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Phospholipase A_2 in macrophage plasma membrane releases arachidonic acid from phosphatidylinositol

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A high level of arachidonic acid release from [2-^{14}C]arachidonylphosphatidylinositol (PI) was observed at neutral pH (6.0–7.0) in the presence of purified plasma membranes of guinea pig peritoneal macrophages. This activity was at least 10-fold higher than that with arachidonylphosphatidylcholine (PC) or phosphatidylethanolamine (PE) as substrate. The accumulation of [^{14}C]diacylglycerol and [^{14}C]phosphatidic acid was not detected at any time, and arachidonic acid release from [^{14}C]arachidonyldiacylglycerol was not detectable either. The data suggest that arachidonic acid release from PI may not occur via the phospholipase C pathway. In this paper, we demonstrate the possibility that arachidonic acid release from PI at neutral pH in the macrophage plasma membrane is dependent on the action of phospholipase A_2 (EC 3.1.1.4) -like activity. The maximum arachidonic acid release was dependent upon both pH and substrate. Particularly, the activity of arachidonic acid release from PI at neutral pH was very high compared with that from PC or PE. We suggest that phosphatidylinositol phospholipase A_2 (EC 3.1.1.52) may play an important role in providing arachidonic acid for subsequent metabolic activity in the macrophages.

Introduction

In the past few years, considerable interest has been focused on the study of cells of the immune system, including macrophages which metabolize arachidonic acid to prostaglandins, thromboxanes and leukotrienes [1]. Because arachidonic acid exists in the esterified form in the *sn*-2-position of membrane phospholipids, the liberation of arachidonic acid from phospholipids is considered to be the rate-limiting step for the biosynthesis of these metabolites [2]. For the liberation of arachidonic acid from phospholipids, several possible pathways have been proposed. One of these is the phospholipase A_2-mediated hydrolysis of phosphatidylcholine (PC) or phosphatidylethanolamine (PE) [3], as phospholipase A_2 has been found in various tissues [4–7] including macrophages [8,9]. However, the details of the mechanism underlying arachidonic acid liberation from phospholipids in the plasma membrane of macrophages are still unknown. In the present study, we determined that arachidonic acid liberation from phosphatidylinositol (PI) at neutral pH in plasma membranes of guinea pig peritoneal macrophages is not dependent on the action of the PI-specific phospholipase C (EC 3.1.4.3) pathway.

Abbreviations: PI, phosphatidylinositol; PC, phosphatidylcholine; PE, phosphatidylethanolamine; Pipes, 1,4-piperazinediethanesulphonic acid; PA, phosphatidic acid.

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Materials and Methods

Materials. Male guinea pigs, weighing 300–350 g, were purchased from Sankyo Labo-Service, Tokyo, Japan. Percoll was obtained from Pharmacia Fine Chemicals, Uppsala, Sweden and Affi-Gel 731 beads were from Bio-Rad Laboratories, California, USA. DC-Plastikfolien Kieselgel 60 plates came from Merck (Darmstadt, F.R.G.). [2-^{14}C]ArachidonylPC, [2-^{14}C]arachidonylPE, [2-^{14}C]arachidonylPI, and [2-^{14}C]arachidonic acid were purchased from Amersham International, Amersham U.K. 1,2-Diacylglycerol (from pig liver lectin) was obtained from Serdary Research Laboratories, Ontario, Canada. All other chemicals were of the highest grade commercially available.

Preparation of macrophage plasma membrane. Resident peritoneal cells were collected from a male guinea pig by washing the peritoneum with phosphate-buffered saline without Mg^{2+} and Ca^{2+}, but containing 0.38% sodium citrate. The cells were then subjected to Percoll density-gradient centrifugation according to the method of Farram and Nelson [10]. Macrophage plasma membranes were purified with Affi-gel 731 beads as follows [11]: isolated macrophage cells were washed with sucrose-acetate buffer (7 parts of 310 mM sucrose and 3 parts of 310 mOsm sodium acetate, pH 5.2). The cell number was adjusted to $1 \cdot 10^8$ cells/ml with sucrose-acetate buffer, then 1 ml of 50% Affi-gel 731 beads previously washed several times with 0.2 M NaCl and resuspended in sucrose-acetate buffer was added. After gentle mixing of cells and beads for 10 min at room temperature, the macrophage-attached beads were washed and resuspended in 2 ml of sucrose-acetate buffer. Next, 0.8 vol of 1 mg/ml dextran sulfate was added in order to prevent the binding of cell debris to the beads after disruption of the macrophages. The macrophage-attached beads was washed twice with sucrose-acetate buffer and resuspended in 2 ml of the same buffer and sonicated for 10 s in an ice water bath. The plasma membrane-coated beads were washed with 1 mM Tris-HCl buffer (pH 7.4) and exposed to 1 mM Tris-HCl buffer (pH 7.4) containing 5 mM EDTA and 1 mM EGTA and then treated with 0.145 M KCl. Finally, they were washed with 0.25 M sucrose and resuspended in 2 ml of 0.25 M sucrose and used for enzyme assays. All reagents were sterilized by autoclaving or filtration (Millipore, 0.45 μm).

