Site-Specific Distribution of Phospholipase D Isoforms in the Rat Pancreas

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Received November 1, 2002; accepted February 14, 2003

Phospholipase D (PLD), one of the signal transducing enzymes, is recognized to play a role in the pancreatic exocrine and endocrine secretion, and yet the distribution of PLD in the pancreas has remained unclarified. We investigated the expression and localization of PLD isoforms, PLD1 and PLD2, in the rat pancreas. Western blot analysis showed that PLD1-immunoreactive band was observed in the islets, but not in the acinar cells. In contrast, PLD2-immunoreactive band was observed in both the islets and the acinar cells. In the immunohistochemistry, PLD1 was evenly distributed throughout the islets while PLD2 immunoreactivity was more intense in the periphery than in the central portion of the islets. To further elucidate the cell-specific localization of PLD2 in the islets, double immunofluorescent staining was performed. PLD2 was mainly localized in A and PP cells, which secrete glucagon and pancreatic polypeptide, respectively. PLD2 was also detected in acinar cells, ductal epithelial cells and intrapancreatic nerve fibers. Meanwhile, PLD1 was detected in ductal epithelial cells and intrapancreatic ganglia, but not in acinar cells. On the basis of this heterogeneity in the distribution of PLD isoforms, it is suggested that PLD might play a specific role in the pancreatic exocrine and endocrine function.

Key words: phospholipase D (PLD), pancreatic islets, acinar cells, A cells, PP cells

I. Introduction

Phospholipase D (PLD) plays an important role in the membrane lipid-mediated signal transduction. PLD catalyzes the hydrolysis of phosphatidylcholine (PC) to choline and phosphatidic acid (PA) [11]. PA has the ability to act directly as a signaling molecule and to be converted into other signaling molecules such as diacylglycerol (DAG) and lysophosphatidic acid (LPA). DAG, which can be formed from PA by phosphatidic acid phosphatase, regulates certain protein kinase C isozymes. LPA, produced from PA through the action of a specific phospholipase A₂, is now recognized as an important extracellular signaling molecule [12]. Although the role of PLD is not fully understood, receptor-mediated PLD activation has been implicated in diverse physiological processes including cell proliferation [1], differentiation [28], cytoskeletal reorganization [7] and the control of protein trafficking and secretion [6, 33].

Recently, two mammalian PLD isoforms, PLD1 and PLD2, have been characterized by molecular cloning and biochemical analysis [13]. In addition, three alternatively spliced forms of PLD1 (hPLD1a–c) and PLD2 (hPLD2a–c) have been characterized in human [14, 38]. In the rat, two alternatively spliced forms of PLD1 (rPLD1a–b) and one type (rPLD2) of PLD2 have been characterized [17, 22, 27, 30].

The pancreas is a unique organ comprising both exocrine and endocrine tissues. The major hormone-producing cells are organized into the islets that are scattered within the exocrine tissue and comprise 1–3% of the total pancreatic mass. These combined exocrine and endocrine components make the pancreas one of the most important and complex organs involved in food metabolism [2, 32, 37, 39].

Information concerning the specific distribution of PLD in the pancreas can provide potential clues to the functional significance of PLD isoforms in the stimulus-secretion coupling. And yet, information on the expression and localization of PLD isoforms is limited. By Northern blot,
both human PLD1 and PLD2 mRNA were abundantly expressed in the human pancreas [26]. Bosch et al. [3] demonstrated that PLD1 protein and PLD2 mRNA were present in the rat pancreatic acini. Though Lainé et al. [23] reported the existence of PLD2 in the islets by indirect immunofluorescence, the specific localization of PLD2 in the islets was not examined. In addition, they mainly focused their interests on the biochemical properties of PLD isoforms.

We recently examined the expression and distribution of phospholipase C and protein kinase C [18, 19], key enzymes in the signal transduction, in the exocrine and endocrine pancreases. Based on the previous studies, we investigated the expression and localization of PLD isoforms in the rat pancreas using specific antibodies.

II. Materials and Methods

Experimental animals

Fifteen male Sprague-Dawley rats (200–250 g, Daehan Biolink Company, Eumsung, Chungbuk, Korea) showing normoglycemia were used throughout the experiment. Ten animals were used to separate the pancreatic islets and acinar cells for Western blot analysis, and the rest for immunohistochemistry. The animals were fasted overnight before the experiment. All experimental procedures performed on the animals were conducted with the approval of the ethics committee of The Catholic University of Korea and conformed to the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publications no. 80–23, revised 1996).

