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Regulation properties of phospholipase Cδ cloned from *Misgurnus mizolepis*

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Phosphoinositide-specific phospholipase Cδ (PLCδ) plays an important role in many cellular responses and is involved in the production of second messenger. The present study was conducted to characterize the catalytic and regulatory properties of the PLCδ of *Misgurnus mizolepis* (ML-PLCδ). The ML-PLCδ gene was cloned and expressed under according to the method of the previous report (Kim *et al*., 2004), and its recombinant protein was purified by successive chromatography using Ni^{2+}-NTA affinity column. The recombinant ML-PLCδ showed a concentration-dependent PLC activity to phosphatidylinositol 4,5-bisphosphate (PIP_2) or phosphatidylinositol (PI). Its activity was absolutely Ca^{2+}-dependence, which was similar to mammalian PLCδ isozymes. The Ca^{2+} concentration yielding maximal activation of ML-PLCδ was 100 μ M. However, the activity was decreased interestingly by a polyamine, such as spermine and spermidine. *In vitro* assay using cholate-micelle cell, ML-PLCδ activity was inhibited in dose-dependent manner by sphingosine but increased by phosphocholine . In the lipid-binding assay, ML-PLCδ was strongly bound to LPA, PI(3)P, PI(4)P, PI(5)P, PI(3,5)P_2, PI(4,5)P_2, PI(3,4,5)P_3 and PA, but it showed the low affinity to S1P, PI(3,4)P_2 and PS. Taken together our results, it is suggested that the general catalytic and regulatory properties of ML-PLCδ are similar with those of mammalian PLCδ1 isozymes, but the N-terminal extended piscine phospholipase Cδ1 (ML-PLCδ) might reflect some distinctions in regulatory properties and inositol-lipid binding specificity between piscine ML-PLCδ and mammalian PLCδ isozymes.

Key words: Phosphoinositide-specific phospholipase Cδ, *Misgurnus mizolepis*, Protein lipid binding assay, PLC activity

Introduction

Phosphoinositide-specific phospholipase C (PI-PLC) is a key enzyme in signal transduction coupled to hormones, growth factors, neurotransmitters and other agonists to cell surface receptors transmits the extracellular signal across the cell membrane resulting in the production of intracellular secondary messengers. PI-PLC families of enzymes are responsible for the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) that results in the generation of second messengers inositol 1,4,5 trisphosphate (IP_3) and diacylglycerol (DAG). IP_3 is a universal calcium (Ca^{2+}) mobilizing second messenger and DAG functions as an activator of protein kinase C (PKC), respectively (Lee *et al*., 1999). Presently, there are at least 14 known mammalian PLC isozymes that are divided into six classes

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(PLC-β1-4, PLC-γ1-2, PLC-δ1-4, PLC-ϵ, PLC-ζ and PLC-η1, 2) (Rhee, 2001; Stewaet *et al*., 2005).

All PLC isozymes contain catalytic X - Y domains as well as various regulatory domains, including the C2 domain, EF-hand domain, and the pleckstrin homology (PH) domain. PLC PH domains have been shown to mediate protein-protein interaction, which indicates that they perform functions beyond that of a simple membrane tether (Wang *et al*., 1999; Thodeti *et al*., 2000; Chang *et al*., 2002). Among these PLC isozymes, the δ-type isozymes are smaller (M_r 85,000) than the PLC-β and PLC-γ (M_r 140,000-155,000) isoforms, and PLCδ isozymes are evolutionally conserved and are thought to be the primary form in mammals. Mammalian PLCδ1 isozyme was activated by G-protein $\beta\gamma$ subunits, thrombin and polyamines *in vitro* and *in vivo* (Banno *et al*., 1994; Lomasney *et al*., 1999), but the regulatory properties of mammalian PLCδ3 differed from those of PLCδ1 which was inhibited by polyamines and sphingosine under *in vitro* conditions (Pawelczyk & Matecki, 1997b, 1998). PLC δ1 is expressed abundantly in most tissues (Lee *et al*., 1999). Because of the presence of the pleckstrin homology (PH) domain that shows a high affinity for PI(4,5)P_2, PLCδ1 is generally distributed at the inner leaflet of the plasma membrane and in the cytoplasm of various cell types (Yagisawa *et al*., 2006). In the lipid binding of recombinant PLCδ1 PH-GFP protein, PLCδ1 PH-GFP was strongly bound to PI(4,5)P_2, but it showed the low affinity to PI(3)P, PI(5)P and PI(3,4)P_2 (Várnai *et al*., 2002).

