

Enhancement of phospholipase A₂ activation by phosphatidic acid endogenously formed through phospholipase D action in rat peritoneal mast cell

Takashi Sato, Tsuyoshi Ishimoto, Satoshi Akiba and Tatsuzo Fujii

Department of Biochemistry, Kyoto Pharmaceutical University, Yamashina-ku, Kyoto 607, Japan

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Contribution of phosphatidic acid (PA) generated by activated phospholipase (PL) D to PLA₂ activation was studied in rat peritoneal mast cells. Exogenous didecanoyl PA induced arachidonate liberation in the permeabilized cells which was inhibited by *p*-bromophenacyl bromide. Upon exposure of the cells to ethanol in a high enough concentration to prevent PA formation, A23187-induced arachidonate liberation was suppressed by 50% and the rest was completely inhibited by *p*-bromophenacyl bromide. In contrast, propranolol, which enhanced PA accumulation, significantly increased the arachidonate liberation. These results suggest that A23187-induced PLA₂ activation may be potentiated, at least in part, by PA generated through PLD action.

Phospholipase A₂; Phospholipase D; Phosphatidic acid; Arachidonic acid; Rat peritoneal mast cell

1. INTRODUCTION

Evidence has recently accumulated showing that phosphatidic acid (PA) which is formed by phospholipase (PL) D-catalyzed hydrolysis of phosphatidylcholine is implicated in the sequence of stimulus-response coupling in various types of cells. This idea has been developed by many observations showing that exogenously added PA or fungal PLD to generate PA mimics physiological agonists causing various kinds of cellular responses: neurotransmitter [1] or hormone release [2,3], enzyme activation such as PLC [4–10], protein kinases [11–13] and phosphatidylinositol-4-phosphate kinase [14], and cell proliferation via promoting DNA synthesis [4,15–18] or increasing Ras activity by inhibiting the GTPase activating protein and stimulating the GTPase inhibiting protein [19,20]. Recently we have reported that exogenous PA stimulates PLA₂ and PLC activity, most notably potentiating PLA₂ activity in its low concentrations in rabbit platelets [21,22]. This suggests that PA generated under physiological stimuli, at least in part, may contribute to enhance preferentially the activation of PLA₂. However, it is not yet clear if endogenous PA plays such a role in the sequence of stimulus-response coupling in agonist-stimulated cells.

In this work, therefore, we examined whether or not

endogenously generated PA by activated PLD can potentiate PLA₂ activation in rat peritoneal mast cells in response to A23187, since it is reported that the mast cells have a substantial PLD activity [23–25] and also that A23187 can activate PLA₂ in the cells [26].

2. MATERIALS AND METHODS

2.1. Materials

Didecanoyl PA was prepared from didecanoyl-phosphatidylcholine (Sigma, St. Louis, MO, USA) by the hydrolytic action of PLD from *Streptomyces chromofuscus* (Boehringer Mannheim, Germany) and purified by thin layer chromatography developed in chloroform/methanol/acetic acid/water (60:30:8:4, by vol.) [21]. We confirmed that the didecanoyl PA did not contain the corresponding lysodecanoyl PA. Propranolol and *p*-bromophenacyl bromide were obtained from Wako Pure Chemical Industries (Osaka, Japan). Bovine serum albumin (BSA, fraction V) was from Sigma Chemical Co. (St. Louis, MO, USA). A23187 was from Calbiochem (La Jolla, CA, USA). [³H]Arachidonic acid ([³H]AA, 76.0 Ci/mmol) was from New England Nuclear (Boston, MA, USA). Phosphatidylethanol used as a standard was prepared according to the method of Comfurius and Zwaal [27]. Other reagents were obtained from commercial sources.

2.2. Preparation of rat peritoneal mast cells and [³H]AA labeling

Mast cells were harvested from peritoneal fluid taken from male Wistar rats of 8 weeks of age. The cells were purified as described previously [28]. Briefly, the peritoneal cells suspended in a medium, composed of 150 mM NaCl, 3.7 mM KCl, 3 mM Na₂HPO₄, 3.5 mM KH₂PO₄, 6 mM glucose, 1 mg/ml BSA, 1 mg/ml gelatin and 10 U/ml heparin (pH 6.8), were centrifuged at 50 × *g* for 6 min at 4°C, and then the pellet was suspended in the medium. The suspension was layered over a 31.5% BSA-saline solution, followed by centrifugation at 300 × *g* for 10 min at 4°C. The pellet was washed twice and then the cells were resuspended at 5 × 10⁶ cells/ml in the medium. The purified mast cells were incubated with [³H]AA (10 μCi/ml) at 37°C for 2 h and then washed twice. The cell number was adjusted to 5 × 10⁵ cells/ml in the same medium without heparin.

