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# Changes of phospholipase D activity of rat peritoneal mast cells in degranulation[1]

Yun- bi LU, Ming WU[2], Han-liang ZHOU[3]

*Department of Pharmacology, School of Medicine, Zhejiang University, Hangzhou 310031; [2]Department of Cardiovascular Thoracic Surgery, Second Affiliated Hospital, School of Medicine, Zhejiang University, Hangzhou 310009, China*

**KEY WORDS** ovalbumin; phospholipase D; immunization; mast cells

## ABSTRACT

**AIM:** To study the changes of phospholipase D (PLD) activity of actively sensitized rat peritoneal mast cells (RPMC) in degranulation. **METHODS:** Degranulation of RPMC was determined by measurement of β-hexosaminidase release. PLD activity assay was carried out by measurement of PLD product, choline, with chemiluminescent oxidation of luminol. **RESULTS:** Actively sensitized RPMC challenged with ovalbumin (0.5-8 mg/L for 120 s, 4 mg/L for 15-120 s) resulted in significant activation of PLD accompanied with the increment of β-hexosaminidase release. PLD activity of sensitized RPMC was increased by more than 2-fold compared with that of unsensitized RPMC which contained low levels of PLD activity [(35±13) pmol choline/min in $1\times10^6$cells], but β-hexosaminidase releases of the sensitized cells were as low as spontaneous releases. After challenge with ovalbumin 4 mg/L for 120 s , PLD activity of sensitized RPMC was increased to (155±43) pmol choline/min in $1\times10^6$cells and β-hexosaminidase release was also elevated significantly (4.5-fold of spontaneous release, $n$=6, $P$<0.05). When unsensitized RPMC were stimulated with antigen, PLD activity and β-hexosaminidase release of the cells were hardly changed. Sensitized RPMC were treated with 1 % 1-butanol or 2,3- disphosphoglycerate 10 mmol/L before challenge with ovalbumin, these drugs induced an inhibition of PLD activity and a reduction of β-hexosaminidase release to basal level. 1-Butanol 0.1 % also worked. **CONCLUSION:** Phospholipase D plays an important role in the regulation of β-hexosaminidase release in actively sensitized rat peritoneal mast cells.

## INTRODUCTION

Mast cells are the main effector cells in allergic diseases. Many stimulants initiate mast cells to release both presynthesized and novo synthesized mediators. These mediators are both redundant and pleiotropic in their capacity to generate tissue inflammation in allergic disease[1]. The mechanism of degranulation in mast cells remains subject to debate. In recent years, lines of evidence provided that phospholipase D (PLD) was involved in degranulation of mast cells[2,3]. PLD is widely distributed in mammalian cells, where it is regulated by a variety of extracelluar signals. The major substrate of PLD is phosphatidylcholine (PC), which is hydrolyzed to phosphatidic acid and choline. The PLD-catalysed PC hydrolysis is an important mechanism of signal transduction in cells[4,5]. Many experiments involving RBL-2H3 cells (a model of mucosal mast cell function) indicated that passively sensitized RBL-2H3

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[3] Correspondence to Prof Han-liang ZHOU.
Phn 86-571-8721-7150. Fax 86-571-8721-7044.
E-mail zhouhl@hzcnc.com
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cells challenged by antigen or pharmacological stimulants, caused degranulation and mediator release accompanied by PLD activation. These degranulation can be attenuated by PLD inhibitor[6]. However, several important questions remain unresolved, including: 1) is PLD activated during degranulation in actively sensitized mast cells? and 2) is PLD activation required for this degranulation?

The present study has been made to demonstrate the role of PLD during the degranulation of actively sensitized RPMC and examine the effect of sensitization on PLD in actively sensitized RPMC.

## MATERIALS AND METHODS

**Rats** Sprague-Dawley (SD) rats of either sex weighing 170±20 g were purchased from Experimental Animal Center of Zhejiang (Grade II, Certificate No 22-9601018 conferred by Zhejiang Medical Laboratory Animal Administration Committee).