We recently cloned PLCδ of Mud loach (*M. mizolepis*; ML-PLCδ) and expressed them in *E.coli* cells using pET vector (Kim *et al*., 2004). The recombinant enzyme was purified to homogeneity using immunoaffinity chromatography and showed a concentration-dependent PLC activity to phosphatidylinositol 4,5-bisphosphate (PIP_2) and its activity was an absolute Ca^{2+}-dependence which was similar to mammalian PLCδ isozymes (Kim *et al*., 2004). The present studies were undertaken to investigate the regulation properties of the expressing enzyme in *E. coli* using PLCδ cloned from *M. mizolepis* (ML-PLCδ).

Materials and Methods

Construction, expression and purification of recombinant PLCδ

The construction of expression vector, recombinant expression and purification of PLCδ was processed by previous report (Kim *et al*., 2004). Briefly, the cDNA of PLCδ was then cloned into the unique *EcoR* Ⅰ and *Hind*Ⅲ sites in pET-self vectors. The pET-PLCδ was used for the expression of PLCδ as a fusion protein with the 6 × His fragment at the N-terminus. For the expression of pET-PLCδ, the BL 21 (DE3) *E. coli* strain was used. Transformed cells were grown in LB medium. At a cell density (A_{600}) of 0.6, gene expression was induced by adding isopropyl-1-β-D-galactopyranoside at a final concentration of 0.4 mM. Transformed cells were harvested after 3 h incubation at 18°C and directly analyzed by SDS-PAGE. Collected cells were resuspended in ice-cold 1× homogenizing buffer (20 mM Tris/pH 7.9, 0.5 M NaCl) and incubated at 30°C for 15 min, followed by centrifugation at 20,000×g at 4°C for 20 min. The supernatant was used for purification and activity assay of active ML-PLCδ. For purification of the expressed ML-PLCδ, the His-bind system (Novagen Co., USA) was employed. The supernatant fraction was applied to the His-bind column. After column washing with 1× wash buffer (20 mM Tris/pH 7.9, 60 mM imidazole, 0.5 M NaCl), ML-PLCδ was eluted in five fractions with 1× elution buffer (20 mM Tris/pH 7.9, 1 M imidazole,

0.5 M NaCl). The pooled fractions were dialyzed and analyzed by SDS-PAGE and PLC activity assay.

Assay of Phosphatidylinositol-4,5-bisphosphate (PIP_2)-hydrolysis

The PIP_2-hydrolyzing activity was assayed with [^{3}H]PtdIns (PI) or [^{3}H]PtdIns-4,5-P_2 (PIP_2) as the substrate. The PI- or PIP_2-hydrolyzing activity was measured with a cholate-mixed micelles, which containing various concentrations of phospholipids, respectively. The lipids in chloroform were dried under a steam of nitrogen gas, suspended in assay buffer containing 50 mM Hepes/pH 7.0, 0.08% sodium deoxycholate, 1 mM ethylene glycol-bis(β-5-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA), 100 mM NaCl, 100 μM $CaCl_2$, and an appropriate amount of enzyme in a total volume of 200 ul. Reactions were initiated by the addition of enzyme, performed for 15 min at 30℃ and terminated by the addition of 1 ml chloroform/methanol /HCl (100:100:0.6, v/v/v), followed by the addition of 0.3 ml of 5 mM EGTA in 1 N HCl. Samples were subjected to vigorous vortex mixing for 30 s and centrifuged at 21,000 × g for 5 min to separate the organic and aqueous phases. The aqueous phase (0.5 ml) was removed, dissolved in 5 ml liquid scintillation fluid, and counted in a liquid scintillation analyzer (Packard Co., USA).

