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Interaction of Phospholipase A of the *E. coli* Outer Membrane with the Inhibitors of Eucaryotic Phospholipases A_2 and Their Effect on the Ca^{2+}-Induced Permeabilization of the Bacterial Membrane

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Abstract Phospholipase A of the bacterial outer membrane (OMPLA) is a β-barrel membrane protein which is activated under various stress conditions. The current study examines interaction of inhibitors of eucaryotic phospholipases A_2—palmitoyl trifluoromethyl ketone ($PACOCF_3$) and aristolochic acid (AA)—with OMPLA and considers a possible involvement of the enzyme in the Ca^{2+}-dependent permeabilization of the outer membrane of *Escherichia coli*. Using the method of molecular docking, it has been predicted that $PACOCF_3$ and AA bind to OMPLA at the same site and with the same affinity as the OMPLA inhibitors, hexadecanesulfonylfluoride and bromophenacyl bromide, and the substrate of the enzyme palmitoyl oleoyl phosphatidylethanolamine. It has also been shown that $PACOCF_3$, AA, and bromophenacyl bromide inhibit the Ca^{2+}-induced temperature-dependent changes in the permeability of the bacterial membrane for the fluorescent probe propidium iodide and suppressed the transformation of *E. coli* cells with plasmid DNA induced by Ca^{2+} and heat shock. The cell viability was not affected by the eucaryotic phospholipases A_2 inhibitors. The study discusses a possible involvement of OMPLA in the mechanisms of bacterial transmembrane transport based on the permeabilization of the bacterial outer membrane.

Keywords OMPLA · *Escherichia coli* · Calcium · Permeabilization

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Introduction

The outer membrane of *Escherichia coli* is a complex and highly ordered structure, the main function of which is to create a barrier between the external medium and the contents of the cell. It includes systems providing both the influx of metabolites and efflux of such macromolecules as bacteriocins. The presence of porin channels in the outer membrane, which give passage to molecules up to 600 Da, allows the bacterial cell to obtain nutrients from the external medium (Dela Vega and Delcour 1996). At the same time, DNA and proteins cannot penetrate into the bacterial cell under normal circumstances; their import is based on either special conditions or special physiological mechanisms. For example, treatment of *E. coli* cells with high concentrations of Ca^{2+} with the following heat shock will change permeability of the bacterial membrane, promoting cells to uptake the plasmid DNA—a phenomenon which underlies the process of bacterial cell transformation (Swords 2003). The phenomenon has been studied extensively, yet there is no clarity concerning the exact molecular mechanisms involved in the import and export of macromolecules in the *E. coli* cell (Mandel and Higa 1970; Cohen et al. 1972; Sabelnikov and Domaradsky 1979; Panja et al. 2008).

Evidently, Ca^{2+} ions exert a wide spectrum of effects on procaryotic cells; in particular, they create conditions which, upon heat treatment, enable the DNA macromolecule to enter the cell. Analysis of literature data allows us to suppose that the underlying mechanisms of Ca^{2+}-induced permeabilization of the outer bacterial membrane are changes in its structure and packing due to the activation of the Ca^{2+}-dependent phospholipase A (Dekker 2000; Bishop 2008). Indeed, phospholipase A of the *E. coli* outer membrane (OMPLA) is shown to be activated in many

stress situations accompanied by the loss of the bacterial membrane integrity: phage-induced lysis, formation of spheroplasts, heat shock, increase in the virulence of pathogenic organisms, release of colicin, etc. (Nelson and Buller 1974; Dekker et al. 1999; Bos et al. 2005; Bishop 2008).

The stress-induced violation of integrity and packing of the outer membrane is believed to be followed by the loss of transmembrane asymmetry: phospholipids are transferred from the inner to the outer membrane leaflet. OMPLA hydrolyzes these phospholipids, restoring the order and integrity of the membrane (Dekker 2000). There are mechanisms of active utilization and recycling of phospholipase reaction products (fatty acids and lysophospholipids) (Bishop 2008), yet it seems a bit strange that such a reparative function of phospholipase A could have something to do with the large-scale alteration of membrane structure and transport of macromolecules, like plasmid DNA.