Chemicals

Trizma base, EDTA, leupeptin, β-mercaptoethanol, sodium dodecyl sulfate (SDS), phenylmethylsulfonyl fluoride (PMSF), Tween 20, bovine serum albumin (BSA, fraction V), paraformaldehyde, collagenase, hyaluronidase, and 3,3′-diaminobenzidine tetrahydrochloride (DAB) were purchased from Sigma (St. Louis, MO, USA). All other chemicals were obtained from other commercial sources.

Preparation of affinity purified anti-PLD1 and PLD2 antibodies

Using New Zealand white rabbit, antisera were raised against the N-terminal peptide of PLD1 and PLD2 corresponding to amino acid residues 1–12 of rat PLD1 (MSLRSEARVNTS) and amino acid residues 1–19 of human PLD2 sequence (MTATPESLFPTGDELDSSQ), respectively. For affinity purification of the antibody, the rat PLD1 N-terminal peptide was coupled to Affi-Gel 15 (BioRad, Hercules, CA, USA) following the manufacturer’s instructions with slight modification. 5 ml of antiserum was incubated with peptide-conjugated Affi-Gel 15 (5 mg of peptide in 1 ml of Affi-Gel 15) overnight at 4°C. The column was then washed with 20 ml of buffer (20 ml HEPES/NaOH, pH 7.0; 200 mM NaCl; 0.1% Triton X-100), and the antibody was eluted with 0.1 M glycine/HCl, pH 2.5 into tubes containing 1 M Tris-HCl, pH 8.0, for neutralization.

Preparation of pancreatic islets and acinar cells

To evaluate the expression of PLD isoforms in the islets and acinar cells of the same pancreas, some portions of the pancreas were used for the isolation of the islets and the rest for acinar cells. The islets were isolated using the collagenase digestion method, as described previously [21]. A suspension of acinar cells was prepared by enzymatic dispersion, according to the previous method [20].

Immunoprecipitation and Western blot analysis

Immunoprecipitation and Western blot for PLD isoforms were performed by the same procedure, as previously described [29]. Tissues of the islets, total pancreas and acinar cells were homogenized in immunoprecipitation assay buffer (20 mM HEPES, pH 7.2; 1% Triton X-100; 1% deoxycholate; 0.1% SDS; 150 mM NaCl; 10 μg/ml leupeptin; 10 μg/ml aprotinin; 1 mM PMSF). The homogenates were centrifuged and then the lysates supernatant were precelared by preimmune IgG and protein A sepharose for 30 min. Subsequently, precelared cell lysates were incubated with anti-PLD1 or anti-PLD2 antibody, and 30 μl of 50% slurry of protein A sepharose for 4 hr. The immune complex was collected and washed five times with ice-cold buffer (20 mM Tris, pH 7.5; 1 mM EDTA; 1 mM EGTA; 150 mM NaCl; 2 mM Na₂VO₃; 10% glycerol; 1% Nonidet P-40), followed by the addition of SDS-sample buffer, and boiled. The recovered protein was resolved on a SDS-PAGE gel, blotted to a nitrocellulose membrane and blocked in 5% non-fat dried milk. The blot was probed with anti-PLD1 or anti-PLD2 antibody, which was diluted in blocking solution (1 μg/ml). Immunoreactive bands were visualized by chemiluminescence using horseradish peroxidase-conjugated goat anti-rabbit IgG and enhanced chemiluminescence (ECL, Amersham Pharmacia Biotech, Bucks, UK). Also, the specificity of the immune reaction was verified by immunoprecipitation and immunoblot analysis using anti-PLD1 or anti-PLD2 antibody (2.5 μg) that had been preadsorbed overnight at 4°C with its specific immunopeptide (2.5 or 25 μg).