Enzyme assay. The reaction mixture for the assay of arachidonic acid release activity contained 0.5 nmol of [2-^{14}C]arachidonylphospholipid (PC, PE or PI, 60 mCi/mmol), 0.1 M buffer (of various types), 2.0 mM $CaCl_2$ and 25 μl of plasma membrane-coated beads (10–20 μg protein/ml) in a final volume of 0.1 ml. The reaction was started by the addition of the substrate sonicated in distilled H_2O and incubation was carried out at 37 °C for 17 h. Control incubations, without plasma membrane-coated beads, were performed simultaneously. The reaction was terminated by the addition of chloroform/methanol (1 : 2) and lipids were extracted twice [12]. The extracted lipid fraction was co-chromatographed with unlabelled arachidonic acid and diacylglycerol using ligroin/diethyl ether/acetic acid (50 : 50 : 1) on a DC-plasticfolien Kieselgel 60 plate and visualized by exposure to iodine vapor. The areas corresponding to authentic arachidonic acid and diacylglycerol on the chromatograms were cut out and the radioactivity of each was measured.

Diacylglycerol lipase (EC 3.1.1.34) activities were assayed with 2 mM $CaCl_2$ and 4.0 nmol of [2-^{14}C]arachidonyldiacylglycerol (6.6 mCi/mmol) for 17 hrs at 37 °C. [2-^{14}C]Arachidonyldiacylglycerol was prepared [13] by incubating [2-^{14}C]arachidonylPC with phospholipase C (from *Bacillus cereus*).

Results

First we investigated the arachidonic acid release from various [^{14}C]arachidonylphospholipids (PC, PE or PI) by highly purified plasma membranes of macrophages. As shown in Fig. 1A, a large amount of arachidonic acid release from PI was observed at pH 6.5. No production of arachidonyldiacylglycerol, which is one of the reaction products of the phospholipase C reaction, was observed. And in the acid pH range, no arachidonic acid release from PI was observed in the Ca^{2+}-free assay system. On the other hand, a small amount of arachidonic acid release from PC was observed at pH 4.5, 7.0 and 9.0 (Fig. 1B).

Also, a small amount of arachidonic acid release from PE occurred at pH 7.5 and 9.0 (Fig. 1C).

Since no arachidonyldiacylglycerol production from PI was observed at neutral pH, the activity of diacylglycerol lipase in plasma membranes was studied, no diacylglycerol lipase activity was observed at any of the pH values tested (data not shown).

As shown in Fig. 2, the arachidonic acid released from [^{14}C]arachidonylPI was significantly increased, by the addition of Ca^{2+}, in a dose-dependent manner. This arachidonic acid release from PI was activated at high free Ca^{2+} concentrations. In the absence of Ca^{2+}, but in the presence of 2 mM EDTA, arachidonic acid release activity was not detected.

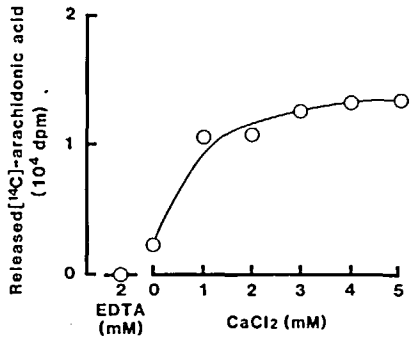

Fig. 2. Effect of Ca^{2+} on [^{14}C]arachidonic acid release from [2-^{14}C]arachidonylPI. Activities were assayed in 0.1 M Pipes-NaOH buffer (pH 6.5) for 17 h at 37°C. The enzyme solution was diluted with 2 vol. of 0.25 M sucrose.

