetic routes present at the cytoplasmic site of the inner membrane was used to demonstrate the transmembrane movement of C_6-NBD-labeled phospholipids from the periplasmic to the cytoplasmic leaflet of the inner membrane of *E. coli*. C_6-NBD-PS and C_6-NBD-PG are converted into C_6-NBD-PE and C_6-NBD-CL, respectively, upon incorporation into *E. coli* cells. This shows that indeed C_6-NBD-PS and C_6-NBD-PG are able to flip across the inner membrane of *E. coli* from the periplasmic side of the membrane, indicating that C_6-NBD-labeled phospholipids can rapidly move across the inner membrane in both directions.

Rapid transmembrane movement of both PC and PC analogues has previously been observed in rat liver microsomes [36–38]. Interestingly this process was also found to be energy-independent and lacked head group specificity [38]. The inner membrane of *E. coli* and the endoplasmic reticulum share one important characteristic, i.e., they are

the main site of de novo synthesis of phospholipids in the cell, and therefore must possess mechanisms for rapid transmembrane movement. The generation time of wild type *E. coli* is about 30 min. In this time the membrane structures and therefore the total amount of phospholipids are doubled. This implies that the half time of transmembrane movement of phospholipids across the inner membrane is 15 min or less, which is close to the observed values.

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