**Drugs** Phosphatidylcholine (C10:0), oleic acid sodium salt , choline oxidase, choline hydrochloride, egg albumin V, *p*-nitrophenol-*N*-acetyl-β-*D*-glucopyranoside,1-butanol, 2,3-disphosphoglycerate (Sigma), HEPES and luminol (Merck), peroxidase (Shanghai Lizhu Dongfeng Biotechnology Co Ltd) were dissolved in double-distilled water. Percoll and inactivated *Bordtella pertussis* organisms were purchased from Amersham Pharmacia Biotech Co Ltd and Shanghai Institute of Biotech, Chinese Academy of Sciences, respectively.

**Protocol** Rat peritoneal mast cells (RPMC) were isolated and purified, then suspended in Tyrode's, HEPES and gelatin buffer (THG) of following composition: NaCl 137 mmol/L, KCl 2.7 mmol/L, $CaCl_2$ 1 mmol/L, $MgCl_2$ 1 mmol/L, glucose 5.6 mmol/L, gelatin 0.1% (w/v), HEPES 10 mmol/L and adjusted to pH 7.4. The cell suspension was divided into 5-7 samples according to cell count. Every sample was warmed in 37 ºC water bathing for 5 min before treatment. To study ovalbumin-induced β-hexosaminidase release and PLD activation in actively sensitized RPMC, mast cells at a concentration of $2\times10^9$ cells /L were challenged with 0.5-8 mg/L of ovalbumin or THG buffer for 15-120 s at 37 ºC in final reaction volume of 250 μL. To investigate the effect of PLD inhibitor, mast cells were treated with 0.1-1% 1-butanol or 10 mmol/L 2,3-disphosphoglycerate (2,3-DPG) or THG for 10 min, then the cells were exposed to 4 mg/L of ovalbumin for 120 s at 37 ºC. Release reaction was stopped by addition of 250 μL ice-cold $Ca^{2+}$, $Mg^{2+}$-free THG and kept the tube on ice. The cell suspension was precipitated at 200×*g*, 10 min, 4 ºC and the supernatant was used for measurement of β-hexosaminidase release. The pellets were lysed in 100 μL buffer (containing 50 mmol/L HEPES, 0.1% Triton X-100, pH 7.4). PLD-catalyzed reaction was carried out within 2 h after the lysis. For each individual study ($n$=1), the RPMC were prepared from five to six sensitized or unsensitized rats.

**Sensitizing procedures**[7,8] SD rats were sensitized by a single sc of ovalbumin 2 mg mixed with 40 mg aluminum hydroxide gel in 0.4 mL saline per animal. To enhance the production of IgE antibodies, $2\times10^{10}$ inactivated *Bordtella pertussis* organisms were injected im as adjuvant at the same time as the antigen. These animals were used 21 d later for isolation and purification of RPMC.

**Isolation and purification of RPMC**[9,10] RPMC were harvested from sensitized or unsensitized rats as described before. Briefly, rats were sacrificed by exsanguinations and the peritoneal cavity was lavaged with 30 mL ice-cold $Ca^{2+}$, $Mg^{2+}$-free THG containing heparin (5 kU/L). Washings were pooled and centrifuged (200×*g*, 10 min, 4 ºC). The cell pellet was resuspended in 1 mL $Ca^{2+}$, $Mg^{2+}$-free THG, then mixed with 4.0 mL 90 % Percoll in isoosmotic $Ca^{2+}$, $Mg^{2+}$-free THG (comprising 3.6 mL Percoll and 0.4 mL 10-fold concentrated THG). The Percoll/cell suspension was overlayed with 1 mL $Ca^{2+}$, $Mg^{2+}$-free THG and then centrifuged (200×*g*, 10 min, 4 ºC). The resulting supernatant was discarded. The mast cell pellet was washed three times (200×*g* , 10 min, 4 ºC) and then resuspended with THG. Greater than 95 % of mast cells were assessed by differential staining with neutral red, and viable mast cells were counted (haemocytometer, Trypan blue exclusion method) with a yield of approximately $0.5\times10^6$ to $0.9\times10^6$ cells per rat.

