Growth Inhibition, Morphological Change, and Ectoenzyme Release of LLC-PK1 Cells by Phosphatidylinositol-specific Phospholipase C of *Bacillus thuringiensis*

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Phosphatidylinositol-specific phospholipase C (PI-PLC) from *Bacillus thuringiensis* added to a culture of LLC-PK1 cells inhibited cell growth by 40%. In contrast with normal cells, the cells cultured in the presence of PI-PLC showed needle-like appendages which seemed to have been formed due to portions of the cell remaining adhered to the culture dish as the cell shrank. When LLC-PK1 cells were treated with PI-PLC, significant amounts of alkaline phosphatase and alkaline phosphodiesterase I were released specifically from the apical surface of the LLC-PK1 cells. Furthermore, PI-PLC treatment caused a delay of enzyme production and dome formation. These data indicate that glycosyl-phosphatidylinositol (GPI)-anchored proteins on the surface of LLC-PK1 cells are important in cell growth and differentiation. Also, the combined use of LLC-PK1 cells and PI-PLC of *B. thuringiensis* is effective for investigating the function of GPI-anchor proteins.

Key words: phosphatidylinositol-specific phospholipase C of *Bacillus thuringiensis*; ectoenzyme release; growth inhibition; morphological change; LLC-PK1 cells

Bacterial phosphatidylinositol-specific phospholipase C (EC 3.1.4.10, monophosphatidylinositol phospholipase D, PI-PLC) was first found in toxic preparations from *Staphylococcus aureus*3 and *Bacillus cereus*.4 Ikezawa et al.5 and Low and Finean6 purified this enzyme from *Bacillus* sp. and *S. aureus*. As a bacterial exotoxin, PI-PLC is not cytolytic, but has been known to inhibit the cell growth of Sarcoma 180,7 KB,8 TN-368,9 and IC-Sofia carcinoma cells.9 Furthermore, PI-PLC releases membrane-bound proteins, which have been reported to be anchored in the plasma membrane by a COOH-terminal GPI moiety.10

In polarized epithelial cells, the apical and basolateral membranes are composed of distinct proteins and lipids.11 The polarized apical distribution of all endogenous GPI-anchored proteins has been reported in the MDCK cell line.12 In this study, using PI-PLC of *B. thuringiensis* and specific antibody for PI-PLC, we examined the growth inhibition, morphological change, and ectoenzyme release of polarized LLC-PK1 cells.

Materials and Methods

**Materials.** PI-PLC was purified from the culture broth of *B. thuringiensis* IAM 12077 by the method of Ikezawa et al.45 Antisera from a rabbit immunized by highly purified PI-PLC was prepared by a method reported previously.13 The anti-PI-PLC IgG fraction was dialyzed against borate-buffered saline (BBS, pH 7.6). The protein concentration was 70 mg/ml. The FPLC system and Superose 12 were products of Pharmacia LKB Biotechnology. Upssala, Sweden; AlamarBlue was from BioSource International, Camarillo, CA. U.S.A.; and all culture media were from Nissui Pharmaceut. Co. Tokyo, Japan. Thymidine 5'-p-nitrophenylphosphate and glycylproline-p-nitroanilide, substrates for alkaline phosphodiesterase I and dipeptidyl peptidase IV were obtained from Boehringer Mannheim GmbH, Germany or the Peptide Institute Inc., Osaka, Japan. All other chemicals were of reagent grade.

