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Urothelial cell platelet-activating factor production is mediated via calcium-independent phospholipase A₂γ

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Abstract

Purpose—To determine the effect of phospholipase A₂ (PLA₂) inhibitors on urothelial cell platelet-activating factor (PAF) production in response to tryptase stimulation.

Materials and Methods—Urothelial cells isolated from normal human ureters were immortalized with HPV type 16E6E7 (TEU-2 cells). PLA₂ activity in TEU-2 cells was measured using (16:0, [³H]18:1) plasmenylcholine and phosphatidylcholine substrates in the presence and absence of calcium. [³H]PAF production was measured in TEU-2 cells prelabeled with [³H] acetic acid. PAF-acetylhydrolase activity was measured by determine the amount of [³H] acetate hydrolysed from [³H]PAF incubated with TEU-2 cellular protein. Adherence of human PMN to TEU-2 cells was assessed by measuring myeloperoxidase activity in adherent PMN following incubation with TEU-2 cells.

Results—The majority of PLA₂ activity measured in TEU-2 cells was determined to be membrane-associated, calcium-independent (iPLA₂) and selective for plasmenylcholine substrate. Stimulation of TEU-2 cells with tryptase results in increased production of platelet-activating factor (PAF) and increased polymorphonuclear leukocyte (PMN) adherence that were inhibited completely by pretreatment with the iPLA₂γ-selective inhibitor (*R*)-bromoenol lactone ((*R*)-BEL). Pretreatment with the cytosolic PLA₂ inhibitor methyl arachidonyl fluorophosphonate (MAFP) results in potentiation of tryptase-stimulated PAF production and PMN adherence to TEU-2 cells that is a result of PAF-acetylhydrolase (PAF-AH) inhibition.

Conclusions—Tryptase stimulation of TEU-2 cells results in activation of iPLA₂γ leading to an increase in PAF production and increased PMN adherence. Inhibition of TEU-2 cell PAF-AH activity with MAFP potentiated tryptase-stimulated PAF production and PMN adherence.

Keywords

inflammation; platelet-activating factor; phospholipase A₂ inhibitors; urothelium

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INTRODUCTION

The inherent barrier function of the urothelial cells lining the ureter and the bladder wall maintains a defense against infection and injury. However, during inflammation there may be a defect in urothelial cell cytoprotection leading to leakage of urinary constituents into the bladder wall. This can contribute to exacerbation of inflammation in the underlying muscle layers, causing a cycle of inflammation and increased urothelial permeability.

In a previous study, we determined that stimulation of human urothelial cells by mast cell tryptase activates a membrane-associated, calcium-independent phospholipase A₂ (iPLA₂), resulting in the increased production of platelet-activating factor (PAF) and prostaglandin E₂ (PGE₂) (1). Platelet-activating factor is an important inflammatory mediator that is expressed on the surface of endothelial and epithelial cells and binds to the PAF-receptor on inflammatory cells (2,3). The biological activities of PAF can be rapidly terminated by PAF-acetylhydrolases (PAF-AH), a family of unique iPLA₂ enzymes that hydrolyze the acetyl group at the *sn*-2 position of PAF to generate biologically inactive lyso-PAF and acetate (4). The efficient degradation of PAF and the abrupt abrogation of its inflammatory actions have led to the characterization of PAF-AH as an anti-inflammatory enzyme (4).

Considerable effort has gone into designing PLA₂ inhibitors to be used therapeutically as anti-inflammatory agents. However, as more information about PLA₂ isoforms has expanded, some PLA₂ inhibitors originally thought to be selective for a single PLA₂ isoform have been found subsequently to inhibit more than one isoform. For example, methyl arachidonyl fluorophosphonate (MAFP) was originally designed as a selective inhibitor of cytosolic PLA₂ (cPLA₂) but has subsequently been described as a potent and irreversible inhibitor of several PLA₂ isoforms, including iPLA₂ and PAF-AH (5,6). Furthermore, PLA₂ inhibitors may inhibit other enzymes involved in membrane phospholipid hydrolysis or remodeling (7,8). Therefore, the development of a PLA₂ inhibitor as a potential anti-inflammatory agent must undergo rigorous evaluation. In the present study, we pretreated an immortalized urothelial cell line, TEU-2, with PLA₂ inhibitors before stimulation with tryptase and measured their effect on PAF production. These data show that pretreatment of TEU-2 cells with the (*R*)-enantiomer of bromoenol lactone ((*R*)-BEL) to selectively inhibit iPLA₂γ results in decreased PAF production and adherence of polymorphonuclear leukocytes (PMN) following tryptase stimulation. However, pretreatment with MAFP increases tryptase-stimulated PAF production and PMN adherence, primarily as a result of PAF-AH inhibition.