The literature practically does not discuss the possibility of stress-related OMPLA activation to be the main factor resulting in the dramatic violation of structure and integrity of the outer bacterial membrane. On the other hand, the products of phospholipase A hydrolytic activity (lysophospholipids and, especially, free fatty acids) will increase membrane permeability.

In this article, we have studied the interaction of two inhibitors of eucaryotic phospholipases A_2—palmitoyl trifluoromethyl ketone ($PACOCF_3$) and aristolochic acid (AA)—with OMPLA and have examined how these inhibitors affect the Ca^{2+}-dependent permeabilization of the outer membrane of *Escherichia coli*. It has been shown that (1) palmitoyl trifluoromethyl ketone and aristolochic acid, which are known as inhibitors of eucaryotic phospholipases A_2, can also be considered—on the basis of computer modeling data—as inhibitors of *E. coli* OMPLA; (2) palmitoyl trifluoromethyl ketone and aristolochic acid inhibit the Ca^{2+}-induced permeabilization of *E. coli* membranes for the fluorescent probe propidium iodide; (3) palmitoyl trifluoromethyl ketone and aristolochic acid suppress the transformation of *E. coli* cells by plasmid DNA.

Materials and Methods

Material and Strains

In this study, we used the *Escherichia coli* strain W3110. The bacteria were cultured in the Luria–Bertani (LB) medium containing 1 % tryptone, 0.5 % yeast extract, and 1 % NaCl. Medium components, all inorganic chemicals, spermine, propidium iodide, bromophenacyl bromide, and aristolochic acid were purchased from Sigma-Aldrich, Palmitoyl trifluoromethyl ketone was purchased from Tocris Bioscience.

Transformation of Bacteria

The transformation of bacteria was performed by heat shock method at 42 °C (2 min) in 100 mM $CaCl_2$ according to the standard protocol (Swords 2003, Yoshida and Sato 2009). The bacterial cells were transformed with the plasmid pGEMAX185, which carries a gene of resistance to ampicillin.

Staining of Permeabilized Cells with Propidium Iodide and Fluorescence Measurements

Propidium iodide (PI) was used to evaluate the effect of Ca^{2+} and phospholipase A_2 inhibitors on cell membrane integrity. PI is a nucleotide-binding probe; it cannot penetrate the membrane of intact cells and is excluded by them. In the cells that lost their membrane integrity, PI stains DNA. Exponential-phase cells were harvested by centrifugation (3000 g, 10 min) and washed in PBS (138 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4 (pH 7.4)). The cell pellets were resuspended in the same buffer, and the cell concentration was adjusted to $A_{600} = 0.15–0.25$. PI was prepared in distilled water and added to cell suspensions to a final concentration of 3 μM (Pagan and Mackey 2000). The time of incubation of cells with PI was 2 min. Experiments were carried out at 37 °C, unless otherwise indicated. Fluorescence was measured using a Cary Eclipse spectrofluorimeter (USA) with a 495-nm excitation filter and a 615-nm emission filter. The slit widths were 10 nm.

Molecular Docking

In this study, we made use of a theoretical approach known as virtual screening of ligands. The analysis was performed on the basis of a series of calculations of flexible molecular docking. For the calculations and subsequent ranking of inhibitor conformers according to their affinity to the target protein, the package Autodock VINA was used, and the data obtained were visualized using the package AutoDockTools (Morris et al. 2009; Trott and Olson 2010). The target protein structure analyzed was that of OMPLA (the pdb 1FW3 sequence); it was included in the calculations in the form of a dimer. The size of the target domain was $62 \times 52 \times 74$ Å; each of the ligands was given the maximal degree of freedom—according to the number of single bonds in the molecule. Before calculation of docking, each of the inhibitors was optimized using the quantum-chemical method PM7 implemented in the MOPAC package

(MOPAC2012, James J.P. Stewart, Stewart Computational Chemistry, Colorado Springs, CO, USA). For each of the inhibitors, we calculated the following parameters.