**Measurement of β-hexosaminidase release**[6,11] Degranulation was determined by measurement of β-hexosaminidase release. β-hexosaminidase hydrolyzed *p*-nitrophenyl-*N*-acetyl-β-*D*-glucosamide to the chromophore, *p*-nitrophenol, as described elsewhere. Absorbance (410 nm) was measured in microtiter (Model DG3022A, HUADONG Vacuum Tube Factory) plate read. The net percentage of release was calculated by following formula: net percentage of release= (Stimulated release-spontaneous release)/(Stimulated release+residual-spontaneous release)×100 %.

**PLD-catalyzed reaction**[12,13] Reaction was carried out in 37 ºC water bathing for 60 min. The 360 μL reaction system was composed of HEPES 22 mmol/L (pH 7.4), $MgCl_2$ 5 mmol/L, $CaCl_2$ 0.1 mmol/L, phosphatidylcholine 2 mmol/L, oleic acid 6 mmol/L. Cell lysate 90 μL was added to initiate the reaction. The reaction was terminated by placing the tube in boiling water for 10 min. After cooling to room temperature, each sample was added 360 μL chloroform and mixed for 1 min (2000 r/min, amplitude was 6 mm) before centrifugation (4000×*g*, 10 min). The supernatant resulting from the centrifugation was used as sample for PLD activity assay.

**PLD activity assay**[12-14] PLD activity was measured by chemiluminescence assay according to previous reports with modifications. The method relies on the measurement of PLD product, choline, by the coupling of two enzyme-catalyzed reaction, the conversion of choline to betain and hydrogen peroxide by choline oxidase and the $H_2O_2$-induced oxidation of luminol by peroxidase. The resulting chemiluminescence was detected with luminometer (Institute of Biophysics, Chinese Academy of Sciences) and a $^{14}C$ standard photon source was used as a reference. The procedure was as following: Choline was oxidized in 500 μL of phosphate buffer 200 mmol/L (pH 8.6) containing luminol 0.02 mmol/L, peroxidase 5 U, choline oxidase 1 U and 5 μL sample or standard. Tubes were placed in the thermostatic chamber (37 ºC). The reaction was initiated in dark cell by added sample or standard. The height of the luminescence peak was used to evaluate choline content. A standard curve was constructed at each study with fresh choline standard (5-100 pmol). The PLD activity can be quantified by calculation of produced choline in a standard curve.

**Statistical analysis** All data were expressed as mean±SD. Difference between groups was analyzed with One-way ANOVA and Dunnett's test using computer software (SigmaStat 1.01 for Windows 95, 1992, Jandel Corp, USA). Difference was accepted as significant at $P<0.05$.

## RESULTS

**Concentration-response studies for ovalbumin-induced β-hexosaminidase release and phospholipase D activation in actively sensitized rat peritoneal mast cells** Concentration-response studies showed that challenge of actively sensitized RPMC with 1 mg/L ovalbumin for 120 s resulted in an elevation of PLD activity from basal level of (83±26) pmol choline/min in $1\times10^6$ cells ($n$=6) to (130±35) pmol choline/min in $1\times10^6$ cells ($n$=7, $P<0.05$), but β-hexosaminidase release was increased when ovalbumin dose was 0.5 mg/L, from unchallenged RPMC of 4.3 %±2.1 % ($n$=6) to 11 %±4 % ($n$=7, $P<0.05$). After exposure of actively sensitized RPMC to 4 mg/L ovalbumin for 120 s, PLD activity was increased from basal levels to (162±46) pmol choline/min in $1\times10^6$ cells ($n$=6, $P<0.05$) and β-hexosaminidase release elevated from spontaneous release to 16 %±4 % ($n$=6, $P<0.05$, Fig 1).