MATERIALS AND METHODS

Materials

Bromoenol lactone (BEL), and methyl arachidonyl fluorophosphonate (MAFP) were purchased from Cayman Chemicals (Ann Arbor, MI). Recombinant human skin β-tryptase was purchased from Promega Corporation (Madison, WI). [³H] acetic acid, [¹⁴C] acetic anhydride, and [acetyl-³H] PAF were purchased from Perkin Elmer Life Sciences (Boston, MA). All other reagents were purchased from Sigma Aldrich (St. Louis, MO).

Cell Culture

TEU-2 cells were generated by immortalization of normal human ureteral cultures with HPV type 16E6E7 (9). Cultures were grown in EpiLife Medium with calcium and growth factor supplements (Cascade Biologics, Inc. Portland, OR), 20 U/ml penicillin and 100 µg/ml streptomycin (Sigma Chemical Company, St. Louis, MO). After reaching confluence, cells were grown in the same medium with 10% fetal bovine serum (FBS) and the addition of 1.0 mM calcium. All experiments were conducted 3 days after calcium and FBS addition.

Measurement of Phospholipase A₂ Activity

Following stimulation, surrounding medium was removed, monolayers were quickly washed with ice-cold PBS and removed from the tissue culture plate in ice-cold buffer containing 250 mM sucrose, 10 mM KCL, 10 mM imidazole, 5 mM EDTA, 2 mM DTT, and 10% glycerol (pH 7.8) (PLA₂ assay buffer). The suspension was sonicated on ice three times for 10 seconds and then centrifuged at $14,000 \times g$ for 10 minutes. The supernatant was then centrifuged at $100,000 \times g$ for 60 minutes to separate the membrane fraction from the cytosol. The membrane fraction was washed twice by resuspending in PLA₂ assay buffer and centrifuging at $100,000 \times g$ for 60 minutes. Phospholipase A₂ activity was assessed by incubating enzyme (8 μ g membrane, 200 μ g cytosol) with 100 μ M (16:0, [³H]18:1) plasmenylcholine or phosphatidylcholine substrate in assay buffer containing 10 mM Tris, 10% glycerol, pH = 7.0 with either 4 mM EGTA or 1 mM CaCl₂ at 37°C for 5 minutes in a total volume of 200 μ l. For determining the sensitivity of membrane activity to (R)- or (S)-BEL the appropriate inhibitor was added to the isolated membrane fraction in the assay buffer for 10 minutes prior to the initiation of the activity assay. Reactions were terminated by the addition of 100 μ l butanol and released radiolabeled fatty acid was isolated by application of 25 μ l of the butanol phase to channeled Silica Gel G plates (Uniplate Inc, Newark, DE), development in petroleum ether/diethyl ether/acetic acid (70/30/1, v/v) and subsequent quantification by liquid scintillation spectrometry with activity normalized to protein content.

Measurement of PAF Production

TEU-2 cells were washed twice with Hanks' balanced salts solution containing 135 mM NaCl, 0.8 mM MgSO₄, 10 mM HEPES (pH=7.4), 1.2 mM CaCl₂, 5.4 mM KCl, 0.4 mM KH₂PO₄, 0.3 mM Na₂HPO₄ and 6.6 mM glucose and incubated with 10 μ Ci [³H] acetic acid/well for 20 minutes. After stimulation with tryptase, lipids were extracted from the cells by the method of Bligh and Dyer (Bligh, E G. and Dyer, W J. 1959). The chloroform layer was concentrated by evaporation under N₂, applied to a silica gel 60 thin layer chromatography plate (Analtech Inc, Delaware), and developed in chloroform/methanol/acetic acid/water [50:25:8:4 (v/v)]. The region corresponding to PAF was scraped and radioactivity quantified using liquid scintillation spectrometry. Loss of PAF during extraction and chromatography was corrected by adding a known amount of [¹⁴C] PAF as an internal standard.