1. Affinity (kcal/mol), an equivalent of energy of the ligand binding to the receptor. Its value depends on the nature and number of the intermolecular bonds formed and, in general, is an integral magnitude characterizing the whole complex of individual interaction contributions to the ligand/receptor binding.
2. Electric dipole moment, a vector magnitude which is sensitive to the changes of the molecular structure. Its value depends on the nature and size of the side groups and the distance between the oppositely charged parts of the molecule.

Results

Interaction of Phospholipase A of the *E. coli* Outer Membrane with Inhibitors of Eucaryotic Phospholipases A_2

Today we know the amino acid sequence as well as the crystal structure of OMPLA (coded by pdb 1FW3). It is shown that the protein consists of 12 anti-parallel beta-strands. OMPLA is Ca^{2+} dependent; in the presence of Ca^{2+} ions, it dimerizes and transforms into the active state (Snijder et al. 1999; Stanley et al. 2006). The catalytic center of the enzyme is built by the triad Asn156–His142–Ser144. OMPLA hydrolyzes various phospholipids, demonstrating both A_1 and A_2 phospholipase activities (Scandella and Kornberg 1971; Kingma et al. 2000; Bishop 2008).

Several inhibitors of OMPLA are discovered (Horrevoets et al. 1991), for example, bromophenacyl bromide, which had been known to inhibit phospholipase A_2 and was also shown to suppress the activity of OMPLA (Homma et al. 1984). The most specific among the known inhibitors is hexadecanesulfonylfluoride, consisting of a long hydrocarbon chain and a polar sulfonyl fluoride moiety; it was synthesized on the basis of the inhibitors of serine hydrolyses which OMPLA belongs to.

It seemed probable that other inhibitors of eucaryotic phospholipases A_2 and compounds similar to hexadecanesulfonylfluoride would suppress the OMPLA activity as well. Correspondingly, we have examined interaction of two of such inhibitors with *E. coli* OMPLA using the methods of computer modeling. Fig. 1 shows that the typical binding site of these inhibitors is a hydrophobic region opposite to the OMPLA catalytic center, with the region and the center being located on different monomers of the dimer. This binding region is also the site where the enzyme binds its substrate palmitoyl oleoyl phosphatidylethanolamine and specific inhibitor hexadecanesulfonylfluoride. As it is shown in Table 1, the parameters of palmitoyl trifluoromethyl ketone ($PACOCF_3$) binding are closest to those of the specific OMPLA inhibitor. Aristolochic acid (AA) has a higher affinity to OMPLA and, on the other hand, a higher dipole moment. At the same time, bromophenacyl bromide has a lower affinity to OMPLA and a lower dipole moment.

The Phospholipase Inhibitors Palmitoyl Trifluoromethyl Ketone and Aristolochic Acid Do Not Affect the Rate of *E. coli* Culture Growth

To study the effect of phospholipase A_2 inhibitors on the survivability of bacteria, we have examined the growth of *E. coli* cells in a liquid culture in the presence of these inhibitors. The inhibitor concentrations used corresponded to those suppressing the phospholipase activity in the eucaryotic cells. Bacteria were cultured in the LB medium in the presence or absence of the inhibitors, with the culture density being measured every 45 min for 7 h. As it can be seen in Fig. 2, neither 20 μM $PACOCF_3$ nor 30 μM aristolochic acid affected the rate of cell biomass growth. At the same time, bromophenacyl bromide (5 μM) slowed down the growth of cellular biomass at the initial phase. The data obtained allow one to refer bromophenacyl bromide to the group of nonspecific OMPLA inhibitors.

Effect of Phospholipase Inhibitors on the Ca^{2+}-induced Permeabilization of Bacterial Membranes

On the basis of our supposition and computer modeling data that inhibitors of eucaryotic phospholipases A_2 can also be considered as OMPLA inhibitors, we examined if they had any effect on the Ca^{2+}-induced permeabilization of the *E. coli* membrane, which was judged by the penetration of PI into the cell.