Protein-lipid overlay assay

The ability of the proteins to bind different phospholipids was examined using Protein-lipid overlay assay. Commercially available PIP-strip (P-6001; Echelon Biosciences, Salt Lake City, Utah, USA) were blocked with 3% non-fat skim milk in TTBS (200 mM Tris/pH 7.0, 1.37 M NaCl, 1% Tween-20) buffer for 1 h. The membrane were incubated with purified ML-PLCδ (0.3 μg/ml), in blocking buffer for 14 h at 4℃. After washes with TTBS buffer, the membranes were incubated with rabbit anti His-taq antibody (Novagen Co., USA) for 2 h at room temperature, rinsed and washed as before, and then incubated with phosphatase labeled goat anti-rabbit IgG antibody (1:1000 dilution, Kirkegard and Perry Laboratories. Co., USA) at room temperature for 90 min. The membranes were washed and expressed proteins were visualized by AP conjugated Kit (Bio-rad. Co., USA).

Protein determination

The protein concentration was determined by the method of Bradford, and bovine serum albumin (BSA) was used to calibrate the assay.

Results and Discussion

Purification and Identification of recombinant ML-PLCδ

The recombinant ML-PLCδ protein was expressed in *E. coli* by the method of Kim *et al* (2004). The expressed recombinant ML-PLCδ protein was purified using Ni^{2+}-NTA affinity column. The purified enzyme was assayed by the cholate-mixed micelle assay containing [^{3}H]PI and immunoblot analysis. After the final chromatographic step, the specific activity of purified recombinant ML-PLCδ was 16.9 nmol/mg/min in the presence of 5 μM PI and 0.1 mM of Ca^{2+}. To investigate the regulation properties of PLCδ, we firstly determined the effect of various concentrations of Ca^{2+}, pH and SDC. As shown in Fig 1A, the Ca^{2+} concentration yielding maximal activation of recombinant ML-PLCδ isozyme was about 96 nmol/mg/min at 3×10^{-4} M Ca^{2+} for [^{3}H]PIP_2 hydorlysis and 18 nmol/mg/min at 3×10^{-4} M Ca^{2+},