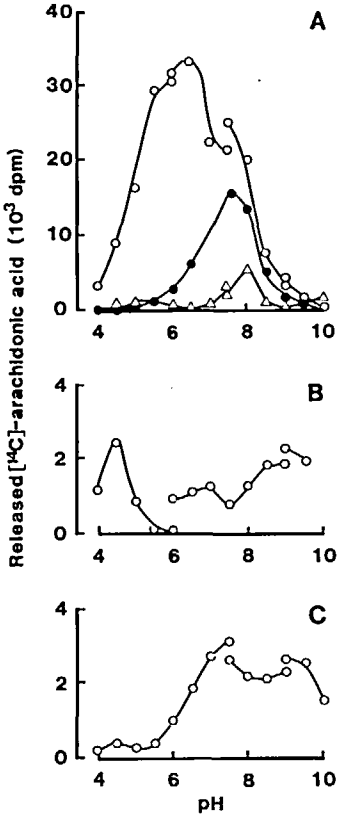

Fig. 1. (A) Optimum pH for hydrolysis of [2-^{14}C]arachidonylPI. Activities were assayed without (●) or with (○) 2 mM $CaCl_2$ for 17 h at 37°C in 0.1 M buffer (acetate (pH 4.0–6.0), Pipes-NaOH (pH 6.0–7.5), Tris-HCl (pH 7.5–9.0), or diethanolamine-HCl (pH 9.0–10.0)). Released [^{14}C]arachidonyldiacylglycerol was determined under the same conditions (△). (B) Optimum pH for hydrolysis of [2-^{14}C]arachidonylPC. (C) Optimum pH for hydrolysis of [2-^{14}C]arachidonylPE.

Discussion

Arachidonic acid release from phospholipids is believed to be controlled by the activation of phospholipase which is probably localized in the plasma membrane [14]. In the arachidonic acid release from phospholipids, several possible pathways have been proposed [15–17]. They include (a) the phospholipase A_2 pathway, which is the simplest and most direct route, (b) the pathway which begins with phospholipase A_1 followed by lysophospholipase and (c) the pathway which begins with phospholipase C followed by the sequential diacylglycerol lipase or phosphatidic acid (PA)-specific phospholipase A_2. However, several questions concerning the mechanism of the arachidonic acid release from phospholipids remain unresolved. Which phospholipase and substrate are required for the arachidonic acid release?

Recently, we reported a method for the preparation of macrophage plasma membranes using Affi-gel 731 beads. With the use of this procedure, a relatively high activity of the plasma membrane marker enzyme Na^+/K^+-ATPase is seen in the plasma membrane-attached bead fraction. In contrast, the activities of marker enzymes of

mitochondria, lysosomes and cytoplasm were significantly low or not detectable in the plasma membrane-attached bead preparation. And purity of the plasma membrane attached to beads was confirmed using a scanning electron microscope [11]. From these data, the enzyme sample used in this experiment should be the highly pure plasma membrane.

Using such purified macrophage plasma membranes, we studied arachidonic acid release from various [2-^{14}C]arachidonylphospholipids. A high degree of arachidonic acid release from PI was observed at neutral pH (6.0–7.0). However, only a small amount of arachidonic acid was released from PC or PE at the same pH. These data thus suggest that PI was mainly used as a substrate for arachidonic acid release at neutral pH by plasma membranes. When macrophages, platelets and neutrophils are exposed to various stimulators, they release a considerable amount of arachidonic acid, and enhancement of PI turnover is observed [18–20]. In the release of arachidonic acid from PI, two pathways involving phospholipase C hydrolysis have been suggested: (a) formation of diacylglycerol by phospholipase C action and its degradation by diacylglycerol lipase and monoacylglycerol lipase to yield arachidonic acid [16] and (b) phosphorylation of the diacylglycerol to PA by 1,2-diacylglycerol kinase and then hydrolysis by the action of PA-specific phospholipase A_2 with arachidonic acid being released [17]. Accordingly, we attempted to determined the production of diacylglycerol and PA in our reaction mixture during incubation. When a large amount of arachidonic acid is released from PI at neutral pH, the accumulation of diacylglycerol and PA were not detected (data not shown). Furthermore, we checked for diacylglycerol lipase activity in the macrophage plasma membrane. When the plasma membranes were incubated with [^{14}C]arachidonyldiacylglycerol, the arachidonic acid release was small, indicating that the macrophage plasma membrane did not have sufficient diacylglycerol lipase activity to cause arachidonic acid release via the PI-specific phospholipase C pathway. Hakata et al. [21] reported that phospholipase A_2 requires a much higher concentration of Ca^{2+} than does phosphatidylinositol phospholipase C. In another report, Hofmann and Majeras [22] showed that the apparent K_m for calcium of phospholipase C was about 3 μM. In our study, arachidonic acid release from PI required 2–3 mM Ca^{2+} for its activation. These results again suggest that the high level of arachidonic acid release from PI is not mainly dependent on the phospholipid C pathway.