The basic level of PI fluorescence in the medium was around zero (2–3 % of the level observed after the Ca^{2+}-dependent cell permeabilization at 37 °C). In the process of incubation of *E. coli* cells with PI, a part of them was getting stained, and the level of fluorescence before the addition of Ca^{2+} was 20–30 % (Figs. 3–5). This initial uptake of PI depends on the physiological state of cells and is characteristic for the bacterial cultures in the phase of exponential growth (Shi et al. 2007).

As it is shown in Fig. 3, addition of 1 mM $CaCl_2$ to the suspension of *E. coli* cells resulted in the rise of PI fluorescence after a small lag period, indicating violation of the integrity of bacterial membrane. The effect of Ca^{2+} was temperature dependent: at 25 °C, no increase in the fluorescence intensity was observed over a 30-min period; at 37 °C, PI fluorescence began to grow after a 5- to 6-min

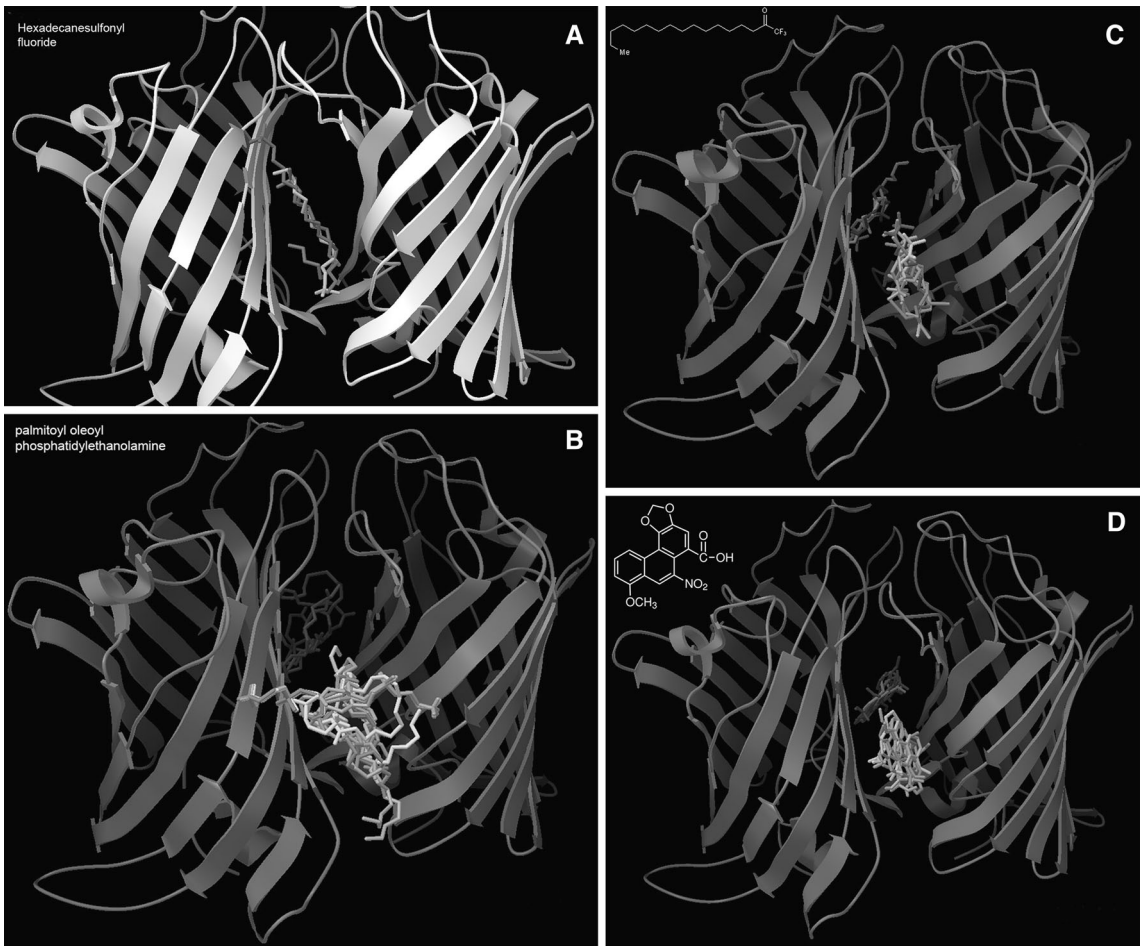

Fig. 1 The calculated docking site of OMPLA for the phospholipase inhibitors: hexadecanesulfonylfluoride (**a**), the phospholipid palmitoyl oleoyl phosphatidylethanolamine (**b**), $PACOCF_3$ (**c**), aristolochic acid (**d**)