Cell lines, cell culture, and measurement of cell proliferation and growth inhibition. The following kidney cells and culture media were used in this study: LLC-PK1 cells (*Sus scrofa domesticus*, pig), Medium 199 with 5% fetal calf serum (FCS); PK (15) cells (*Sus scrofa domesticus*, pig), Eagle’s minimum essential medium (MEM) with non-essential amino acids (NEAA), 1 mM sodium pyruvate, and 5% calf serum (CS); 293 cells (*Han Biosensa*, human), MEM with 10% heat-inactivated horse serum; VERO cells (*Cercopithecus aethiops*, African green monkey), MEM with NEAA and 5% FCS; COS-1 cells (*Cercopithecus aethiops*, African green monkey), Dubecco’s modified MEM (DMEM) with 10% FCS, CRFK cells (*Felis catus*, cat), DMEM with NEAA and 10% FCS; NRK-49F cells (*Rattus norvegicus*, rat), DMEM (high glucose concentration: 4.5 g/liter) with 5% CS; LLC-RK1 cells (*Oryctolagus cuniculus*, New Zealand white rabbit), Medium 199 with 10% horse serum; MDBK cells (*Bos taurus*, bovine), Ham’s F-12 with 10% FCS, MDCK cells (*Canis familiaris*, dog), MEM with 10% FCS. These kidney cells were generous gifts from the Japanese Cancer Research Resources Bank. Cells were maintained in 25 cm² plastic tissue culture flasks at 37°C in humidified 95% air and 5% CO₂.

To measure cell growth, LLC-PK1 cells were cultured in 0.1 ml of medium in flat-bottomed 96-well tissue culture plates (Becton Dickinson Labware, Lincoln Park, NJ, U.S.A.) that were incubated at 37°C for 2 h. According to the manufacturer’s instructions, AlamarBlue was added at 10 ml/well, equal to 10% of the culture volume. The 96-well plates were then incubated in a CO₂ incubator for several hours. Colorimetric reactions in the AlamarBlue-treated plate were measured from the difference of 570/630 nm using an Iwaki microplate reader SMM 3400, Tokyo, Japan. To study the effects of PI-PLC on cell growth, LLC-PK1 cells (1 x 10⁴ cells/well) were cultured at 37°C in a 96-well plate with or without the presence of 1 unit/ml of PI-PLC.

Ectoenzyme release from LLC-PK1 cells. Ectoenzyme release from LLC-PK1 cells was measured by a method reported previously.14 Ten percent suspensions (0.5 ml, 5 x 10⁴ cells in BBS) of LLC-PK1 cells and

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Abbreviations: GPI, glycosyl-phosphatidylinositol; PI-PLC, phosphatidylinositol-specific phospholipase C; FCS, fetal calf serum; MEM, Eagle’s minimum essential medium; NEAA, non-essential amino acids; CS, calf serum; DMEM, Dulbecco’s modified Eagle’s minimum essential medium; BBS, borate-buffered saline; N-CAMs, neural cell adhesion molecules.
an appropriate amount of PI-PLC were incubated at 37°C. The 400,000 x g supernatants of the reaction mixtures obtained using an Optima TL Ultracentrifuge (Beckman Instruments Inc., Palo Alto, CA, U.S.A.) were assayed for ectoenzyme activity. Total activities of ectoenzymes assayed in the presence of 0.1% Triton X-100 after homogenization were taken as 100%. The inhibition of ectoenzyme release by anti-PI-PLC antibody was measured by the method of Nakabayashi et al.18 For separate access of PI-PLC to apical or basolateral membrane domains to study the localization side of GTP-anchored proteins, LLC-PK1 cells were seeded at high density (3 x 10^5 cells/ml) on six filter chambers with cell inserts (Becton Dickinson Labware, Lincoln Park, NJ, U.S.A.) and cultured for 5 days to allow the development of a tight monolayer. After the cells were washed with BBS, 1 ml of BBS and/or 100 munits of purified PI-PLC was added to the upper or lower chamber. After incubation at 37°C for 30 min, each reaction mixture was collected and the 400,000 x g supernatant was obtained as described above.

