RESEARCH COMMUNICATION

The localization of phospholipase A₂ in the secretory granule

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A heat-resistant phospholipase A₂ has been detected in the secretory granules of the mast cell [Chock, Rhee, Tang and Schmauder-Chock (1991) *Eur. J. Biochem.* **195**, 707–713]. By using ultrastructural immunocytochemical techniques, we have now localized this enzyme to the matrix of the secretory granule. Like the cyclo-oxygenase [Schmauder-Chock and Chock (1989) *J. Histochem. Cytochem.* **37**, 1319–1328], this enzyme also adheres tightly to the ribbon-like granule matrix components. The results from Western-blot analysis suggest that it has a molecular mass of about 14 kDa. The localization of the

phospholipase A₂, the presence of a phospholipid store with millimolar concentrations of calcium and the localization of the enzymes of the arachidonic acid cascade make the secretory granule a natural site for lipid-mediator synthesis. The packaging of phospholipase A₂, together with its substrate and the components of the arachidonic acid cascade, in the secretory granule represents a physical arrangement by which the initiation of the cascade and the release of mediators can be directly linked to the stimulation of cell-surface receptors.

INTRODUCTION

Phospholipase A₂ (PLA₂) hydrolyses the glyceryl–fatty-acid ester bond at the *sn*-2 position of phospholipid molecules. This action gives rise to a lysophospholipid and a non-esterified fatty acid. Since arachidonic acid is mostly found esterified at the *sn*-2 position of glycerophospholipid, the action of PLA₂ is responsible for the release of arachidonic acid. In this sense, PLA₂ controls the availability of substrate for the arachidonic acid cascade, leading to the production of eicosanoids which are potent mediators of inflammation [1,2].

Lysophospholipid, the other product of phospholipid hydrolysis by PLA₂, is a potent membrane detergent capable of causing cell lysis. The cytolytic action of PLA₂ may be responsible for the tumour-cell killing and the bactericidal activity of the immune cells [3]. The hydrolysis of 1-*O*-alkyl-2-acyl-*sn*-glycero-3-phosphocholine by PLA₂ yields lyso-(platelet activating factor) (lyso-PAF), which, on acetylation, produces PAF (1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine). PAF is one of the most potent lipid mediators known to date and has been implicated in the pathogenesis of diseases such as bronchial asthma [4,5].

A group II PLA₂ of molecular mass about 14 kDa has been purified from the synovial fluid of arthritic patients, and its presence may be an important factor in the pathogenesis of the disease [6,7]. Besides being implicated in many inflammatory diseases such as rheumatoid arthritis, pancreatitis, adult respiratory-distress syndrome, tuberculosis, acute renal failure and septic shock etc. [8–11], PLA₂ has also been suspected of being able to stimulate cell proliferation [12]. As PLA₂ becomes recognized as a key enzyme in cellular regulation, the need to understand its site of action becomes increasingly crucial.

Recently we have detected a PLA₂ activity co-existing with a non-bilayer phospholipid pool and with the enzymes of the arachidonic acid cascade within the secretory granule of the mast cell [13–16]. However, a question was raised concerning its subcellular origin. Here we present immunocytochemical evidence which confirms the presence of a PLA₂ in the matrix of the

secretory granule. The presence of PLA₂ as a secretory-granule component not only provides the physiological basis for linking PLA₂ activity to the initiation of the arachidonic acid cascade [17], but it also explains how PLA₂ and lipid mediators can be released into the extracellular medium after a receptor-mediated event. This finding provides a general mechanism for the accumulation of extracellular PLA₂ and lipid mediators.

MATERIALS AND METHODS

Rat peritoneal mast cells and mast-cell granules were routinely prepared from peritoneal lavages according to published procedures [13,14]. For the immunoblot experiment, granules were heated at 100 °C for 5 min and stored at –80 °C until use. SDS/PAGE was performed as described by Laemmli [18], using 15% (w/v) polyacrylamide gel. Protein molecular masses were estimated using Gibco BRL's low-molecular-mass protein standards. Coomassie Blue staining was used to detect protein bands. Protein bands from unstained gel were transferred to poly(vinylidene difluoride) ('PVDF') membrane (Immobilon, Millipore) as described by Burnette [19]. Blots were stained by using an immunogold–silver staining kit (Janssen) according to the manufacturer's instructions. Polyclonal antibodies to PLA₂ were produced in rabbits using oligopeptides from purified porcine pancreatic PLA₂ as immunogens. Antibodies were purified and characterized as reported in [20]. Only those antibodies which are specific against the epitope containing the calcium-binding loop were selected and used in this experiment. Since the amino acid sequence of the Ca²⁺-binding loop (amino acid residues 21–40) is highly conserved for both the group I and group II enzymes, this antibody cross-reacts with both enzymes.

The procedure for the localization of PLA₂ in whole cells was similar to that already published for cyclo-oxygenase (prostaglandin H synthase) [15]. This procedure labels antigens on exposed surfaces. Intracellular PLA₂, which was inaccessible to the antibody, could not be labelled. Since rat mast-cell-granule matrices do not dissociate and become soluble immediately after

being exteriorized, granules which are released as the result of ionophore A23187 stimulation become available for antibody labelling.