PAF-AH Activity

PAF-AH activity in TEU-2 cells was assessed as described previously (6). Briefly, cellular protein (25 μ g) was incubated with 0.1 mM [acetyl-³H]PAF (10 mCi/mmol) for 30 min at 37°C. The reaction was terminated by the addition of acetic acid and sodium acetate. Released [³H]acetic acid was isolated by passing the reaction mixture through a C₁₈ silica gel column (J. T. Baker, Phillipsburg, NJ), and eluted radioactivity was measured using a liquid scintillation counter.

Adherence of PMN to TEU-2 cells

Human adult peripheral blood was collected from volunteers in vials containing 3.8% sodium citrate to inhibit coagulation. Neutrophils were isolated using Polymorphprep (Axis-Shield PoC AS, Oslo, Norway) and resuspended in MEM + 10% FBS at 1×10^6 cells/ml. 500 μ l of PMN suspension were added to each of the TEU-2 monolayers and incubated for 10 minutes at room temperature. Media and unbound neutrophils were removed and discarded. Adherent PMN and TEU-2 cells were lysed with 500 μ l 0.2% Triton X-100 and neutrophil peroxidase activity was assessed as described in detail previously (6)

Statistical analysis

Data were analyzed using the Student's t-test. ANOVA was used for comparison between multiple groups where applicable. p-values of <0.05 were considered statistically significant (shown as *); p-values of <0.01 were considered highly statistically significant (shown as **).

RESULTS

Phospholipase A₂ activity was measured in the cytosol and membrane fractions obtained from TEU-2 cells. As seen in Table 1, the majority of activity was observed in the membrane fraction in the absence of calcium (4 mM EGTA). Higher iPLA₂ activity measurements were obtained when plasmenylcholine was used as substrate when compared to phosphatidylcholine. Thus, the majority of TEU-2 PLA₂ activity was found to be membrane-associated, calcium-independent PLA₂ (iPLA₂). Isolated membrane fractions were incubated with increasing concentrations of the R and S enantiomers of the iPLA₂-selective inhibitor bromoenol lactone (BEL, 10 mins). In previous studies, (S)-BEL was shown to be an order of magnitude more selective for iPLA₂β and (R)-BEL an order of magnitude more selective for iPLA₂γ (11). (R)- and (S)-BEL were incubated with TEU-2 membrane protein for 10 mins and iPLA₂ activity was measured in the absence of calcium using (16:0 [³H]18:1) plasmenylcholine substrate. As shown in Figure 1, left panel, membrane-associated iPLA₂ activity is inhibited to a greater extent when TEU-2 cells are incubated with (R)-BEL than at the corresponding concentration of (S)-BEL, suggesting that the majority of iPLA₂ activity in TEU-2 cells is iPLA₂γ. At higher concentrations, (S)-BEL inhibits TEU-2 iPLA₂ activity, demonstrating the loss of selectivity at higher concentrations. TEU-2 cells were stimulated with tryptase (20 ng/ml, 10 mins) and iPLA₂ activity was measured in the absence of calcium using (16:0 [³H]18:1) plasmenylcholine substrate (Figure 1, right panel). Tryptase stimulation resulted in a significant increase in membrane-associated iPLA₂ activity that was completely inhibited by pretreatment with (R)-BEL (1 μM, 10 mins). Pretreatment with MAFP or (S)-BEL had no significant effect on iPLA₂ activity in tryptase-stimulated or unstimulated TEU-2 cells. These data suggest that iPLA₂γ is activated in tryptase-stimulated TEU-2 cells.

When TEU-2 cells were stimulated with tryptase (20 ng/ml, 10 mins), an increase in PAF production was observed (Figure 2, left panel). Pretreatment of TEU-2 cells with (R)-BEL (1 μM, 10 mins) resulted in a complete inhibition of tryptase-stimulated PAF production (Figure 2, left panel). Pretreatment of TEU-2 cells with 1 μM (S)-BEL had no significant effect on tryptase-stimulated PAF production in TEU-2 cells (Figure 2, left panel). These data suggest that PAF production in response to tryptase stimulation of TEU-2 cells is a result of iPLA₂γ activation. When TEU-2 cells were pretreated with MAFP (5 μM, 10 mins), there was a potentiation of tryptase-stimulated PAF production. Additionally, there was a significant increase in PAF production when TEU-2 cells were incubated with MAFP alone (Figure 2, left panel).