**Molecular weight measurement of ectoenzymes in culture medium by gel filtration.** Two ml suspensions containing 5 x 10^6 LLC-PK1 cells were seeded into 25 cm^2^ culture dishes containing Media 199 with 5% FCS and cultivated at 37°C. After 1 day, the culture fluid was removed and 2 ml of medium containing 2 units of PI-PLC was added to each dish. To the control dishes, 2 ml of medium without PI-PLC was added. After 4 days, the culture medium was removed and centrifuged at 400,000 x g for 15 min. The resulting supernatant was concentrated by ultrafiltration and 200-ml portions were put on a Superoxel 12 column (16 x 500 mm) equilibrated with 50 mM Tris-HCl buffer (pH 7.5) containing 150 mM NaCl and then eluted with the same buffer using the FPLC System. Fractions (0.5 ml) were collected and assayed for alkaline phosphatase and phosphodiesterase I activities. The molecular weights of alkaline phosphatase and phosphodiesterase I were estimated as described by Andrews,15 using the molecular weight marker proteins.

**Enzyme assays.** The activities of alkaline phosphatase, alkaline phosphodiesterase I, γ-glutamyl transpeptidase, and dipeptidyl peptidase IV were measured by a spectrophotometric assay method reported previously.14 Protein was measured by the method of Smith et al.,15 using BCA protein assay reagent (Pierce, Rockford, IL, U.S.A.) with bovine serum albumin as a standard.

**Statistical analysis.** The results were analysed for statistical significance by Student's t-test.

**Results**

**Specific activities of ectoenzymes in homogenates of various cultured kidney cells.**

As shown in Table I, LLC-PK1 cells showed the highest specific activities for alkaline phosphatase and alkaline phosphodiesterase I, but not for γ-glutamyl transpeptidase or dipeptidyl peptidase IV. In particular, the value of the specific activity for alkaline phosphodiesterase I was 1.7 times as much as that of rat small intestine, which was the highest value of the organs reported previously.18 We therefore selected LLC-PK1 cells for the following experiments.

**Growth inhibition and morphological change in LLC-PK1 cells by PI-PLC of B. thuringiensis**

Figure 1A shows the relationship between cell number and OD 570/630 using AlamarBlue. The AlamarBlue assay is a new rapid and non-radioactive assay for monitoring and measuring the proliferation of various cultured cell lines. As 3 h of incubation gave a higher value of OD 570/630 than 2 h, we used the 3-h incubation to measure cell proliferation after addition of AlamarBlue. When one unit/ml of PI-PLC was added to 2-day cultures of LLC-PK1 cells, the cell growth was inhibited by 40%, as compared with the control culture without PI-PLC (Fig. 1B). Furthermore, the growth inhibition by PI-PLC was completely abolished when it was heated at 100°C for 30 min before addition to the culture medium (data not shown). These findings suggest that growth inhibition must be due to a direct action of PI-PLC on cell membranes.

Normal cells cultivated in the absence of PI-PLC had an epithelial-like appearance under phase contrast microscopy (Fig. 2A). After cultivation in the presence of PI-PLC, needle-like appendages appeared, probably due to portions of the cell remaining adhered to the culture dish as the cell shrank (Fig. 2B). Also, there were many detached cells that had been released into the culture medium.

**Ectoenzyme release from LLC-PK1 cells by PI-PLC of B. thuringiensis**

Figure 3 shows the courses of alkaline phosphatase and alkaline phosphodiesterase I activities released from LLC-PK1 cells by the action of PI-PLC. As shown in Fig. 3A, the modes of alkaline phosphatase release from the cell suspension of LLC-PK1 resembled that of alkaline phosphodiesterase I (Fig. 3B), although the rate of release was about five times higher than alkaline phosphodiesterase I. At the maximum, approx. 95% of the alkaline phosphatase

<table>
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<tr>
<th>Table I. Specific Activities of Ectoenzymes in Homogenates of Various Kidney-derived Cell Lines</th>
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<tbody>
<tr>
<td><strong>Cell line</strong></td>
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<tr>
<td></td>
</tr>
<tr>
<td>LLC-PK1</td>
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<tr>
<td>PK (15)</td>
</tr>
<tr>
<td>293</td>
</tr>
<tr>
<td>VERO</td>
</tr>
<tr>
<td>COS-1</td>
</tr>
<tr>
<td>CRFK</td>
</tr>
<tr>
<td>NRK-49F</td>
</tr>
<tr>
<td>LLC-RK1</td>
</tr>
<tr>
<td>MDRK</td>
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<tr>
<td>MDCK</td>
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</tbody>
</table>

Cells were suspended in 0.1% Triton X-100 containing 10 mM Tris-HCl buffer (pH 7.4) and homogenized in a Dounce homogenizer by 20 strokes with a B pestle. Next, the ectoenzyme activities and protein contents were assayed as described in the text.