Furthermore, there are reports showing that (1) in [^{14}C]arachidonic acid-labelled platelets, arachidonic acid release occurred following thrombin stimulation in spite of the presence of diacylglycerol lipase inhibitor [23]; (2) PA was not an intermediate in release of arachidonic acid from PI, since thrombin stimulation was not associated with increased PA catabolism as would be required by phospholipase A_2 [24]; (3) with calcium ionophore stimulation, PI levels of macrophage cells were lowered and the level of lysoPI was increased [25]; (4) the exaggerated stimulation of arachidonic acid release by calcium ionophores tends to support the involvement of a calcium-dependent phospholipase A_2 in the release of [^{14}C]arachidonic acid release from PI and PC in the labelled human neutrophil membrane [26]. These studies, along with our data showing little diacylglycerol lipase activity and the lack of accumulation of PA and [2-^{14}C]arachidonyl lysoPI at any time, strongly indicate that arachidonic acid release from PI occurs via the phospholipase A_2 pathway.

Another possibility remains; the pathway of phospholipase A_1 and lyso phospholipase. To solve this problem, we measured the accumulation of [^{14}C]arachidonyl lysoPI in our reaction mixture containing [^{14}C]arachidonylPI at neutral pH. As a result, no [^{14}C]arachidonyl lysoPI was detected. However, because lysophospholipase activity is reported to be very strong in the macrophage-like cell line P388D1 [27], this result does not lend weight to the argument ruling out the phospholipase A_1/lysophospholipase pathway. Since it is well known that rat intestinal mucosal phospholipase A_2 is significantly activated by the addition of diacylglycerol [28], we examined the effect of diacylglycerol addition to the reaction mixture. Diacylglycerol significantly enhanced the arachidonic acid release from PI by the macrophage plasma membranes (data not shown), once more suggesting that the high level of arachidonic acid release from PI in the macrophage plasma

membranes might occur via phospholipase A_2-like action.

Wightman et al. [29] reported that homogenates of peritoneal resident macrophages of mice have two types of phospholipase A_2 when PC and PE are used as the substrate. The first is active at pH 4.5 and is not dependent on Ca^{2+}. The second is optimally active at pH 8.5 and is Ca^{2+}-dependent. Both phospholipases A_2 are capable of hydrolysing [^{14}C]arachidonic acid from [^{14}C]arachidonyl-phospholipids. On the other hand, Lanni and Franson [30] reported in a study using autoclaved *E. coli*. Phospholipids labelled in the 2-acyl position with [^{14}C]oleate that the Ca^{2+}-dependent and membrane-bound phospholipase A_2 from *Bacillus Calmette-Guérin*-induced alveolar macrophages is activated at pH 7.0. In our study with purified macrophage plasma membrane, the release of arachidonic acid from PC showed peaks at pH 4.5, 7.0 and 9.0, and the optimum pH values of arachidonic acid release from PE were pH 7.5 and 9.0. Unlike the high level of release at neutral pH, the release of arachidonic acid from PI at acidic or alkaline pH was small; and, under these conditions, the arachidonic acid release from PC or PE was greater than that from PI. Therefore, except at neutral pH, the phospholipase A_2 of the macrophage plasma membrane appears to react better with PC or PE.

In conclusion, we have demonstrated the possibility that arachidonic acid release from PI at neutral pH in the macrophage plasma membrane is dependent on phospholipase A_2-like activity. Also, the maximum arachidonic acid release from phospholipids was dependent upon both pH and substrate. Particularly, the level of arachidonic acid release from PI at neutral pH was very high compared with that from PC or PE. We suggest that phosphoinositol phospholipase A_2 may play an important role in providing arachidonic acid for subsequent metabolic activity in the macrophage.

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