The localization of PLA₂ in purified mast-cell granules was also carried out using the pre-embedding method, except that purified granules were used in place of the stimulated mast cell. Secretory granules were purified according to a published procedure [13] and heated for 3 min at 100 °C in 1 ml of pH 7 buffer containing 0.25 mg/ml digitonin, 0.05 mg/ml leupeptin, 0.025 mg/ml phenylmethanesulphonyl fluoride, 4 mM MgCl₂, 5 mM Hepes, 100 mM cacodylate and 2.5% glutaraldehyde before antibody labelling. After dehydration and embedding, thin sections were examined with a Hitachi-7000 electron microscope. The presence of PLA₂ was shown by the appearance of dark grains, which reflects the electron density of the bound ferritin. As usual, control experiments were carried out using pre-immune serum in place of the anti-PLA₂ immunoglobulin.

RESULTS

When purified secretory granules were analysed by Western blot, a 14 kDa band similar to the purified porcine pancreatic PLA₂ was observed (Figure 1). In addition, immunogenic bands of higher molecular masses, about 25 and 30 kDa, could also be seen. For purified porcine PLA₂, the main 14 kDa band is accompanied by a 30 kDa band.

The presence of PLA₂ in mast-cell secretory granules was confirmed by the labelling of the granule matrices released by ionophore A23187 stimulation. A newly released granule (Figure 2, arrowhead) still surrounded by its membrane in a vacuole and an unravelling granule matrix (Figure 2, arrow) are seen labelled with anti-PLA₂ antibodies (Figure 2). The newly released granule exhibits antibody labelling on its periphery, while the dispersed granule is heavily labelled on its ribbon-like strands. This suggests that the loosening of the granule matrix has exposed more PLA₂ sites.

As in the case of cyclo-oxygenase localization [15], we did not observe any specific antibody labelling on the plasma membrane of stimulated mast cells. This is true even in the vicinity of the released granule (Figure 2), where the plasma membrane could have been exposed to the granule contents.

Figure 1 Antibody detection of PLA₂ in mast-cell granules by immunoblot

The left lane represents the anti-PLA₂ immunoblot of 5 µg of purified porcine pancreatic PLA₂. The right lane shows anti-PLA₂ antibody labelling of a blot obtained by SDS/PAGE of about 100 µg of granule protein. Abbreviation: M, molecular mass.

The presence of PLA is further confirmed by the localization of PLA₂ in purified granules (Figure 3). Like in the whole cell (Figure 2), granule PLA₂ is bound to the dispersed strand-like granule-matrix-backbone materials which we have previously called 'proteo-heparin'. This suggests that much of the PLA₂ remains bound to the granule matrix.

DISCUSSION

It was realized long ago that the synthesis and the release of eicosanoids via the arachidonic acid cascade were coupled to the secretory process which, in turn, is the direct result of cell-surface-receptor stimulation [21–23]. Since arachidonic acid is mainly esterified at the *sn*-2 position of phospholipid, a role for PLA₂ in providing the substrate for eicosanoid synthesis via the arachidonic acid cascade was implicated [24]. Recently, evidence which indicates the presence of a PLA₂ along with its phospholipid substrate and the enzymes of the arachidonic acid cascade within the membrane-delimited secretory granule has also been published [13–16]. The current localization of the PLA₂ to the secretory granule further supports the idea that the secretory granule may serve as the site for the initiation of arachidonic acid cascade during a receptor-mediated event.

On the basis of immunoblots (Figure 1) it appears that the granule PLA₂ has a molecular mass of about 14 kDa, similar to that of the secretory enzymes (e.g. group I pancreatic type). It also has a tendency to form aggregates of higher molecular mass such as those represented by the 25 and the 30 kDa bands. The formation of multimeric forms of PLA₂ on SDS/PAGE has been reported [25]. The presence of a high concentration of proteolytic enzyme which degrades granule proteins, and the natural affinity between PLA₂ and the granule matrix components, may have contributed to the formation of complex band patterns. The presence of a higher-molecular-mass band (30 kDa) is also seen in the purified porcine pancreatic PLA₂. The presence of multiple bands on SDS/PAGE appears to be a common characteristic of PLA₂.

The polyclonal anti-PLA₂ immunoglobulin used in the present study is specific against the Ca²⁺-binding loop, which shares sequence similarity with both the group-I and group-II enzymes. This makes it impossible for us to classify the granule enzyme. However, we are inclined to think that the granule enzyme may be a group-II enzyme. This reasoning is based on published evidence which suggests that the group-II enzymes are mainly pro-inflammatory and are found in neutrophils, platelets, mast cells and macrophages, which normally participate in the inflammatory process [26–29]. So far, only a group-II enzyme has been purified from arthritic synovial fluid, and it must have been released by immune cells, including mast cells, which infiltrate the inflamed synovium [30]. Recently, it was suggested that mast cells can release a type II PLA₂, and its presence could enhance the synthesis of eicosanoids [31,32].