PAF promotes the aggregation, chemotaxis, granule secretion and oxygen radical generation from leukocytes and the adherence of leukocytes to endothelial and epithelial cells. To determine whether increased TEU-2 cell PAF production is associated with increased adherence of PMN, we measured PMN adherence to tryptase-stimulated TEU-2 cells in the presence or absence of PLA₂ inhibitors (Figure 2, right panel). Accompanying the increase in PAF production, we observed a significant increase in PMN adherence to tryptase-stimulated TEU-2 cells (Figure 2, right panel). The PMN adherence was inhibited when tryptase-stimulated TEU-2 cells were pretreated with (R)-BEL, suggesting the involvement of iPLA₂γ activation and increased PAF production. Treatment of TEU-2 cells with MAFP resulted in a significant increase in PMN adherence to unstimulated TEU-2 cells and

increased adherence to tryptase-stimulated cells (Figure 2, right panel). Increased PAF production by endothelial or epithelial cells is expressed on the cell surface and interacts with the PAF receptor on circulating inflammatory cells resulting in tethering, activation and subsequent transmigration. We pretreated PMN with the PAF receptor antagonist CV 3988 (10 μ M, 10 mins) prior to addition to unstimulated or tryptase-stimulated TEU-2 cells and observed complete inhibition of PMN adherence (Figure 2, right panel), suggesting that the PAF-PAF receptor interaction was important.

Incubation of TEU-2 cells with MAFP does not alter iPLA₂ activity in unstimulated or tryptase-stimulated TEU-2 cells (Figure 1, right panel), but increases PAF production under both conditions (Figure 2, left panel). Since PAF is hydrolyzed by PAF-acetylhydrolase, a PLA₂ isoform, we treated TEU-2 cells with (R)-BEL and MAFP and measured PAF-AH activity to determine whether the increase in PAF observed when TEU-2 cells were incubated with MAFP was due to PAF-AH inhibition (Figure 3). Treatment with MAFP significantly inhibited PAF-AH activity at concentrations as low as 0.1 μ M, whereas treatment with (R)-BEL did not significantly inhibit PAF-AH at concentrations up to 10 μ M (Figure 3). Taken together, these data suggest that TEU-2 cell iPLA₂ γ is activated by tryptase, resulting in increased PAF production and PMN adherence. Conversely, inhibition of PAF-AH by MAFP results in increased PAF production and PMN adherence to TEU-2 cells under stimulated or non-stimulated conditions. These data highlight the need for rigorous evaluation of PLA₂ inhibitors as possible anti-inflammatory therapeutic agents.

DISCUSSION

PAF is a highly potent phospholipid metabolite requiring picomolar concentrations to exert its physiological effects (2). As such, the synthesis and degradation of PAF are tightly regulated within the cell. Endothelial cell PAF synthesized in response to agonist stimulation contributes to the progression of inflammatory disorders by increasing vascular permeability and the expression of endothelial cell surface adhesion molecules that facilitate transmigration of circulating inflammatory cells such as polymorphonuclear leukocytes (PMN) across the endothelial cell monolayer (12). The synthesis of PAF in endothelial cells occurs via the remodeling pathway, activated during inflammation and hypersensitivity responses, as opposed to the de novo pathway in which PAF is synthesized for physiologic functions (2,3). In tryptase-stimulated bladder endothelial cells, we have shown that the remodeling pathway begins with iPLA₂ activation (13). This catalyzes the hydrolysis of membrane plasmenylethanolamine to yield lysoplasmenylethanolamine that acts as an acceptor for an *sn*-2 fatty acid in a CoA-independent acylation reaction with alkyl acyl glycerophosphorylcholine to generate lyso-PAF. Finally, lyso-PAF is acetylated at the *sn*-2 position in a reaction catalyzed by lyso-PAF acetyltransferase (13). In the present study, we have demonstrated that tryptase stimulation of urothelial cells results in iPLA₂ γ activation and increased PAF production.