Immunohistochemistry

The pancreas was taken out following anesthesia by an intraperitoneal injection of chloral hydrate (400 mg/kg). The pancreatic specimens were immersed overnight at 4°C in 4% paraformaldehyde solution, and subsequently embedded in polyethylene glycol (Polysciences Inc., Warrington, PA, USA). The immunohistochemical procedure was performed based on the avidin-biotin-peroxidase complex (ABC) method [15]. Prior to labeling, tissue sections were processed through routine procedures. After blocking with normal goat serum for 30 min, the sections were incubated overnight at 4°C with the primary antibodies against PLD isoforms (PLD1, 1:200; PLD2, 1:300) diluted in PBS containing 0.1% ovalbumin (10 mM phosphate buffer; 105 mM NaCl, pH 7.4). Then, the secondary biotinylated goat anti-rabbit antibody (1:300) was applied to these sections for 1 hr at room temperature. After washing with PBS, the sections
were incubated in ABC solution (Vector Laboratories, Burlingame, CA, USA) for 1 hr, washed in 0.1 M Tris-HCl buffer (TB, pH 7.4), and then incubated in 0.05% DAB in TB containing 0.01% H2O2 to show the degree of immunostaining. After immunostaining, these sections were counterstained with hematoxylin and mounted. Control sections were processed as described above except that the primary antibody was omitted, or preabsorbed with its specific antigen. To illustrate the location of PLD2 immunoreactive cells in the islets, double immunofluorescent staining was applied on the same sections. The first primary antibody used was either anti-insulin (1:1,000), anti-glucagon (1:500), antisomatostatin (1:1,000), or anti-pancreatic polypeptide (PP, 1:250). The associated secondary antibody was fluorescein (FITC)-conjugated and consistent with the source of each primary antibody. Then, the second primary antibody, anti-PLD2 antibody was applied. The associated secondary antibody used was a Texas Red-conjugated donkey anti-rabbit. FITC and Texas Red fluorescence was visualized by using 490 nm and 570 nm filters on a Zeiss Axiopt photomicroscope (Carl Zeiss, Oberkochen, West Germany). Specificity and crossreactivity were checked for double fluorescent staining by applying control sections with the primary antibody preabsorbed with its specific immunopeptide or by running control sections that lacked primary or fluorochrome-labeled antibodies. No significant background staining was seen, nor was any crossreactivity between the two fluorochrome-labeling systems. Antibodies were obtained from the following sources: mouse monoclonal anti-insulin antibody, mouse monoclonal anti-glucagon antibody, goat polyclonal anti-somatostatin antibody, and sheep polyclonal anti-PP antibody were purchased from BioGenex (San Ramon, CA, USA), Sigma, Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA), and Serotec (Oxford, UK), respectively. FITC and Texas Red-conjugated secondary antibodies were purchased from Jackson ImmunnoResearch Laboratories, Inc. (West Grove, PA, USA).

III. Results

Western blot analysis

As shown in Fig. 2A, the immunoreactive band of PLD1 protein, approximately 120 kDa, was strongly expressed in the islets of Langerhans, and was not detectable in acinar cells. A mild expression of PLD1 protein appeared in whole pancreatic tissue, reflecting the existence of PLD1 protein in the islets and ductal epithelium, which are the components of the whole pancreas. In contrast, the PLD2 protein, approximately 105 kDa, was detected in both the islets and acinar cells. The expression level of PLD2 protein in the islets was somewhat greater than that in acinar cells (Fig. 2B).

Immunohistochemistry

The results of immunohistochemistry are summarized in Table 1. PLD1 immunoreactivity was strongly detected in the cytoplasm of endocrine cells throughout the islet (Fig. 3A1). Furthermore, PLD1 immunoreactivities were observed in the ductal epithelial cells (Fig. 3A2) and in the intrapancreatic ganglia (Fig. 3A3). However, PLD1 immunoreactivity was not detected in acinar cells consistent with the result from Western blot analysis. Presumably, PLD1 expression level in acinar cells might be very low and not detectable by anti-PLD1 antibody.

As shown in Fig. 3B, PLD2 immunoreactivity was detected throughout the whole pancreas. PLD2 immunoreactivity was more intense in the periphery than in the central portion of the islet (Fig. 3B1). PLD2 immunoreactivities were also weakly observed in the ductal epithelial cells (Fig. 3B2) and in the intrapancreatic nerve fibers (Fig. 3B3). While there was no detectable immunoreactivity of PLD1 in acinar cells, PLD2 immunoreactivity was easily detected in tele-insular acinar cells.

Based on the localization pattern of endocrine cells in rodents, the cells strongly stained by anti-PLD2 antibody in the islet periphery suggest that they were not insulin-producing B cells. To confirm this, double immunofluorescent stainings with anti-PLD2 and each hormone-directed antibody were performed. The immunofluorescence of PLD2 was visualized by the red color of Texas Red, whereas the reaction of antibodies directed against each hormone was displayed by the green fluorescence of FITC.