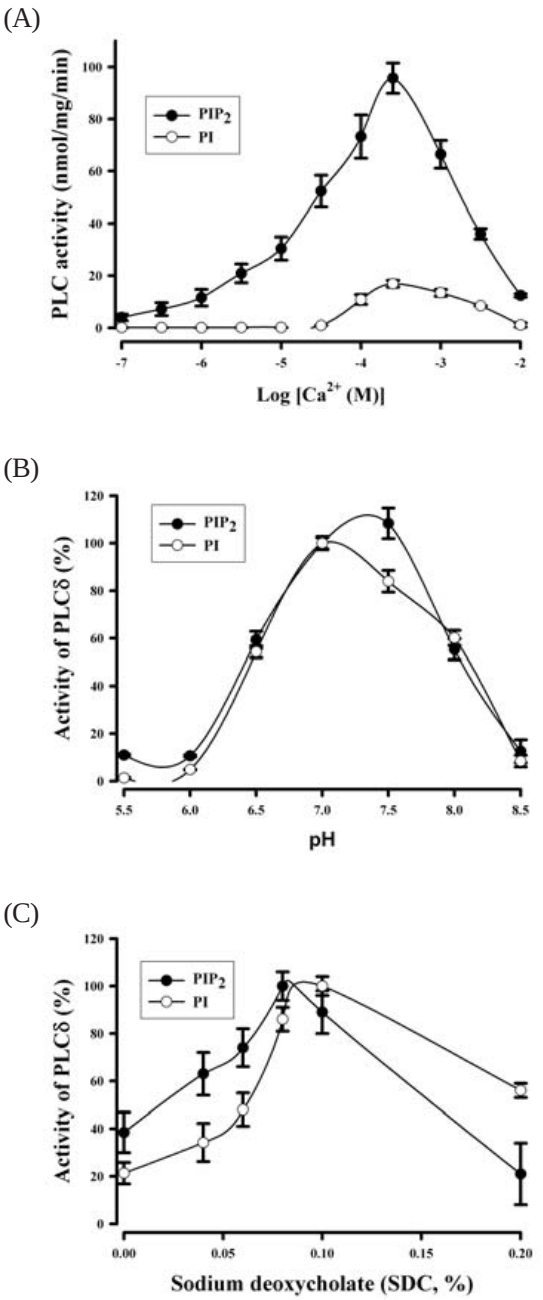

Fig. 1. Effects of calcium, pH, and sodium deoxycholate (SDC) of PLCδ. The activity of ML-PLCδ was assayed by the cholated-mixed micelles assay as described in Materials and Methods. Reaction mixture containing purified enzyme, 1 mM EGTA and various concentrations of calcium (A), pH (B) or SDC (C) in a total volume of 200 $\mu\ell$ was incubated at 30℃ for 15 min. One hundred percent of PLC δ activity was 16 nmol/min/mg with PI-hydrolysis (○) and 98 nmol/min/mg with PIP_2 hydrolysis (●), respectively. Error bars represent S.E.M. of triplicates.

for [^{3}H]PI hydrolysis, respectively (Fig. 1A). The optimum pH of ML-PLCδ was between 7.0 and 7.5, and the PLCδ activity was rapidly decreased at lower pH than 6.5 or higher than 7.8 (Fig. 1B). In addition, the optimum concentration of SDC for PIP_2 hydrolysis was 0.08% in the final concentration, which was slightly higher than that observed for the PI hydrolysis (0.1%) (Fig. 1C). Enzymatic properties of the recombinant ML-PLCδ for PIP_2 hydrolysis were similar to those of other enzymes that were obtained from various mammalian tissues.

Effects of phospholipids on ML-PLCδ activity

Membrane phospholipids are the basic structural components of eukaryotic cells which also play an important role in the control of diverse cellular responses. Phospholipid-derived signaling is thought to constitute a complex signal network that is mediated by not only phospholipase C (PLC) but also by PLA and PLD. One of the best characterized membrane-derived phospholipids is phosphatidylinositol-4,5-bisphosphate (PIP_2), whose hydrolysis by a phosphoinositide (PI)-specific phospholipase C (PI-PLC) results in the generation of diacylglycerol (DAG) and inositol-1,4,5-trisphosphate (IP_3). Phospholipase Cδ1 bound weakly to phospholipid vesicles composed of phosphatidylserine (PS) or phosphatidylcholine (PC) or phosphatidylethanolamine (PE) + PC, and even more weakly to vesicles composed of phosphatidylinositol. The phospholipase Cδ1 enzyme bound strongly to phospholipid vesicles composed of PE + PC and phosphatidylinositol 4,5-bisphosphate (PIP_2) or sphingomyelin (SM) (Pawelczyk & Lowenstein, 1993). Wheather the different phospholipids may affect PIP_2 hydrolysis activity of PLCδ under the conditions of cholate-mixed micelle assay, we were used, the mixed and dried