Correspondence address: T. Sato, Department of Biochemistry, Kyoto Pharmaceutical University, Misasagi, Yamashina-ku, Kyoto 607, Japan.

Abbreviations: AA, arachidonic acid; DAG, 1,2-diacylglycerol; PA, phosphatidic acid; PL, phospholipase.

2.3. Measurement of lipid metabolism

[³H]AA-labeled mast cells or the cells permeabilized with 15 μg/ml saponin [28] were incubated with BW755C (80 μM: 3-amino-*m*-(trifluoromethyl)-phenyl]-2-pyrazoline, a lipoxygenase and cyclooxygenase inhibitor [29] at 37°C for 2 min, and subjected to various experiments. After the reaction was terminated by the addition of ice-cold chloroform/methanol/HCl (200:200:1, by vol.), lipids were extracted and separated by thin layer chromatography on a Silica Gel G plate (Merck) with a developing solvent system of petroleum ether/diethyl ether/acetic acid (80:80:1.5, by vol.) or the combination of chloroform/methanol/7 M NH₄OH (60:45:7.3, by vol.) in the first dimension, and chloroform/methanol/acetic acid/water (60:30:8:4, by vol.) in the second dimension. Each fatty acid, PA and phosphatidylethanol fraction, identified on the basis of comigration with authentic standards was scraped off and the radioactivity was determined by liquid scintillation counting.

3. RESULTS

3.1. Didecanoyl PA-induced PLA₂ activation

To confirm whether or not PA can activate PLA₂ in mast cells, we examined the effect of exogenous addition of didecanoyl PA to mast cells on PLA₂ activation. In this experiment we used saponin-permeabilized mast cells to exclude the Ca²⁺ ionophoretic effect of the PA [30], since these permeabilized cells are not influenced by Ca²⁺ permeation through membranes. Fig. 1A shows that an addition of didecanoyl PA to [³H]AA-labeled mast cells liberated dose-dependently [³H]AA in the presence of Ca²⁺. Didecanoyl PA also induced dose-dependent [³H]AA liberation from [³H]AA-labeled intact mast cells in the presence of Ca²⁺ (data not shown). Furthermore, as seen in Fig. 1B, the didecanoyl PA-

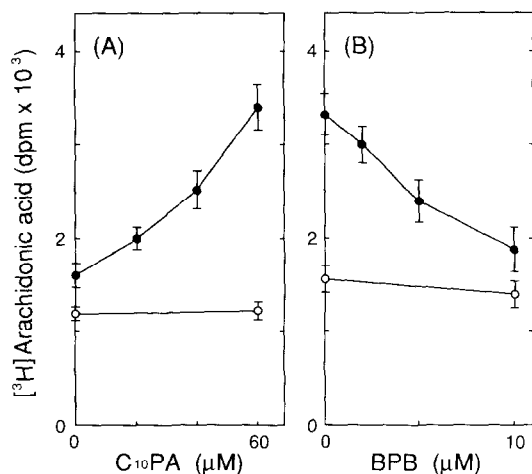


Fig. 1. Didecanoyl PA-induced AA liberation (A) and the inhibition by *p*-bromophenacyl bromide (BPB) (B). Various concentrations of didecanoyl PA (C₁₀PA) were added to [³H]AA-labeled, saponin-permeabilized mast cells in the presence of 50 μM CaCl₂ (●) or 1 mM EGTA (○) and the mixture was incubated at 37°C for 10 min (A). [³H]AA-labeled, saponin-permeabilized mast cells were treated with various concentrations of BPB at 37°C for 10 min and then 60 μM C₁₀PA (●) or buffer (○) was added and incubated in the presence of 50 μM CaCl₂ as above (B). The radioactivity of [³H]AA liberated was determined. Each point represents mean ± S.E.M. of three separate experiments.