As shown in Fig. 4, there was no colocalization of insulin and PLD2 within the same cells of the islet at a detectable level. The distribution of glucagon-immunoreactive cells paralleled that of PLD2-immunoreactive cells (Fig. 5). Since the results of these antibodies, we tried to investigate the expression pattern of PLD isoforms in the pancreas. As shown in Fig. 2A, the immunoreactive band of PLD1 protein, approximately 120 kDa, was strongly expressed in the islets of Langerhans, and was not detectable in acinar cells. A mild expression of PLD1 protein appeared in whole pancreatic tissue, reflecting the existence of PLD1 protein in the islets and ductal epithelium, which are the components of the whole pancreas. In contrast, the PLD2 protein, approximately 105 kDa, was detected in both the islets and acinar cells. The expression level of PLD2 protein in the islets was somewhat greater than that in acinar cells (Fig. 2B).

Table 1. The immunoreactive distribution of PLD isoforms in the rat pancreas

<table>
<thead>
<tr>
<th>Islet</th>
<th>A Cell</th>
<th>B cell</th>
<th>D cell</th>
<th>PP Cell</th>
<th>Acinar Cell</th>
<th>Ductal Epithelial Cell</th>
<th>Intrapancreatic Ganglia and Nerve</th>
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<tr>
<td>PLD1</td>
<td>++</td>
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<td>PLD2</td>
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The degree of immunoreactivity is arbitrarily expressed as: –, not labeled or tissue was negative; ±, weak labeling above background or only traces of the tissue were stained; +, moderate labeling above background or less than one third of the tissue was stained; ++, intense labeling above background or more than one third of the tissue was stained.
Specificity of PLD immunoreactivity. Extracts of rat brain and pancreas were prepared, and the lysates (2 mg) were immunoprecipitated (IP) using anti-PLD1 (A) or anti-PLD2 (B) antibodies (2.5 μg) preincubated in the absence (−) or presence (1x or 10x) of its specific immunopeptide (2.5 μg or 25 μg) and then immunoblotted (Blot) with anti-PLD1 or anti-PLD2 antibodies, respectively. Br, brain; Ps, total pancreatic tissue. Results shown are from a representative experiment and were reproduced in at least three other identical experiments.
Fig. 2. Expression of PLD isoforms in the rat pancreas by Western blot analysis. Each sample containing 1 mg of lysate protein from the islets, whole pancreas, and acinar cells was immunoprecipitated (IP) with anti-PLD1 or anti-PLD2 antibody. Immunoprecipitated proteins were subjected to an 8% SDS-PAGE and immunoblotted (Blot) with anti-PLD1 (A) or anti-PLD2 antibody (B). I, the islets of Langerhans; W, whole pancreatic tissue; A, acinar cells. Results shown are from a representative experiment and were reproduced in at least three other identical experiments.
red and green colors combine to produce a yellow color under dual exposure of the section, cells that appear yellow contain both PLD2 and glucagon. This finding suggests that the expression of PLD2 was high in glucagon-producing A cells. In contrast, the double staining of PLD2 and somatostatin did not show such a coincident pattern (Fig. 6). Also, all pancreatic polypeptide-immunoreactivity was easily detected in PLD2-immunoreactive cells (Fig. 7).
Fig. 4. Immunofluorescent colocalization of PLD2 with insulin in the rat pancreatic islet. Insulin (FITC) immunoreactivity is mainly detected in the central portion of the islet (A). Meanwhile, PLD2 (Texas Red) immunoreactivity is highly expressed in the peripheral portion of the islet (B). Colocalization of PLD2 and insulin is hardly observed in the same cells (C). The experiment was performed at least three times with identical methods: one representative example is shown. Bar=20 μm.

Fig. 5. Immunofluorescent colocalization of PLD2 with glucagon in the rat pancreatic islet. Colocalization of glucagon (FITC, A) and PLD2 (Texas Red, B) is observed in the same cells of the islet periphery, demonstrated by yellow color (C). The experiment was performed at least three times with identical methods: one representative example is shown. Bar=20 μm.
Fig. 6. Immunofluorescent colocalization of PLD2 with somatostatin in the rat pancreatic islet. Somatostatin (FITC, A) immunoreactive cells are localized in the islet periphery, similar to the pattern of PLD2 (Texas Red, B). However, somatostatin and PLD2 are hardly colocalized in the same cells after double exposure (C). The experiment was performed at least three times with identical methods: one representative example is shown. Bar=20 μm.