Table 1 Parameters of affinity of phospholipase A_2 inhibitors to OMPLA (coded by pdb 1FW3) obtained by the method of flexible molecular docking and PM7-calculated dipole moments of the inhibitor molecules

Agent	Affinity (kcal/mol)	Electrical dipole moment
Hexadecanesulfonylfluoride	−7.3	3.10
Phospholipid POPE	−7.0	14.70
Palmitoyl trifluoromethyl ketone	−7.2	2.73
Aristolochic acid	−9.8	7.06
Bromophenacyl bromide	−6.6	2.60

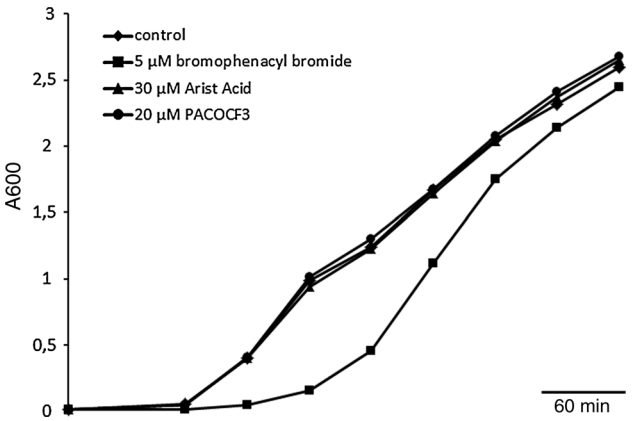

Fig. 2 Growth of *E. coli* biomass in the LB medium in the presence of phospholipase A inhibitors. The graph represents the average for two experiments. Symbols: control (*diamond*), aristolochic acid (*triangle*), $PACOCF_3$ (*circle*), bromophenacyl bromide (*square*)

lag phase; and at 45 °C, the lag phase shortened to 2–3 min. In the absence of Ca^{2+}, incubation of *E. coli* cells at 45 °C did not lead to any visible changes of PI fluorescence. It should be noted that preincubation of *E. coli* cells with 1 mM Sr^{2+} resulted in the complete suppression of Ca^{2+}-induced membrane permeabilization, which suggests a competition between these two cations for the binding sites on the membrane proteins.