As is the case for the cyclo-oxygenase and prostaglandin E₂ [15], the rat mast-cell PLA₂ remains sequestered to the granule matrix after release from the cell. Although the released granules will eventually disperse and solubilize, many are taken up whole by neutrophils, macrophages and other cells attracted to the site of inflammation by chemotactic factors disseminate from the granules. The inherent affinity between the heparin-rich granule matrix and the enzyme has been exploited in the application of the heparin-Sepharose affinity column as an important step in the purification of PLA₂ and other phospholipases [33,34]. It should also be mentioned here that a granule phospholipase C has also been preliminarily localized to the mast-cell secretory granule [35].

Figure 2 Localization of PLA₂ in a stimulated mast cell

On the left is an electron micrograph of a mast cell after treatment with the secretagogue A23187. On the right is the high magnification of the released granules showing extensive anti-PLA₂ antibody binding as seen by the dark grains adhering to the surface of the released granules. A granule (arrowhead) in the process of being extruded has taken on antibody labelling on its periphery. Another granule (arrow), which was released earlier, has become unravelled and its matrix is intensely labelled with anti-PLA₂ antibody. The bars represent 1 μm.

Figure 3 Localization of PLA₂ in the matrix of a purified mast-cell granule

Like the granules released from stimulated mast cells, purified granules also exhibit intense anti-PLA₂-antibody labelling on the matrix strands of unravelled granules. The bar at the lower right represents 0.5 μm.

For many years the secretory PLA₂ was suggested by many workers to be the enzyme responsible for providing the arachidonic acid for the synthesis of eicosanoids, a step assumed by many to be integral to the mechanism of signal transduction [36,37]. Recently, a high-molecular-mass "cytosolic phospholipase A₂" was thought to assume that role [38]. However, owing to the lack of information concerning its subcellular site of action, the physiological function of the cytosolic PLA₂ remains unclear. Furthermore, in experiments in which whole cells are used, it is impossible to tell if the arachidonic acid released can be attributed to the enzyme activity in the granules, the plasma membrane, or to any other compartment in the cytoplasm. After all, the secretory granule is also in the cytosolic compartment.

The simultaneous localization of PLA₂ in the matrices of granules released by stimulated mast cells (Figure 2) and in the matrices of purified mast-cell granules (Figure 3) further confirms the presence of the PLA₂ in the secretory granules. This finding also argues against the possibility that the PLA₂ activity previously detected in our granule preparations [13] could have been due to contamination by enzyme from the plasma membrane or from other cytoplasmic sources. The presence of cyclo-oxygenase in the granule matrix has already been demonstrated [15]. Barring the possibility that different enzymes may occur exclusively on different granule matrix strands, the current result suggests that PLA₂ and the enzymes of the arachidonic acid cascade may co-exist in close proximity to each other within the membrane-delimited secretory granule. Together with the presence of a phospholipid store and millimolar concentrations of Ca²⁺ [14,39], not only is the secretory granule a preformed mediator storage depot, but it can also function as a generator of lipid mediators. This arrangement has eliminated the need for enzyme translocation and avoids the inefficiency associated with the diffusion of metabolic components across the undefined cytosolic medium. As such, the secretory granule is an example for the packaging of complete metabolic cascades within a membrane-delimited organelle poised for activation on demand.

The co-packaging of PLA₂ with phospholipid and with the components of the arachidonic acid cascade bodes well with the scenario that, during granule activation, the hydrolysis of granule phospholipid by the PLA₂ is what provides the precursors for the formation of lipid mediators. This explains why purified mast-cell granules can rapidly synthesize eicosanoids following their disruption in aqueous buffer [16,17]. As a secretory-granule component, PLA₂ is released along with various preformed and newly formed mediators during a stimulus-secretory response. Thus the presence of extracellular PLA₂ may reflect the secretory activity of granule-bearing cells at the site of inflammation, with its ability to degrade phospholipid, the presence of PLA₂ in the extracellular fluid, if not contained, may exacerbate an inflammatory condition. The understanding of the fate of the secreted PLA₂ may further shed light on the mechanism of inflammation.

The ultrastructural localization of PLA₂ to the secretory granule has provided the physical basis underlying the biochemical detection of the PLA₂ activity reported previously in purified granules [13]. The placement of PLA₂ and the arachidonic acid cascade in the secretory granule provides the physiological basis for the release of PLA₂ and lipid mediators following a receptor-mediated event. This arrangement also explains how hydrophobic molecules such as arachidonic acid and its metabolites, which normally do not easily diffuse across the plasma membrane [40], can be rapidly released into the extracellular medium. The localization of PLA₂ and the components of the

arachidonic acid cascade in the secretory granule has highlighted the granule as an important source of lipid mediators and other messenger molecules during a receptor-mediated event. This finding should shed new light on the mechanism of the immune response, of which inflammation is an important aspect.

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