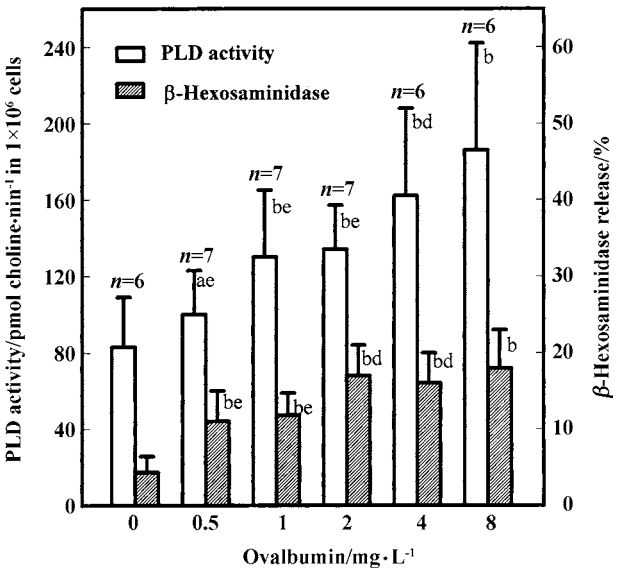


**Fig 1. Concentration-response studies for ovalbumin-induced β-hexosaminidase release and phospholipase D activation in actively sensitized rat peritoneal mast cells. Mean±SD. [a]$P>0.05$, [b]$P<0.05$ *vs* 0 mg/L. [d]$P>0.05$, [e]$P<0.05$ *vs* 8 mg/L.**

**Time-course studies for ovalbumin-induced β-hexosaminidase release and phospholipase D activation in actively sensitized rat peritoneal mast cells** After challenge of actively sensitized RPMC with 4 mg/L ovalbumin for 15 s, PLD activity of RPMC was increased from unchallenged levels of (93±19) pmol choline/min in $1\times10^6$ cells ($n$=6) to (113±13) pmol choline/min in $1\times10^6$ cells ($n$=6, $P<0.05$), accompanied by an increase in β-hexosaminidase release from spontaneous release of 3.7 %±1.7 % ($n$=6) to 11 %±5 % ($n$=6, $P<0.05$). Compared with the challenge of cells with antigen for 15 s, the challenge of cells with 4 mg/L ovalbumin for 120 s resulted in an elevation of β-hexosaminidase release to 18 %±5 % ($n$=6, $P<0.05$, Fig 2),

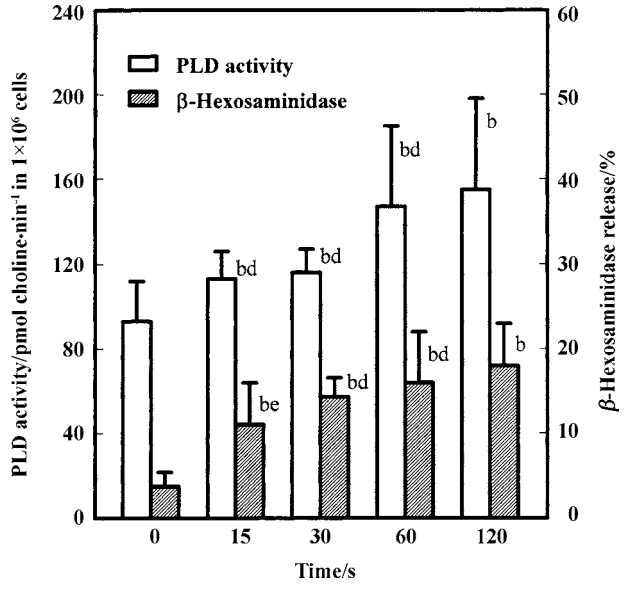


**Fig 2. Time-course studies for ovalbumin-induced β-hexosaminidase release and phospholipase D activation in actively sensitized rat peritoneal mast cells. *n*=6. Mean±SD. [b]*P*<0.05 *vs* 0 s. [d]*P*>0.05, [e]*P* <0.05 *vs* 120 s.**