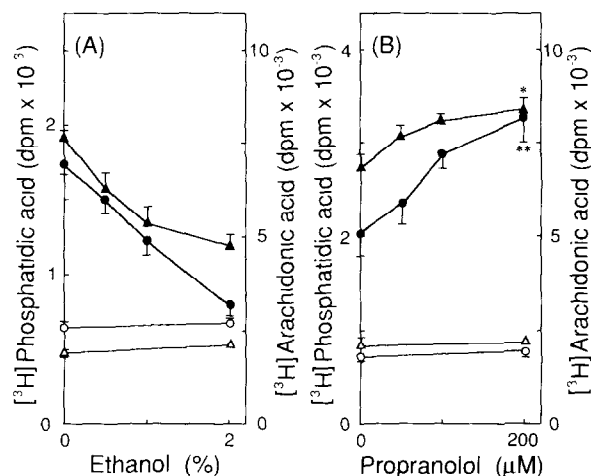


Fig. 2. Effect of ethanol (A) or propranolol (B) on A23187-induced PA formation and AA liberation. [³H]AA-labeled mast cells were treated with various concentrations of ethanol (A) or propranolol (B) at 37°C for 10 min, and then stimulated with 30 nM A23187 (●,▲) or DMSO (○,△) in the presence of 1.3 mM CaCl₂ for additional 10 min. The radioactivity of [³H]PA (●,○) or [³H]AA (▲,△) was determined. Each point represents mean ± S.E.M. of three separate experiments. ***Significantly different from the corresponding values without propranolol ($P < 0.05$ and $P < 0.02$, respectively).

induced AA liberation was inhibited by *p*-bromophenacyl bromide, a PLA₂ inhibitor. These results indicate that PA can activate PLA₂.

3.2. A23187-induced activation of PLD

A23187, a Ca²⁺ ionophore, is reported to stimulate endogenous PLD activity to form PA and 1,2-diacylglycerol (DAG) in many cell types [31–34]. Therefore, we examined A23187-induced PLD activation in mast cells. As shown in Fig. 2A, when [³H]AA-labeled mast cells were exposed to A23187 in the presence of various concentrations of ethanol, [³H]PA formed by the stimulation was decreased in a dose-dependent manner and at 2% ethanol the PA formation was almost completely inhibited. In contrast, [³H]phosphatidylethanol that was formed by the PLD-catalyzed transphosphatidyl reaction increased as a function of the ethanol concentration (128 ± 10 dpm at 0%, 2,912 ± 349 dpm at 1%, and 5,876 ± 717 dpm at 2% of ethanol, the mean ± S.E. ($n = 3$)). These observations indicate that stimulation with A23187 activates PLD in mast cells.

3.3. Contribution of PLD-derived PA to PLA₂ activation

Since exogenously added PA could activate PLA₂ (Fig. 1), we further studied the effect of endogenous PA that is generated by PLD on PLA₂ activation. The result in Fig. 2A shows that addition of ethanol to [³H]AA-labeled mast cells suppressed dose-dependently A23187-induced AA liberation but the suppression was up to approximately 50% at the concentration of 2% ethanol. To the contrary, when the mast cells had been

treated with propranolol, an inhibitor of PA phosphohydrolase, to accumulate PLD-derived PA, [^3H]AA liberation gradually increased dose-dependently. Under the condition [^3H]PA formation was observed to increase significantly and at 200 μM of propranolol it reached approximately twice that in the absence of propranolol, as indicated in Fig. 2B. We examined the effect of *p*-bromophenacyl bromide to inhibit PLA₂ activity on AA liberation that is independent on ethanol treatment. As shown in Fig. 3, ethanol suppressed AA liberation to about half level of the control 10 min after stimulation. However, when the cells had been pretreated with *p*-bromophenacyl bromide, the AA liberation in the presence of ethanol was completely inhibited. Under the condition, it is confirmed that ethanol completely inhibited the PA accumulation in any time of incubation examined (data not shown).

4. DISCUSSION

In our previous work we reported that exogenous PA could activate PLA₂ as well as PLC in platelets [22]. In the present work, we confirmed that didecanoyl PA, when added exogenously in the presence of Ca²⁺, dose-dependently induced AA liberation that was inhibited by *p*-bromophenacyl bromide (Fig. 1). This indicates that the PA is involved in control of PLA₂ activation in mast cells.