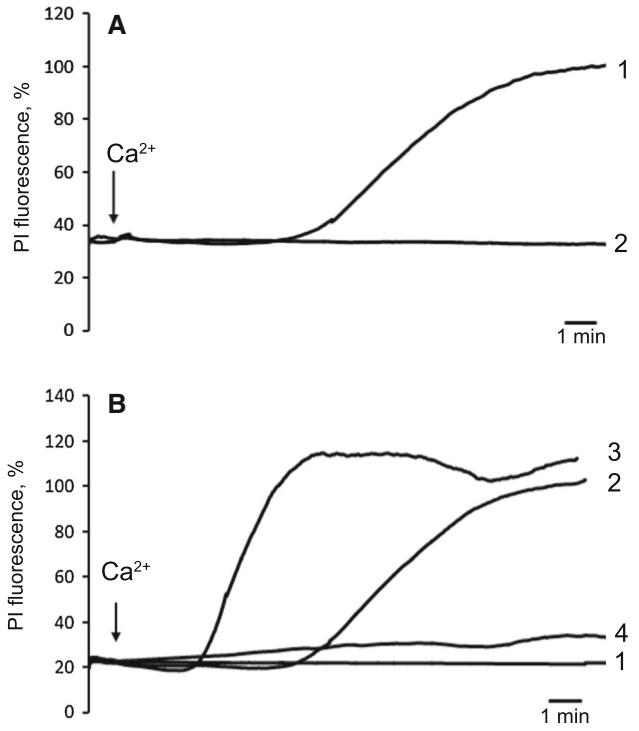

Fig. 3 Ca^{2+}-induced changes in the permeability of *E. coli* membranes for propidium iodide (PI). The fluorescence level observed after the Ca^{2+}-dependent cell permeabilization at 37 °C was taken as 100 %. A Sr^{2+} (2) suppresses the Ca^{2+}-induced change (1) in membrane permeability for PI. B Temperature dependence of Ca^{2+}-induced permeabilization of *E. coli* membranes (1–25 °C, 2–37 °C, 3–45 °C, 4–45 °C, no Ca^{2+}). Medium composition: 138 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 3 μM PI pH 7.4. Additions: (**a**) 1 mM $CaCl_2$ (1) and 1 mM $SrCl_2$ + 1 mM $CaCl_2$ (2); (**b**) 1 mM $CaCl_2$

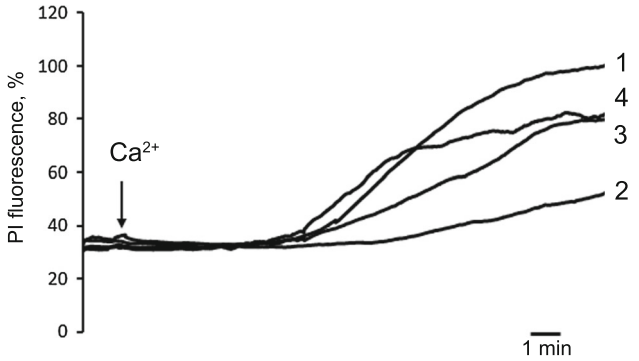

Fig. 4 Effect of phospholipase A_2 inhibitors on the Ca^{2+}-induced change in the permeability of *E. coli* membranes for PI. The fluorescence level observed after the Ca^{2+}-dependent cell permeabilization at 37 °C was taken as 100 %. Medium composition was the same as in Fig. 3. The permeabilization of *E. coli* membrane for PI was induced by 1 mM $CaCl_2$. Additions: 1, control; 2, 20 μM $PACOCF_3$; 3, 30 μM aristolochic acid; 4, 5 μM bromophenacyl bromide

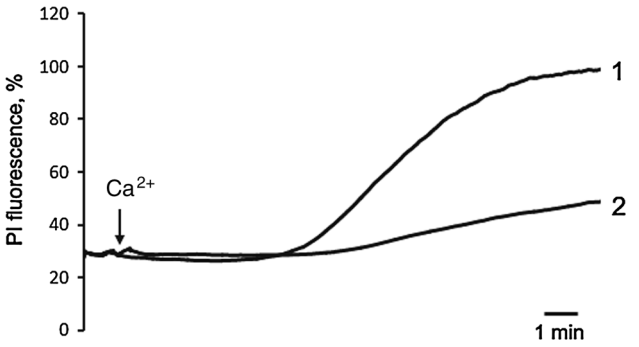

Fig. 5 Effect of the inhibitor of porin channels spermine on the Ca^{2+}-induced permeabilization of *E. coli* membranes. The fluorescence level observed after the Ca^{2+}-dependent cell permeabilization at 37 °C was taken as 100 %. Medium composition was the same as in Fig. 3. The permeabilization of *E. coli* membrane for PI was induced by 1 mM $CaCl_2$. Additions: 1, control; 2, 0.8 mM spermine

Figure 4 shows that preincubation of *E. coli* cells with $PACOCF_3$ (20 μM) leads to the inhibition of Ca^{2+}-induced changes in the permeability of bacterial membranes for PI. Other inhibitors, aristolochic acid (30 μM) and bromophenacyl bromide (5 μM), exerted only a slight inhibitory effects. It should be noted that, in some experiments, bromophenacyl bromide accelerated the beginning of Ca^{2+}-induced permeabilization (data not shown).

