Korean J Physiol Pharmacol
Vol 7: 211–215, August, 2003

Comparative Analysis of Phospholipase D2 Localization in the Pancreatic Islet of Rat and Guinea Pig

Gyeong Ryul Ryu, Myung-Jun Kim, Chan-Hee Song, Do Sik Min, Duck-Joo Rhie, Shin-Hee Yoon, Sang June Hahn, Myung-Suk Kim, and Yang-Hyeok Jo

Department of Physiology, College of Medicine, The Catholic University of Korea, Seoul 137–701, Korea

To examine the localization pattern of phospholipase D2 (PLD2) in the pancreatic islet (the islet of Langerhans) depending on species, we conducted a morphological experiment in the rat and guinea pig. Since individual islets display a typical topography with a central core of B cell mass and a peripheral boundary of A, D, and PP cells, double immunofluorescent staining with a panel of antibodies was performed to identify PLD2-immunoreactive cells in the islets PLD2 immunoreactivity was mainly present in A and PP cells of the rat pancreatic islets. And yet, in the guinea pig, PLD2 immunoreactivity was exclusively localized in A cells, and not in PP cells. These findings suggest a possibility that PLD2 is mainly located in A cells of rodent pancreatic islets, and that the existence of PLD2 in PP cells is not universal in all species. Based on these results, it is suggested that PLD2 may play a significant role in the function of A and/or PP cells via a PLD-mediated signaling pathway.

Key Words: Phospholipase D2 (PLD2), Islet of Langerhans, A cell, PP cell

INTRODUCTION

Much attention has been focused on the role of PLD in a variety of cell and tissue types, and 2 mammalian isoforms of phospholipase D (PLD), PLD1 and PLD2, have been characterized by molecular cloning and biochemical analysis (Hammond et al, 1997; Kodaki & Yamashita, 1997; Min et al, 1998b). Information concerning cell specific expression of PLD can provide potential clues to the functional significance of PLD isoforms.

The endocrine pancreas (the islet of Langerhans) shows a characteristic cell-type organization. In the rodents, the islets are organized as a core of insulin-producing B cells, surrounded by the three other non-B cells; A, D, and PP cells, which secrete glucagon, somatostatin, and pancreatic polypeptide, respectively (Orci & Unger, 1975). Since the early 1990s, many studies have been focused on the functional role of PLD in the islet of Langerhans. In the presence of a carbohydrate, activation of PLD is mediated by protein kinase C in the isolated rat islets (Dunlop & Metz, 1992). In addition to the islets, mouse-derived insulinoma and glucagonoma cell lines have been shown to secrete glycosylphosphatidylinositol-specific phospholipase D (GPI-PLD) (Metz et al, 1991). A recent study by Chen et al, (2000) demonstrated the involvement of PLD induction in a early stimulatory effect of IL-1β on islet insulin release.

Recently, we reported the site-specific distribution of PLD isoforms, PLD1 and PLD2, in the rat pancreas (Ryu et al, 2003). In the study, both isoforms were abundantly present in the islets compared to acinar cells. Interestingly, PLD2 immunoreactivity was exclusively localized in A and PP cells, while PLD1 was evenly distributed throughout the islets. Therefore, the present study was undertaken to comparatively analyze distribution pattern of PLD2 in the islets of rat and guinea pig.

Corresponding to: Yang-Hyeok Jo, Department of Physiology, College of Medicine, The Catholic University of Korea, 505 Banpo-dong, Seocho-gu, Seoul 137-701, Korea. (Tel) 82-2-590-1170, (Fax) 82-2-532-9575, (E-mail) yhjo@catholic.ac.kr

METHODS

Experimental animals

Fifteen Sprague-Dawley male rats (*b.w.* 200~250 g) were obtained from Daehan Biolink Company (Eumsung, Chungbuk, Korea). Ten rats were used for Western blot analysis, and the rest for immunohistochemistry. Six Dunkan-Hartley male guinea pigs (*b.w.* 300 g, Harlan company, Indianapolis, IN, USA) were used for immunohistochemistry. Both 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 were consistent with 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

ABBREVIATIONS: PLD2, Phospholipase D2; PC, Phosphatidylcholine; DAG, diacylglycerol; LPA, lysophosphatidic acid.

(PMSF), Tween 20, bovine serum albumin (BSA, fraction V), paraformaldehyde, and 3,3'-diaminobenzidine tetrahydrochloride (DAB) were purchased from Sigma (St. Louis, MO, USA). All other chemicals were obtained from various commercial sources.

Preparation of affinity purified anti-PLD2 antibody

Antiserum was raised against the N-terminal peptide of PLD2 corresponding to amino acid residues 1-19 of the human PLD2 sequence: MTATPESLFPTGDELDSSQ. For affinity purification of the antibody, the 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.