The activation of PLD is detected by the formation of phosphatidylethanol under stimulation with agonists in the presence of ethanol, due to a PLD-catalyzed transphosphatidylation reaction [35]. Our results showed that A23187-induced PA formation was markedly diminished with concomitant increase in phosphatidylethanol as ethanol concentration increases, and in the presence of 2% ethanol no PA accumulation was observed (Fig. 2A). Furthermore, propranolol, an inhibitor of PA phosphohydrolase, elevated A23187-induced PA formation dose-dependently (Fig. 2B). These results clearly indicate that most of the PA is formed by endogenous PLD-catalyzed hydrolytic action on membrane phospholipids, presumably phosphatidylcholine, and therefore PLC/DAG kinase pathway may not be involved in the PA formation under the condition used.

On the other hand, A23187-induced AA liberation was decreased by ethanol treatment dose-dependently but the decrease was up to 50% even in the presence of 2% ethanol which inhibited completely the PA formation (Fig. 2A). Therefore, the amount of AA released that was inhibited by ethanol might possibly be derived by lipase action on DAG that arises from PA through the action of PA phosphohydrolase. However, the treatment with propranolol to inhibit the conversion of PA to DAG did not decrease, but rather enhanced the AA liberation (Fig. 2B). This evidence suggests the possibility that the PA, accumulated by preventing the conversion, might potentiate the PLA₂ activity, resulting in

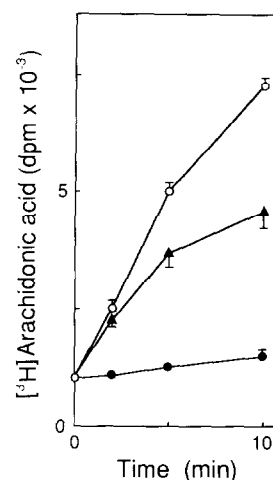


Fig. 3. Effect of ethanol and *p*-bromophenacyl bromide on time-dependent AA liberation in response to A23187. [^3H]AA-labeled mast cells were treated with 2% ethanol in the absence (\blacktriangle) or presence of 10 μM *p*-bromophenacyl bromide (\blacktriangle), or buffer (\circ) at 37°C for 10 min, and stimulated with 30 nM A23187 in the presence of 1.3 mM CaCl₂ for the period of time indicated. The radioactivity of [^3H]AA was determined. Each point represents mean \pm S.E.M. of three separate experiments.

further AA liberation. Consequently, it is reasonable to consider that the ethanol-sensitive AA liberation upon stimulation with A23187 is evoked by both the potentiated PLA₂ action by PA generated through PLD and DAG lipase action on DAG derived from the PA. The AA liberation that was not inhibited by ethanol (approximately 50% of the total liberation) was almost completely inhibited by further treatment with *p*-bromophenacyl bromide (Fig. 3), suggesting that the liberation arises from the hydrolytic action of PLA₂ on membrane phospholipids.

Recently, it was reported that three distinct PLA₂ species, 14-kDa group II PLA₂, 85-kDa cytosolic PLA₂ and PLA₂ having substrate specificity for phosphatidylserine, were detected in rat mastocytoma RBL-2H3 cells and bone marrow-derived mast cells [36]. Although it is unknown yet whether or not rat peritoneal mast cells have all of these three PLA₂ species, it is suggested that prostaglandin D₂ generation on IgE-mediated activation of the cells might be mediated by 85-kDa cytosolic PLA₂, not by 14-kDa group II PLA₂ [37]. In our recent publication we have shown that the activity of partially purified 85-kDa cytosolic PLA₂ from rabbit platelets which exhibits specificity for arachidonoyl residue of phospholipids, is significantly potentiated by PA [22]. These observations lead to the suggestion that the activation of 85-kDa cytosolic PLA₂ might be involved in the endogenous PA-enhanced AA liberation found in the present work.

The mechanism by which endogenously generated PA potentiates PLA₂ activation is not yet clear. The possibility exists that the accumulated PA in mem-

branes may increase the affinity of the membrane to associate with the enzyme or calcium, due to the anionic charge of PA, leading to enhancement of the enzyme activity [38]. Another mechanism may be that PA may act *via* its specific receptor-mediated process, although existence of the PA receptor has been reported so far only on 3T3 fibroblasts by Nakamura and Ui [5].

In conclusion, we suggest that while the PA formed by PLD action is converted to DAG that is hydrolyzed by DAG lipase to liberate free AA, it has a role for potentiating PLA₂ action to contribute to further AA formation upon stimulation of mast cells with A23187.

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