Although PI can be referred to the molecules that do not pass through the porin channels of the bacterial outer membrane (molecular mass of PI is 668 Da, and porins give passage to molecules up to 600 Da), the inhibitor of porins spermine (0.8 mM) also suppressed changes in the Ca^{2+}-induced permeability of *E. coli* membranes for PI (Fig. 5).

Phospholipase Inhibitors Suppress the Transformation of *E. coli* Cells with Plasmid DNA

The experiments described above showed that the inhibitors of phospholipases A_2–$PACOCF_3$ and AA–suppressed Ca^{2+}-induced changes in the permeability of bacterial membranes for a small molecule of PI. As it is known, the use of high Ca^{2+} concentrations is the basis for the conventional technique of chemical transformation of bacteria with plasmid DNA (Swords 2003). In the next series of experiments, we tested if phospholipase A_2 inhibitors affected the transformation of *E. coli* with plasmid DNA induced by the treatment of cells with 100 mM $CaCl_2$ followed by heat shock (42 °C for 2 min). The data of Table 2 show that phospholipase A_2 inhibitors significantly reduced the effectiveness of *E. coli* transformation with plasmid DNA.

Table 2 Effect of phospholipase A_2 inhibitors on the effectiveness of *E. coli* transformation with plasmid DNA

Inhibitor	Effectiveness of transformation (number of transformants/μg DNA·10^3)
Control [$CaCl_2$ competent cells treated with heat shock (2 min)]	7.200 ± 1.300
Cells treated with heat shock (2 min)	0
$CaCl_2$ competent cells without heat shock	0.755 ± 0.152*
$PACOCF_3$ (20 μM) + $CaCl_2$ competent cells treated with heat shock (2 min)	1.013 ± 0.225*
Aristolochic acid (30 μM) + $CaCl_2$ competent cells treated with heat shock (2 min)	1.035 ± 0.205*
Bromophenacyl bromide (5 μM) + $CaCl_2$ competent cells treated with heat shock (2 min)	0.088 ± 0.028*

Mean values ± SD are presented ($n = 6$)

* The difference between control and experiment is statistically significant; $P < 0.05$

Discussion

At present, there is a large number of work studying participation of *E. coli* OMPLA in stress-related processes (see reviews Dekker 2000; Bishop 2008). As a rule, it is considered as a kind of "restorer" of membrane structure (Dekker 2000). At the same time, the role of OMPLA as a constitutive enzyme under normal conditions is not quite clear. In this study, we consider the possibility of *E. coli* OMPLA to be involved in the mechanisms that lead to the permeabilization of the bacterial membrane.

As mentioned above, the amino acid sequence and crystal structure of OMPLA are known today, and now researchers are making a wide use of computer modeling to study the interaction of this enzyme with various ligands (Stanley et al. 2006; Stanley et al. 2007; Fleming et al. 2012). On the basis of general principles of operation of phospholipases A, we supposed that inhibitors of eucaryotic phospholipases A_2 would also inhibit phospholipase A of the *E. coli* outer membrane. We selected two inhibitors of different nature, one of them (palmitoyl trifluoromethyl ketone) being an analog of the specific OMPLA hexadecanesulfonylfluoride. The modeling revealed that all three inhibitors had the same docking spot on the enzyme—the binding site also used by the specific OMPLA inhibitor and the phospholipid palmitoyl oleoyl phosphatidylethanolamine (Fig. 1; Table 1). Evidently, palmitoyl trifluoromethyl ketone is a full analog of the specific inhibitor hexadecanesulfonylfluoride. At the same time, aristolochic acid has a higher affinity to the enzyme and a higher dipole moment as well, whereas bromophenacyl bromide shows lower values of these two parameters. It is, therefore, possible that these inhibitors (especially, bromophenacyl bromide) will interact not only with the target enzyme but also with other proteins. The higher dipole moment of AA should also result in a higher hydration energy of its polar head and, possibly, in the interaction of the inhibitor with Ca^{2+}. Consequently, the interaction of AA with OMPLA may be hindered, which would explain why AA turned out to be a lesser potent inhibitor of OMPLA than $PACOCF_3$.