Immunoprecipitation and Western blot analysis

Immunoprecipitation and Western blot analysis were performed according to the procedures described previously (Lee et al, 2000; Min et al, 2000). The pancreas was 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 the lysate supernatant was then precleared with preimmune IgG and protein A sepharose for 30 min. Subsequently, precleared cell lysates were incubated for 4 h with anti-PLD2 antibody, and 30 μl of a 50% slurry of protein A sepharose. 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 Na_3VO_4, 10% glycerol, 1% Nonidet P-40), followed by the addition of SDS-sample buffer and boiling. The recovered protein was resolved on an 8% SDS-PAGE gel, transferred to a nitrocellulose membrane and blocked in 5% solution of skim milk powder. The blot was probed with anti-PLD2 antibody, which was diluted in blocking solution (1 μg/ml). Immunoreactive bands were visualized using horseradish peroxidase-conjugated goat anti-rabbit IgG and enhanced chemiluminescence (ECL, Amersham Pharmacia Biotech, Bucks, UK). Specificity of the immune reaction was verified by the use of anti-PLD2 antibody (2.5 μg) that had been preadsorbed overnight at 4°C with its specific immunopeptide (2.5 μg or 25 μg).

Immunohistochemistry

The pancreas was removed 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 (Hsu et al, 1981). Before labeling, tissue sections were processed through routine procedures. After blocking with normal goat serum, the sections were incubated overnight at 4°C with anti-PLD2 antibody (1 : 300) diluted in PBS (10 mM phosphate buffer; 105 mM NaCl, pH 7.4). The secondary biotinylated goat anti-rabbit antibody and ABC solution (Vector Laboratories, Burlingame, CA, USA) were applied to these sections for 1 h at room temperature. Thereafter, the immunocytochemical reaction was carried out using DAB solution. The sections were then counterstained with hematoxylin and mounted. To assure consistency in the immunohistochemical findings, at least three identical experiments were conducted. Control sections were processed as described above, except that the primary antibody was preadsorbed with its antigen.

To illustrate the relation of PLD2 immunoreactive cells to the islet endocrine cells, 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), anti-somatostatin (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 Axiophot photomicroscope (Carl Zeiss, Oberkochen, West Germany).

To reveal the relation of PP and PLD2 in the guinea pig, double immunostaining using DAB/DAB-Nickel substrates was performed as previously described (Min et al, 1998a), because the sheep anti-PP antibody, used in the rat, could not react in the guinea pig tissue. Instead, rabbit anti-PP antibody was employed to unravel the PP cells. Also, this rabbit anti-PP antibody was confirmed to be able to detect the identical PP cells that sheep anti-PP antibody detected in the rat tissue. After immunostaining for PLD2 as described above, the tissue sections were treated for 1 min with vigorous shaking in a solution composed of 0.25% $KMnO_4$ (1 vol.), 5% H_2SO_4 (1 vol.) and distilled water (140 vol.) to elute tissue-bound PLD2 antibody as well as the linked antibody. The sections were then immunostained with rabbit anti-PP antibody. The immune reaction was developed by DAB-Nickel substrate which resulted in a dark brown color product, easily discriminated from the light brown coloration of the DAB substrate alone.

Antibodies were obtained from the following sources: mouse monoclonal anti-insulin antibody, mouse monoclonal anti-glucagon antibody, goat polyclonal anti-somatostatin antibody, sheep polyclonal anti-PP antibody, and rabbit polyclonal anti-PP antibody were purchased from BioGenex (San Ramon, CA, USA), Sigma, Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA), Serotec (Oxford, UK), and Chemicon International Inc. (Temecula, CA, USA), respectively. FITC and Texas Red-conjugated secondary antibodies were purchased from Jackson ImmunnoResearch Laboratories, Inc. (West Grove, PA, USA).

RESULTS

PLD2 immunoreactivity: specificity of staining

The immunoreactive band of PLD2, approximately 105 kDa, was specifically recognized in whole rat pancreas as well as in the control rat brain tissue. Recognition of the protein by anti-PLD2 antibody was specifically blocked, when the blot was incubated with anti-PLD2 antibody that