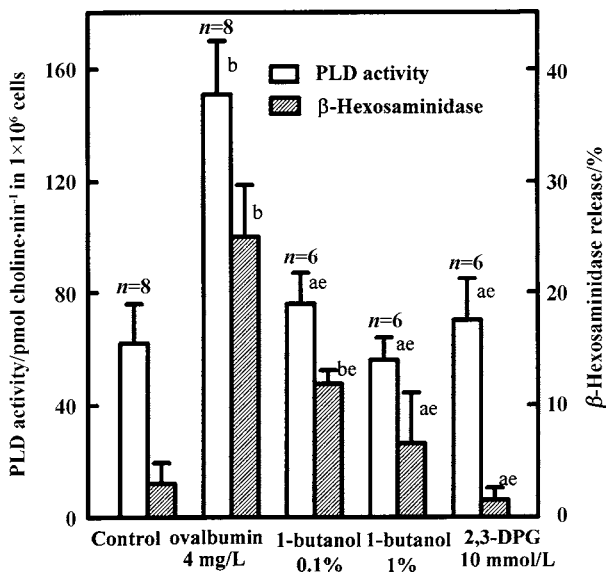


**Fig 3. Effects of phospholipase D inhibitors on β-hexosaminidase release in actively sensitized rat peritoneal mast cells. Mast cells were pretreated with 0.1%-1% 1-butanol or 2,3-disphosphoglycerate (2,3-DPG) 10 mmol/L for 10 min, then exposed to ovalbumin 4 mg/L for 120 s at 37 ºC. Mean±SD. [a]*P*>0.05, [b]*P*<0.05 *vs* Control. [d]*P*>0.05, [e]*P*<0.05 *vs* ovalbumin 4 mg/L.**

but the changes of PLD activity were small.

**Effects of phospholipase D inhibitors on β-hexosaminidase release in actively sensitized rat peritoneal mast cells** Pretreatment of actively sensitized RPMC with 0.1 % 1-butanol for 10 min before exposure to ovalbumin 4 mg/L for 120 s, produced a small stimulation of the PLD activity [(76±11) pmol choline/min in $1\times10^6$ cells] that did not reach statistical significance ($P$>0.05, compared with control value of (64±14) pmol choline/min in $1\times10^6$ cells). The same concentration of 1-butanol inhibited ovalbumin-induced β-hexosaminidase release to 11.9 %±1.2 % ($P$<0.05, compared with that of RPMC challenged by ovalbumin directly, 25 %±5 %), but remained significantly above control levels (3.0 %±1.9 %, $P$<0.05, Fig 3).

Pretreatment of actively sensitized RPMC with 1 % 1-butanol or 2,3-DPG 10 mmol/L, respectively, for 10 min before addition of antigen, resulted in inhibition of PLD activity and β-hexosaminidase release to basal level.

**Effects of sensitization on β-hexosaminidase release and phospholipase D activation in rat peritoneal mast cells challenged with ovalbumin** Before challenge with antigen, actively sensitized RPMC contained 2.7-fold levels of PLD activity greater than unsensitized RPMC did [(93±19) *vs* (35±13) pmol choline/min in $1\times10^6$cells, $P$<0.05] but β-hexosaminidase release (3.7 %±1.7 %) of sensitized RPMC were low as spontaneous releases ($P$>0.05, *vs* unsensitized RPMC). After challenge with ovalbumin 4 mg/L for 120 s , both PLD activity and β-hexosaminidase release in sensitized RPMC were elevated significantly. However, PLD activity and β-hexosaminidase release had hardly changes in unsensitized RPMC exposed to ovalbumin ($P$>0.05, Fig 4).

## DISCUSSION

These results indicated that PLD activation is associated with mast cell degranulation.

When actively sensitizied RPMC were challenged with ovalbumin, the elevation of β-hexosaminidase release appeared in a biphasic fashion. The initial elevation was at 15 s, the secondary elevation was at 120 s. However, PLD activity was only increased at 15 s. The concentration-response studies with the antigen ovalbumin showed that both β-hexosaminidase release and activation of PLD were in a concentration-dependent manner and β-hexosaminidase release was increased significantly at concentration of ovalbumin ≥0.5 mg/L, however, PLD activity was elevated till the concentration of ovalbumin ≥1 mg/L.