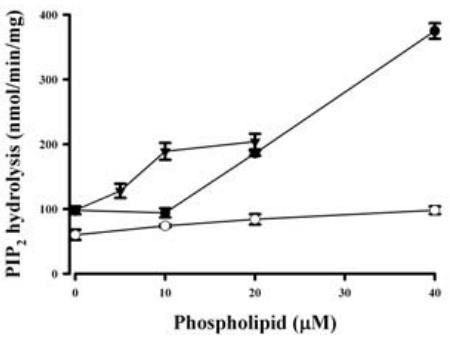

Fig 2. Effect of nonsubstrate phospholipid on PIP_2 hydrolysis catalyzed by PLCδ. The reaction mixture for cholated-mixed micelles assay contained 5 μM [^{3}H]PtdIns-4,5-P_2 (PIP_2) and various concentrations of phospholipids as indicated in the figure legends, 50 mM Hepes/pH 7.0, 0.08% sodium deoxycholate, 1 mM ethylene glycol-bis(β-5-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA), 100 mM NaCl, 100 μM $CaCl_2$. (●) Vesicles were composed of 5 μM [^{3}H]PtdIns-4,5-P_2 (PIP_2) and various concentrations of PC; (○) Vesicles were composed of 5 μM [^{3}H]PtdIns-4,5-P_2 (PIP_2) and various concentrations of PE; (▼) Vesicles were composed of 5 μM [^{3}H]PtdIns-4,5-P_2 (PIP_2) and various concentrations of PS. Error bars represent S.E.M. of triplicates.

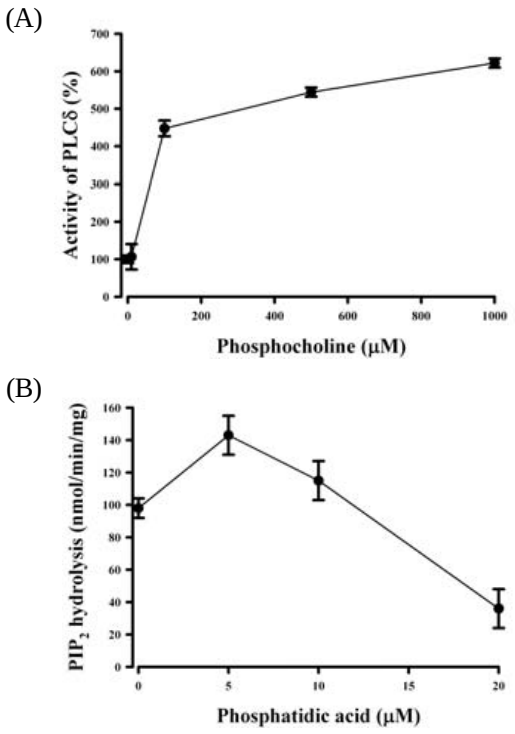

Fig 3. Effects of phosphocholine and phosphatidic acid on activation of PLCδ. (A) The activity of ML-PLCδ was assayed by the cholated-mixed micelles assay as described in Materials and Methods. Reaction mixture containing purified enzyme, 50 mM Hepes/pH7.0, 1 mM EGTA, 0.08% SDC, 0.3 mM Ca^{2+} and various concentrations of phosphocholine in a total volume of 200 μℓ was incubated at 30°C for 15 min. One hundred percent of PLCδ activity was 98 nmol/min/mg with PIP_2 hydrolysis. (B) The reaction mixture for cholated-mixed micelles assay contained 5 μM [^{3}H]PtdIns-4,5-P_2 (PIP_2), 40 μM phosphatidylethanolamine (PE) and various concentrations of phosphatidic acid (PA) as indicated in the figure legends, 50 mM Hepes/pH 7.0, 0.08% sodium deoxycholate, 1 mM ethylene glycol-bis(β-5-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA), 100 mM NaCl, 100 μM $CaCl_2$. Error bars represent S.E.M. of triplicates.

several phospholipids to make the cholate-mixed liposome with various concentrations. As shown in Fig 2, the enzymatic activity of ML-PLCδ was increased in concentration-dependent manner by phospholipids, such as phosphatidyl ethanolamine (PE), phosphatidylcholine (PC) and phosphatidylserine (PS). We also examined the substrate specificity of ML-PLCδ. It showed the preferential hydrolyzing activity for PIP_2, but did not hydrolyze PC or PE (data not shown). The PIP_2-hydrolyzing activity of ML-PLCδ was increased dose-dependent manner by phosphocholine but not phosphatidic acid (Fig. 3).