Phospholipase A₂ enzymes catalyze the hydrolysis of the *sn*-2 fatty acid from membrane phospholipids, producing a lysophospholipid and a free fatty acid. They are classified into three main classes, secretory (sPLA₂), cPLA₂ and iPLA₂ and are further divided based on their amino acid sequences (14). The three types of PLA₂ coexist in mammalian cells and can interact with each other (14). Calcium-independent PLA₂ isoforms are ubiquitously expressed in a wide variety of cells and tissues and can be preferentially distributed in the membrane fraction (15–17). The major iPLA₂ isoforms found in mammalian cells are iPLA₂ β and iPLA₂ γ (15–17). Several pharmacological inhibitors have been developed with the goal of inhibiting a specific PLA₂ isoform or group. Bromoenol lactone is the most isoform-specific inhibitor developed to date (10). It demonstrates 1000-fold selectivity for iPLA₂ and the separation of racemic BEL into its R and S enantiomers demonstrates a 10-

fold selectivity of (*S*)-BEL for iPLA₂β and of (*R*)-BEL for iPLA₂γ (11). We used the *R* and *S* enantiomers of BEL to determine whether tryptase-stimulated PAF production in TEU-2 cells was mediated via activation of iPLA₂γ or iPLA₂β respectively. We show that treatment of TEU-2 cells with (*R*)-BEL inhibits membrane-associated iPLA₂γ activity and completely inhibits tryptase-stimulated PAF production and PMN adherence.

The biological activities of PAF can be rapidly terminated by PAF-acetylhydrolases (PAF-AH), a family of unique iPLA₂ enzymes that hydrolyze the acetyl group at the *sn*-2 position of PAF to generate biologically inactive lyso-PAF and acetate (4). The efficient degradation of PAF and the abrupt abrogation of its inflammatory actions have led to the characterization of PAF-AH as an anti-inflammatory enzyme (4). In addition, evidence suggests that the inhibition of PAF-AH activity contributes to PAF-mediated proinflammatory effects. Deficiency in PAF-AH is the mechanism implicated in several pathological conditions and has been shown to be caused by a number of genetic mutations, each resulting in inactivation of PAF-AH activity (18–20). In animal studies, administration of recombinant human PAF-AH resolved many of the PAF-induced inflammatory effects (21). These observations highlight the tight regulation of PAF bioactivities mediated by PAF-AH. When TEU-2 cells were pretreated with MAFP, increased PAF production and PMN adherence was observed in both non-stimulated and tryptase-stimulated TEU-2 cells. We measured PAF-AH activity in the presence and absence of MAFP and determined that MAFP is a potent inhibitor of TEU-2 cell PAF-AH. Coupled with activation of iPLA₂γ by tryptase stimulation, inhibition of PAF-AH by MAFP pretreatment leads to potentiation of PAF production and PMN adherence. Pretreatment of PMN with CV3988 completely inhibited PMN adherence to TEU-2 cells, demonstrating the importance of the PAF-PAF receptor interaction.

Mast cells are present in the subepithelium of the normal human ureter and are activated by urine *in vitro* (22,23). Activation of mast cells in the ureter has been implicated in pathological conditions such as renal colic and vesicoureteral reflux (24). In conditions where the urothelium is damaged and urine can penetrate the bladder wall, activation of mast cells may contribute to inflammation by releasing several inflammatory mediators such as histamine and tryptase. Increased urinary concentrations of PAF are thought to be of renal origin and have been detected in patients with acute kidney injury, glomerulonephritis and nephrotic syndrome (25,26). Additionally, increased urinary PAF concentrations have been observed in patients with non-insulin dependent diabetes mellitus prior to overt albuminuria occurs and may serve as an indicator of renal disease in diabetes (27). To our knowledge, this is the first study to demonstrate PAF production in urothelial cells isolated from the ureter. Increased PAF production can facilitate adherence and transmigration of inflammatory cells when expressed on the cell surface. We have demonstrated that endothelial cell PAF production remains cell-associated and is not released into the surrounding medium, thus an increase in urothelial cell PAF production may or may not result in increased urinary PAF concentrations. Bijuklic and coworkers have demonstrated an increase of neutrophils in the urine of interstitial nephritis patients and shown that the transepithelial migration of PMN through the proximal tubule epithelial cell line HK-2 cells stimulated with TNF-α is dependent on ICAM-1 expression (28). The same research group also determined that PMN migration across renal proximal tubular LLC-PK1 cells was twice as efficient in a basolateral to apical direction (29). An increase in urothelial cell PAF production and adherence of inflammatory cells may result in increased concentrations of inflammatory cells in the urine and may represent a mechanism to minimize or resolve inflammation by the removal of inflammatory cells from the urinary tract.