Values are means ± S.E. of five measurements.
or 20% of the phosphodiesterase I activity was liberated from LLC-PK1 cells. Throughout the experiments, the enzyme release was suppressed when purified anti-PI-PLC antibody was added to the reaction mixture. Furthermore, the enzyme-releasing activity of PI-PLC was completely abolished by heating at 100°C for 30 min. These findings suggest that the enzyme release must be due to the direct action of PI-PLC on brush border membranes. However, regardless of the PI-PLC treatment, none of the γ-glutamyl transpeptidase or dipeptidyl peptidase IV, which is known as a transmembrane protein, was solubilized in the 400,000 × g supernatant (data not shown).

When LLC-PK1 cells were incubated with 10–50 munits of PI-PLC, the alkaline phosphatase or alkaline phosphodiesterase I release was proportional to the PI-PLC concentration (data not shown). However, the release of both ectoenzymes reached maximum at 75 munits of PI-PLC with no further substantial increase at higher concentrations.

**Molecular weight measurements of ectoenzymes in culture medium by gel filtration**

The molecular weight of alkaline phosphatase or alkaline phosphodiesterase I was estimated by gel filtration on Superose 12. In the presence of PI-PLC, alkaline phosphatase or alkaline phosphodiesterase I activity released from LLC-PK1 cells by PI-PLC was detected as a single peak, corresponding to a molecular mass of about 140 or 280 kDa, respectively (Fig. 4). However, no activity was detected in the fractions from the control culture (data not shown).

**Ectoenzyme distribution in LLC-PK1 cells**

To assess the polarized distribution of ectoenzymes, LLC-PK1 cells were grown to confluence (5 days) in filter chambers containing cell inserts which allow separate access to the apical and the basolateral surfaces. LLC-PK1 cells grown on such permeable supports were then selectively attacked either on the apical or the basolateral surface using PI-PLC. After incubation, each reaction mixture in the upper or lower chamber was collected and the 400,000 × g supernatant was assayed for ectoenzyme. As shown in Table II, alkaline phosphatase or alkaline phosphodiesterase I activity was selectively released from the apical surface of LLC-PK1 cells by the action of PI-PLC.

**Ectoenzyme production after PI-PLC treatment in LLC-PK1 cells**

To study the ectoenzyme production, LLC-PK1 cells treated with or without PI-PLC were cultured for 8 days and assayed for ectoenzyme activity. Figure 5 shows the courses of production of alkaline phosphatase and phos-
phodiesterase I activities. Although the reason remains obscure, both enzyme activities markedly decreased after seeding, then increased with cultivation. With alkaline phosphatase (Fig. 5A), the rate of increase in activity was higher in cells without PI-PLC treatment. No difference in the curve was noted for alkaline phosphodiesterase I activity (Fig. 5B). Also, in contrast to Fig. 2, no difference of morphology was observed in control or PI-PLC treated cells (data not shown). In the case of γ-glutamyl transpeptidase and dipeptidyl peptidase IV, which are not GPI-anchored enzymes, the PI-PLC treatment had no effect on the production (data not shown). In the polarized epithelial cell monolayer, dome formation is a typical phenomenon. 

As shown in Table III, the cell culture with PI-PLC treatment showed a delay of dome formation, although there was no difference in the cell numbers with or without PI-PLC treatment. When the level of specific activity in alkaline phosphatase reached a plateau after 7 days of cultivation, the decrease in the number of dome formations in PI-PLC treated cells disappeared to reach that of the nontreated cells.