Effects of basic protein, polyamines and sphingosine on ML-PLCδ activity

The regulation of PLCδ1 critically depends on phospholipid, polyamines, and Ca^{2+}. Sphingomyelin (SM) is the most effective of the phospholipids tested for its ability to inhibit PLCδ1. Sphingosine, which is on the pathway of SM degradation, activates PLCδ1 moderately. Previously it has been reported that sphingosine moderately activates PLC δ1 in the absence of spermine (Pawelczyk & Lowenstein, 1997). Also, in the presence of 1 mM

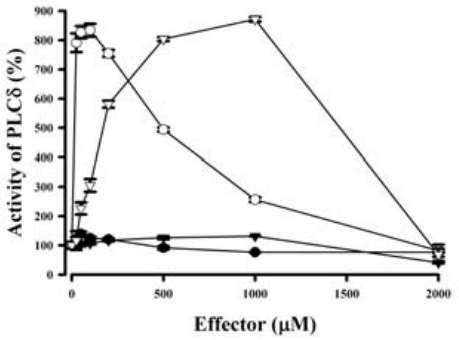

Fig. 4. Effect of polyamines on activation of ML-PLCδ. The activity of ML-PLCδ was assayed by the cholated-mixed micelles assay as described in Materials and Methods. One hundred percent of PLCδ activity was 16 nmol/min/mg with PI-hydrolysis (●, ▼) and 98 nmol/min/mg with PIP_2 hydrolysis (○, ▽) respectively. Reaction mixture containing purified enzyme, 50 mM Hepes/pH7.0, 1 mM EGTA, 0.08% SDC, 0.3 mM Ca^{2+} and various concentrations of polyamine in a total volume of 200 $\mu\ell$ was incubated at 30℃ for 15 min. Sperimine (●, ○) and spermidine (▼, ▽) was present at the concentrations indicated.
Error bars represent S.E.M. of triplicates.

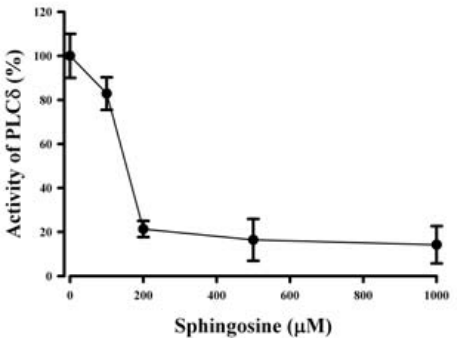

Fig. 5. Effect of sphingosine on activation of ML-PLCδ. The activity of ML-PLCδ was assayed by the cholated-mixed micelles assay as described in Materials and Methods. Reaction mixture containing purified enzyme, 50 mM Hepes/pH7.0, 1 mM EGTA, 0.08% SDC, 0.3 mM Ca^{2+} and various concentrations of sphingosine in a total volume of 200 $\mu\ell$ was incubated at 30℃ for 15 min. One hundred percent of PLCδ activity was 98 nmol/min/mg with PIP_2 hydrolysis. Error bars represent S.E.M. of triplicates.

Ca^{2+}, the hydrolysis of phosphatidylinositol by phospholipase C of human amnion tissue was increased greatly by spermine and spermidine (Sagawa *et al*., 1983). Recently it has been reported that PLCδ1 activity is significantly enhanced by both guanosine thiotriphosphate (GTPγS) and *Clostridium botulinum* exoenzyme C3 (C3) but not by aluminium fluoride (Hodson *et al*., 1998). To study the regulatory properties of ML-PLCδ by basic protein (lysozyme, thrombin), small G protein (GTPγS), polyamines (spermine, spermidine) and sphingosine, we determined the enzymatic activity of ML-PLCδ using the cholate-mixed micelle assay *in vitro*.