Fig. 7. Immunofluorescent colocalization of PLD2 with pancreatic polypeptide in the rat pancreatic islet. The number of pancreatic polypeptide immunoreactive cells (PP, FITC, arrows in A) in the islet periphery are fewer than that of PLD2 immunoreactive cells (Texas Red, B). Cells rich in both PP and PLD2 are easily demonstrated as yellow color (arrows in C). The experiment was performed at least three times with identical methods: one representative example is shown. Bar=20 μm.
IV. Discussion

Recently, the functional role of PLD in the pancreas has been suggested. Cerulein or growth factors stimulated PLD-induced PC hydrolysis in the rat pancreatic acini [34, 36]. PLD was also reported to promote insulin release from the islets [5, 9, 10]. To know the function of PLD, we examined the site-specific distribution of PLD isoforms, PLD1 and PLD2, in the rat pancreas. The functional properties of PLD isoforms are different in that PLD1 is activated by PKC-α, ADP-ribosylation factor (ARF) and small GTP-binding proteins [25, 35], whereas PLD2 is constitutively active, but less sensitive to ARF and RhoA [7, 22, 25].

We observed PLD isoforms mainly in the cytoplasm, not on the plasma membrane. Others also showed that PLD1b predominantly existed in the cytosolic fraction of acinar cells by Western blot analysis [3] and that PLD2 was detected in the cytosol of acinar cells on immunohistochemistry [23]. The intracellular localization of PLDs is diverse depending on cell types [4, 8, 16, 41].

In our immunohistochemistry, PLD1 was evenly distributed throughout all the islet cells. In the rodent, the islets consist of central insulin-producing B cells and peripheral non-B cells, viz., A, D, and PP cells [31]. Both rPLD1a and rPLD1b mRNAs were expressed in insulin cells such as primary rat B-cells and RINm5F cells and PLD1 expression was early induced by exposure to IL-1β [5]. Thus, the induction of PLD1 might contribute to the early stimulation of insulin release implying PLD1 might play a role in the pancreatic hormones secretion including insulin. Although Lainé et al. [23] confirmed the existence of PLD1 in the membranous and microsomal fraction of the whole pancreas by Western blot analysis, they could not observe PLD1 by indirect immunofluorescence. The discrepancy between our and their immunohistochemical findings may reflect the differences of tissue section used (wax embedding vs frozen tissue), staining method (ABC vs immunofluorescence), and antibody used (N-terminal vs C-terminal residues).

In our study, the PLD2 immunoreactivity was intensely observed in the islet periphery as in others’ [23]. Chen et al. [5] observed no detectable PLD2 mRNA expression in the primary rat B-cells. Especially, double staining showed the enrichment of PLD2 in A and PP cells, which release glucagon and pancreatic polypeptide, respectively. The abundance of PLD2 in A cells was also observed in mouse and guinea pig (our unpublished data). Along with the report on the involvement of PLD in bradykinin-induced glucagon release [40], our results provide a clue to understand the role of PLD2 in A cells.

In the acinar cells, especially of tele-insular portion, PLD2 was easily detected while PLD1 immunoreactivity was not detected. Others also reported that PLD2 was distributed throughout the acinar cells by indirect immunofluorescence [23] and identified the PLD2 transcript using RT-PCR [3]. These results suggest the possible involvement of PLD2 in the acinar cell function. In contrast to our result, Bosch et al. [3] suggested the existence of rPLD1b in pancreatic acini by Western blot analysis following the observation of rPLD1b transcript by RT-PCR. The discrepancy between our and their results is obscure, but it may be due to the binding capacity of each antibody or source of immunopeptide (rat vs human). Because our antibody was raised against the amino acid residues common to two rPLD1 isoforms, it could have recognized PLD1 isoforms.

PLD1 was expressed in the intrapancreatic ganglia which are likely cholinergic. PLD2 was also observed in intrapancreatic nerve fibers. Considering that cholinergic neurons are involved in acetylcholine synthesis by PLD-catalyzed PC hydrolysis [24], PLD may control pancreatic secretion through modulation of acetylcholine synthesis.

In conclusion, we identified the site-specific localization of PLD isoforms in both acinar cells and the islets, and these findings suggest the involvement of PLD-mediated signaling in the pancreatic exocrine and endocrine functions.

V. Acknowledgment

This work was supported by a grant (200106101) from the Good Health R&D Project, the Ministry of Health and Welfare, Korea.

VI. References