As it can be seen in Fig. 2, neither $PACOCF_3$ nor aristolochic acid affected the growth of cell biomass. At the same time, bromophenacyl bromide inhibited the initial stages of cell biomass growth. Moreover, bromophenacyl bromide seems to have a cytotoxic effect on *E. coli* cells. In spite of the earlier report that bromophenacyl bromide inhibits the activity of the isolated OMPLA (Homma et al. 1984), this agent should not be considered for application in vivo as a specific OMPLA inhibitor. The same conclusion was made by Horrevoets with coauthors (Horrevoets et al. 1991). The other two inhibitors (especially, palmitoyl trifluoromethyl ketone) can be applied to suppress phospholipase A of the *E. coli* outer membrane. Moreover, being added to the suspension of *E. coli* cells, palmitoyl trifluoromethyl ketone was also found to inhibit the Ca^{2+}-dependent increase in the fluorescence of Bis-BODIPY® FL C_{11}PC, a probe for phospholipase activity (unpublished data). This indicates that palmitoyl trifluoromethyl ketone is indeed an inhibitor of OMPLA.

As mentioned above, phospholipase A of the *E. coli* outer membrane is a Ca^{2+}-dependent enzyme (Kingma et al. 2000; Dekker 2000). In this connection, we have examined the changes of the Ca^{2+}-dependent permeability of *E. coli* cells for the non-penetrating fluorescent probe propidium iodide. As it is shown in Fig. 3, 1 mM Ca^{2+} induces, after a lag period, a change in the bacterial membrane permeability for PI. The duration of the lag period depends on temperature (Fig. 3) and Ca^{2+} concentration (data not shown), as well as the presence of other divalent cations. This indicates activation of an enzyme reaction and accumulation of the products of this reaction, leading to the increase in the permeability of the bacterial membrane for PI. As it is seen in Fig. 4, the reaction may be the one catalyzed by OMPLA. The Ca^{2+}-induced permeabilization of *E. coli* membranes was significantly inhibited by palmitoyl trifluoromethyl ketone. A slight inhibitory effect was also observed in the presence of aristolochic acid.

The suppression of Ca^{2+}-induced changes in the permeability of *E. coli* membranes for PI by the inhibitor of porin channels (OmpF and OmpC) spermine (Dela Vega and Delcour 1996) (Fig. 5) indicates complexity of the processes of regulation of bacterial membrane permeability. On the one hand, PI—as mentioned above—is unable to pass through porin channels, so the inhibitory effect of spermine is somewhat unexpected. On the other hand, applied at a relatively high concentration (0.8 mM), spermine could affect OMPLA as well, since both OMPLA and porin channels belong to the same family of β-barrel proteins of the outer *E. coli* membrane.

The data of the current study show that phospholipase inhibitors suppress the transformation of *E. coli* cells by plasmid DNA (Table 2), which can indicate a possible involvement of OMPLA into the process of transformation. Hence, one can assume that OMPLA activates during transformation, resulting in the penetration of plasmid DNA into the cell. The data obtained allow one to suppose that OMPLA is an important factor in the processes related to the transport of macromolecules. Facilitating permeabilization of the bacterial membrane, OMPLA stimulates both the release of bacteriocins (Dekker et al. 1999) and the uptake of exogenous DNA.

There may be different mechanisms by which membrane permeability could change upon Ca^{2+}-induced activation of phospholipase A. The membrane organization, i.e., the packing of membrane components, may be disturbed, and non-bilayer structures may appear in the membrane (Tarahovsky et al. 1995, 1998). In addition, lipid pores may form. As it was shown in our earlier work, saturated fatty acids are able to induce, in the presence of Ca^{2+}, the formation of lipid pores—by the mechanism of chemotropic phase transition in the lipid bilayer (Agafonov et al. 2003; Belosludtsev et al. 2005; 2010). Taking into account that saturated acids dominate in the pool of esterified fatty acids of *E. coli* membranes (Cronan 1968; de Siervo 1969) and that stress conditions (like those used in the procedure of transformation) favor transitions in the phase state of lipids, the "phase transition" mechanism of membrane permeabilization could, indeed, take place in the outer membrane of the *E. coli* cell.

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