It appeared that the enzymatic activity of ML-PLCδ was not directly affected by the basic protein, such as thrombin and lysozyme or GTPγS (data not shown). However, the enzymatic activity of ML-PLCδ was increased in a dose-dependent manner by spermine and spermidine (Fig. 4). In addition, it was inhibited in a dose-dependent manner by sphingosine (Fig. 5). Our results were different from the previous report that the activity of mammalian PLC δ1 and δ3 is stimulated polyamines and basic proteins (Haber *et al*., 1991; Pawelczyk & Matecki, 1997a, 1997b), and mammalian PLCδ1 isozyme was also activated by G-protein $\beta\gamma$ subunits, thrombin and polyamines *in vitro* and *in vivo* (Banno *et al*., 1994; Lomasney *et al*., 1999).

Protein - lipid binding assay

PLC contains various domains, X -Y, C2, EF-hand, PH domains. PLC PH domains have also been shown to mediate protein-protein interaction, which indicates that they perform functions beyond that of a simple membrane tether (Wang *et al*., 1999; Thodeti *et al*., 2000; Chang *et al*., 2002). The PLCδ PH domains bind specifically and with high

affinity to membranes containing PIP_2 (Rebecchi *et al*., 1992; Pawelczyk & Lowenstein, 1993; Ferguson *et al*., 1995; Garcia *et al*., 1995). Deletion analysis of PLCδ1 and studies of its isolated PH domain indicate that this domain is responsible for direct membrane tethering by binding to $PI(4,5)P_2$. Among the PI-PLC isozymes, only the PH domain from PLCδ1 has been studied in terms of its phospholipid binding properties; it was shown to have high affinity, stereospecific binding to IP_3 and $PI(4,5)P_2$. In the lipid binding of recombinant PLCδ 1 PH-GFP protein, PLCδ1 PH-GFP was strongly bound to $PI(4,5)P_2$, but it showed the low to PI(3)P, PI(5)P and $PI(3,4)P_2$ (Várnai *et al*., 2002). However, ML-PLCδ interestingly revealed the presence of a 22-residue amino-terminal extension that is lacking in the mammalian PLCδ isozymes (Kim *et al*., 2004). Investigating the lipid dependence of ML-PLCδ, we used lipid-binding assay. In the lipid-binding assay, ML-PLCδ was strongly bound to LPA, PI(3)P, PI(4)P, PI(5)P, $PI(3,5)P_2$, $PI(4,5)P_2$ and PA, but it was showed the low affinity to S1P, $PI(3,4)P_2$ and PS (Fig. 6). The PH domain of human PLCδ1 was strongly bound to $PI(4,5)P_2$ and $PI(3,4)P_2$ but it was slightly lower affinity to PI, PI(4)P and $PI(3,4,5)P_3$ (Várnai *et al*., 2002). This difference might reflect some distinctions in inositol-lipid binding specificity or subcellular localization between piscine ML-PLCδ and mammalian PLCδ isozymes.

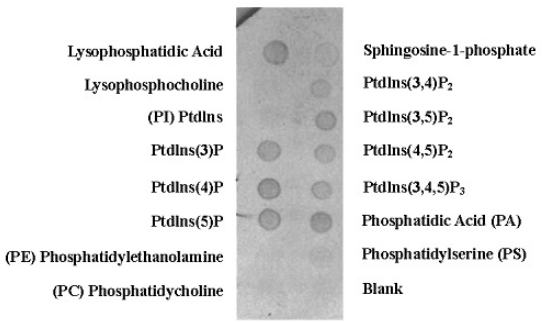

Fig 6. Effects of binding on phospholipids by ML-PLCδ. The phospholipids binding assay of ML-PLCδ was assayed by the protein-lipid overlay assay .

Acknowledgements

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