Highly specific PLA₂ inhibitors have been proposed as valuable anti-inflammatory drugs, since they would have the ability to regulate eicosanoid production and minimize

inflammatory cell recruitment by targeting PAF production. However, facilitation of the removal of inflammatory cells across the urothelium may contribute to resolution of inflammation in urinary tract infections or cystitis and thus the use of this type of anti-inflammatory may be contraindicated in these diseases.

CONCLUSION

Tryptase stimulation of TEU-2 cells results in activation of iPLA₂γ and increased PAF production and PMN adherence. We propose that mast cells in the ureter are activated during inflammation and could play a role in resolution of the inflammatory episode by facilitating the removal of inflammatory cells from the urinary tract. Pretreatment with PLA₂ inhibitors can target either the synthesis or degradation of urothelial cell PAF production and inhibit or facilitate the removal of inflammatory cells across the epithelial cell barrier. Taken together, these data highlight the importance of rigorous studies to determine the therapeutic potential for PLA₂ inhibitors as anti-inflammatory agents.

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FIGURE 1.

Membrane-associated iPLA₂ activity in TEU-2 cells. Left Panel: iPLA₂ activity was measured in the presence of increasing concentrations of (*R*)-BEL or (*S*)-BEL (10 mins). Right Panel: TEU-2 cells were pretreated with MAFP (5 μM, 10 mins) or (*R*)-BEL or (*S*)-BEL (1 μM, 20 mins) prior to tryptase stimulation. Results represent mean ± SEM. N=6. **p<0.01 when compared to unstimulated values. ++p<0.01 when comparing corresponding results in the presence or absence of inhibitor.

FIGURE 2.

Platelet-activating factor (PAF) production (left panel) and PMN adherence (right panel) in TEU-2 cells in response to tryptase stimulation (20 ng/ml, 10 mins). Urothelial cells were pretreated with MAFP (5 μ M, 10 mins) or (*R*)-BEL or (*S*)-BEL (1 μ M, 20 mins) prior to tryptase stimulation. Where indicated, PMN were treated with CV 3988 (10 μ M, 10 mins) prior to addition to TEU-2 cells. ** $p < 0.01$ when compared to unstimulated values. + $p < 0.05$, ++ $p < 0.01$ when comparing corresponding results in the presence or absence of inhibitor. Results represent mean $\pm$ SEM. N=6.

FIGURE 3.

PAF- acetylhydrolase activity in TEU-2 cells in response to tryptase stimulation (20 ng/ml, 10 mins). Cells were pretreated with MAFP (5 μM, 10 mins) or (R)-BEL (1 μM, 20 mins) prior to tryptase stimulation. ++ $p < 0.01$ when compared to untreated cells. Results represent mean $\pm$ SEM. N=6.

TABLE 1

Phospholipase A₂ activity (nmol-mg protein⁻¹.min⁻¹) in TEU-2 cells defined using plasmenylcholine or phosphatidylcholine substrates in the absence (4 mM EGTA) or presence (1 mM Ca²⁺) of calcium. Values represent mean + SEM for separate measurements from 6 different cell cultures.

Cell Fraction	Substrate ^a	EGTA	Ca ²⁺
Cytosol	16:0,[³ H]18:1		
	Plasmenylcholine	0.73 ± 0.02	0.53 ± 0.02**
	16:0,[³ H]18:1		
	Phosphatidylcholine	0.11 ± 0.01	0.07 ± 0.02*
Membrane	16:0,[³ H]18:1		
	Plasmenylcholine	37.27 ± 1.20	33.32 ± 4.81
	16:0,[³ H]18:1		
	Phosphatidylcholine	6.74 ± 0.35	4.73 ± 0.22*

**
p<0.01 compared to corresponding value obtained in the presence of EGTA.

^a substrate composition is represented as a:b, c:d where a:b and c:d represent the chain length: number of double bonds for the aliphatic groups at the *sn*-1 and *sn*-2 positions, respectively, of the corresponding phospholipid substrate molecule.