So, the increment of PLD activity was not in parallel with that of β-hexosaminidase release when ac-

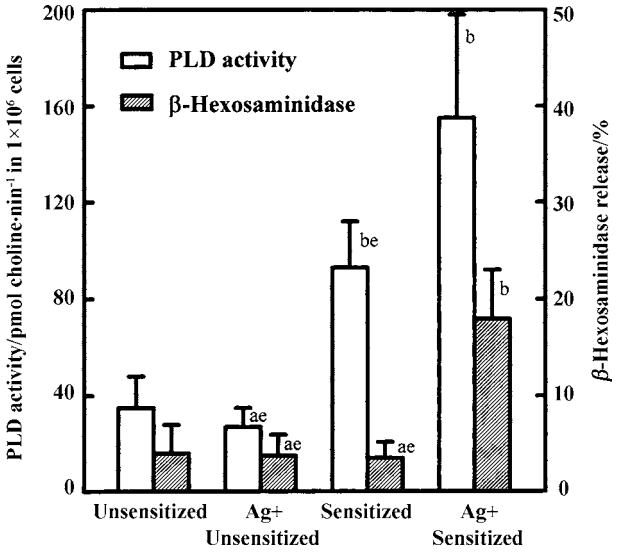


**Fig 4. Effects of sensitization on β-hexosaminidase release and phospholipase D activation in rat peritoneal mast cells challenged with ovalbumin. *n*=6. Mean±SD. [a]*P*>0.05, [b]*P*<0.05 *vs* unsensitized. [c]*P*<0.05 *vs* sensitized+antigen (Ag).**

tively sensitized RPMC were challenged with antigen. However, the data of PLD inhibitor effects showed that inhibition of PLD activation was in parallel with that of β-hexosaminidase release when RPMC were exposed to ovalbumin.

No specific inhibitor of PLD is currently available. In the present study, we utilized two complementary inhibitors of PLD-mediated signal transduction, 1-butanol and 2,3-DPG. Phospholipase D catalyzes a transphosphatidylation reaction and, 1-butanol, the physiological inhibitor of PLD[4], is included in reaction system, which will result in the formation of phosphatidylbutanol at the expense of phosphatidic acid. The 2,3-DPG acted as a competitive inhibitor of PLD which has low toxicity to intact cells[15]. The results showed that 1 % 1-butanol and 2,3-DPG 10 mmol/L produced an inhibition of PLD activation and a concominitant reduction of β-hexosaminidase release to basal level.

How to explain the unparallel appeared in concentration-response studies and time-course studies for ovalbumin-induced PLD activation and β-hexosaminidase release in RPMC? It was suspected that PLD activity of actively sensitized RPMC was increased before the cells were exposed to antigen. The PLD activity of unsensitized RPMC and actively sensitized RPMC were determined in the absence or presence of antigen, respectively. It was observed that, before the actively sensitized RPMC were exposed to ovalbumin, the PLD activity of the cells was increased significantly, but the β-hexosaminidase release remained at the basal level. So, the 'unparallel' was in appearance. Actually, the increment of PLD activity was also biphasic, the first increase of PLD activity was induced by sensitization and the second was induced by challenge with antigen. As to say, PLD activation is earlier than β-hexosaminidase release. It was proposed that RPMC was primed by actively sensitization.

The ability of cells to adopt an increased functional status under certain conditions or in a defined environment is referred to as priming. Priming represents a general phenomenon and is functional option for many cells including neutrophils, basophils, lymphocytes, eosinophils, *etc*[16]. The upregulated PLD activity may be the mechanism by which neutrophil was priming[17]. On these bases, it was presumed that PLD activation appeared to be target on RPMC priming, when the primed RPMC were exposed to antigen, the PLD activity was increased again and the β-hexosaminidase release increment was a concomitant. However, further investigation is needed to elucidate the relationship between PLD activation and the mast cell priming.

Taken together, the results support that ovalbumin-induced β-hexosaminidase release in actively sensitized RPMC may be mediated via PLD activation.

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