**Discussion**

The bacteria that produce PI-PLC are more or less virulent; *S. aureus* and *Clostridium novyi* are known to cause food poisoning and infectious diseases of mammals, and *B. cereus* and *B. thuringiensis* are well-known insect pathogens. In the infection by PI-PLC-producing bacteria of the host eukaryotes, PI-PLC may play some important but unknown role as one of the exotoxins. However, bacterial PI-PLCs are not highly toxic and can be administered to animals or added to a culture medium of cell lines without causing cell lysis, especially for studies on the biological role of GPI-anchoring proteins. It is well known that bacterial PI-PLCs cause growth inhibition and morphological change. In fact, PI-PLCs from *B. cereus* and *B. thuringiensis* inhibited growth of transplanted Sarcoma 180 ascites tumor cells in vivo causing concavity of the cells, and *B. thuringiensis* PI-PLC inhibited growth of TN-368 and IC-Sofia carci-

![Graph](image-url)

**Fig. 3.** Course of Ectoenzyme Release from LLC-PK1 Cells by PI-PLC.

Ten percent suspensions (0.5 ml, 5 x 10^6 cells in 3BRS) of LLC-PK1 cells and 50 min of PI-PLC were incubated at 37°C for 3.45 min. The 400,000 x g supernatants of the reaction mixtures were assayed for ectoenzyme activity. Total activities of the enzymes in LLC-PK1 cells were assayed in the presence of 0.1% Triton X-100 after homogenization and taken as 100% (A); alkaline phosphatase release from LLC-PK1 cells; (B) alkaline phosphodiesterase I release from LLC-PK1 cells. (C), control; (○), before use, PI-PLC was heated at 100°C for 30 min; (●), before use, PI-PLC was mixed with anti-PI-PLC antibody, in the ratio of 67mU PI-PLC vs. 5.8 mg anti-PI-PLC antibody stored at 4°C for 1 h to neutralize the PI-PLC activity. Next, 100 μl of the reaction mixture was added to the reaction mixture. (△), after PI-PLC treatment for 3 min, 4.3 mg (61 μl) of anti-PI-PLC antibody was added to the reaction mixture. (A), PI-PLC treatment. The results are the means ± S.E. (n =3).

![Graph](image-url)

**Fig. 4.** Estimation of Molecular Weight of Alkaline Phosphatase and Alkaline Phosphodiesterase I of Culture Medium Obtained from LLC-PK1 Cells by PI-PLC Treatment by Gel Filtration on Superose 12.

The concentrated culture medium was fractionated by gel filtration on Superose 12 column (16 x 500 mm) equilibrated with 0.2 M Tris- HCl buffer (pH 7.5) containing 150 mM NaCl and eluted with identical buffer at a flow rate of 1 ml/min, using the FPLC System. The eluent was monitored at 280 nm. Fractions (0.5 ml) were collected and assayed for alkaline phosphatase and phosphodiesterase I activities, molecular mass marker proteins (thyroglobulin, M.W. 660 KD; ferritin, 450 KD; catalase, 240 KD; IgG, 155 KD; bovine serum albumin, 68 KD). (○), alkaline phosphodiesterase I; (●), alkaline phosphatase.

**Table II.** Ectoenzyme Release from the Apical or Basolateral Surface of LLC-PK1 Cells by the Action of PI-PLC

<table>
<thead>
<tr>
<th>PI-PLC</th>
<th>Surface</th>
<th>Enzyme release (%)</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td>Alkaline phosphatase</td>
</tr>
<tr>
<td>+</td>
<td>Apical</td>
<td>90.9±2.9</td>
</tr>
<tr>
<td>+</td>
<td>Basolateral</td>
<td>2.3±0.2</td>
</tr>
<tr>
<td>−</td>
<td>Apical</td>
<td>1.3±0.2</td>
</tr>
<tr>
<td>−</td>
<td>Basolateral</td>
<td>2.0±0.1</td>
</tr>
</tbody>
</table>

Values are means ± S.E. of three measurements.
developed brush border, and the presence of glucose (Nadependent) and phosphate transport systems. The polarized apical distribution of all endogenous GPI-anchored proteins was found in MDCK or LLC-PK1 cell lines by using biotinylation. Analysis of LLC-PK1 showed nine GPI-anchored proteins of molecular masses from 30 to 180 kDa. As shown in Table II, apical distribution of GPI-anchored enzymes such as alkaline phosphatase or alkaline phosphodiesterase I was shown by the direct action of PI-PLC in LLC-PK1 cells. Alkaline phosphatase or alkaline phosphodiesterase I are known to be dimers composed of equal weight subunits and biotinylated protein having a molecular mass of 70 or 150 kDa may be considered to be the monomer of each, respectively. If these values are doubled, they coincide with the molecular masses shown in Fig. 4.

To investigate the physical and chemical properties of GPI-anchored proteins in the membrane, bacterial PI-PLCs have been widely used as an important tool. However, some of the GPI-anchored proteins present in different organs are resistant to the action of bacterial PI-PLC. As shown in Fig. 3B and Table II, a maximum of approximately 20% of alkaline phosphodiesterase I activity was released from the apical surface of LLC-PK1 cells by the action of PI-PLC. Based on the difference in PI-PLC susceptibility, two types of enzymes are present on the surface of LLC-PK1 cells, i.e., a PI-PLC-sensitive type and a PI-PLC-resistant type. Neural cell adhesion molecules (N-CAMS) are cell surface glycoproteins that appear to mediate cell-cell adhesion in many tissues, including neural cells. In vertebrates, N-CAMS of 180 and 140 kDa span the plasma membranes with a transmembrane region while the 120 kDa form is anchored by a GPI-moiety. Recently, the purified protein responsible for alkaline phosphodiesterase I activity was found to be a type II membrane protein, which consists of a short NH₂-terminal cytoplasmic tail, a signal-anchor domain and an extended stem region which is followed by a large COOH-terminal catalytic domain. Alkaline phosphodiesterase I of the PI-PLC-resistant type may be considered to be a transmembrane enzyme.

GPI-anchored enzymes such as alkaline phosphatase are reported to be differentiation markers in cultured cells. The dome formation has been attributed to ion and water transport by well-differentiated polarized epithelial cells. As shown in Fig. 5 or Table III, a short period of PI-PLC treatment caused delay of alkaline phosphatase production and dome formation. In the presence of PI-PLC, GPI-anchored enzymes were released into the culture medium from LLC-PK1 cells, causing growth inhibition (Figs. 1 and 4). These data indicate that GPI-anchored proteins on the surface of LLC-PK1 cells are important in cell growth and differentiation. Also, the combined use of LLC-PK1 cells and PI-PLC of B. thuringiensis is effective for investigating the function of GPI-anchor proteins.

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Table III. Effects of PI-PLC on Dome Formation in LLC-PK1 Cells

<table>
<thead>
<tr>
<th>Cultivation days</th>
<th>Dome number (number/cm²)</th>
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<tbody>
<tr>
<td></td>
<td>Control</td>
</tr>
<tr>
<td>5</td>
<td>28.6 ± 3.5</td>
</tr>
<tr>
<td>6</td>
<td>46.5 ± 5.8</td>
</tr>
<tr>
<td>7</td>
<td>52.9 ± 8.0</td>
</tr>
<tr>
<td>8</td>
<td>56.2 ± 6.8</td>
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Values are means ± S.E. of six measurements.
* p < 0.001: Statistical difference from the control group by Student’s t-test.

nomal cells leading to swollen and globular deformation, loss of protoplasmic extensions, or fibrosis. From the differences of growth inhibition and morphological change induced by PI-PLC, there seem to be at least two types of cultured cells. With the first type, both growth inhibition and morphological change occur simultaneously in the presence of PI-PLC (Type I). With the second type, as seen with KB cells, only growth inhibition occurs (Type II). As shown in Figs. 1B and 2, LLC-PK1 cells showed both growth inhibition and morphological change, indicating their classification as Type I.

LLC-PK1 cells are typical polarized epithelial cells originating from the proximal tubule as indicated by a well-
References