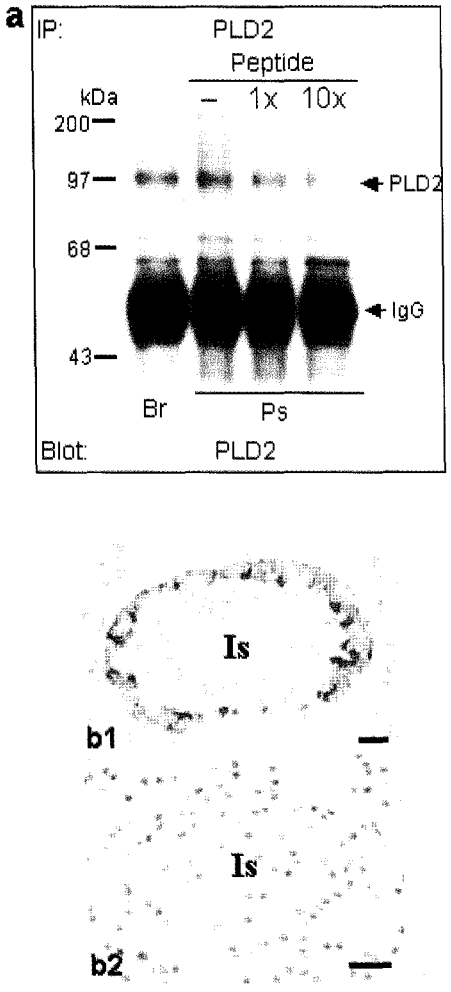

Fig. 1. Specificity of PLD2 immunoreactivity. (a) Extracts of rat brain and pancreas were prepared, the lysates (500 μg) were immunoprecipitated using anti-PLD2 antibody (2.5 μg) which had been preincubated in the absence (−) or presence (1X or 10X) of its specific immunopeptide (2.5 μg or 25 μg), and then immunoblotted with anti-PLD2 antibody. Br, brain; Ps, whole pancreatic tissue. (b1) The immunostaining of PLD2 is easily observed, especially in the peripheral portion of the islet (Is). (b2) Controls, in which the primary antibody was preadsorbed with antigen, gave negative staining. The experiment was performed at least three times with similar results: one representative example is shown. Fig. 1a on top panel was previously published (Ryu et al, 2003). Scale bars: 20 μm.

had been preadsorbed with an increased antigen concentration (Fig. 1a), indicating the specificity of PLD2 immunoreactivity on Western blot.

Immunocytochemical staining of the rat pancreas sections with anti-PLD2 antibody showed a specific pattern of PLD2 distribution. Cells at the islet periphery exhibited strong immunoreactivity, compared with the weak staining in the islet center (Fig. 1b1). However, the sections incubated with anti-PLD2 antibody that had been preadsorbed with its antigen showed negative immunostaining, indicating high specificity of PLD2 immunoreactivity (Fig. 1b2).

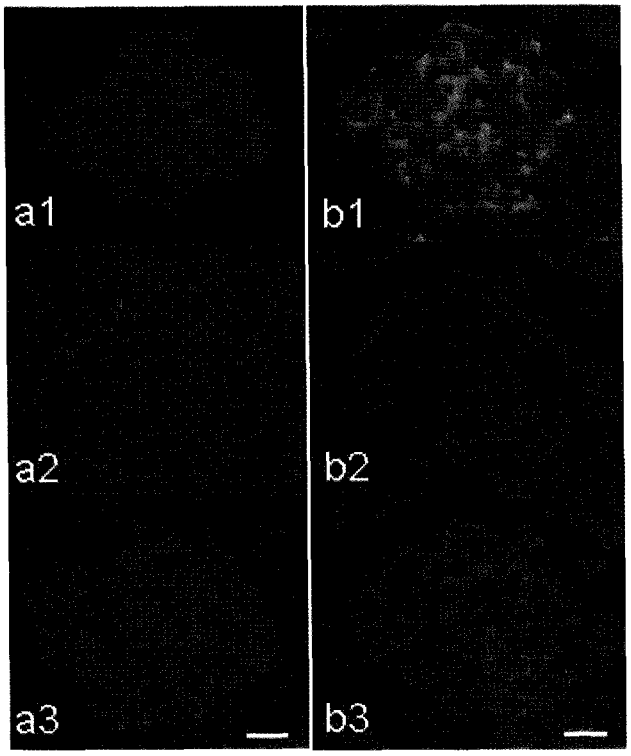

Fig. 2. Immunofluorescent colocalization of PLD2 with insulin in rat (a1-a3) and guinea pig (b1-b3) pancreatic islet. (a1, b1) Insulin (FITC) immunoreactivity is mainly detected in the central portion of the islet. (a2, b2) Meanwhile, PLD2 (Texas Red) immunoreactivity is highly expressed in the peripheral portion of the islet. (a3, b3) Colocalization of PLD2 and insulin is hardly observed in the same cells. The experiment was performed at least three times with similar results: one representative example is shown. Figs 2a1-a3 on left panel were previously published (Ryu et al, 2003), however, for comparison with guinea pig, we again presented them. Scale bars: 20 μm.

Double staining

Based on the localization pattern of endocrine cells, distribution of the cells strongly stained by anti-PLD2 antibody in the islet periphery suggested that they were not insulin-producing B cells. To confirm this assumption, double immunostainings with anti-PLD2 and each hormone-directed antibody were conducted. 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. 2, in both rat and guinea pig, there was no detectable colocalization of insulin and PLD2 within the same cell. Interestingly, distribution of glucagon-immunoreactive cells coincided with that of PLD2-immunoreactive cells (Fig. 3). Since red and green colors combine to produce a yellow color under dual exposure of the section, cells appearing 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 a coincident pattern (Fig. 4). PLD2 immunoreactivity was easily observed in PP cells of the rat pancreas, whereas never detected

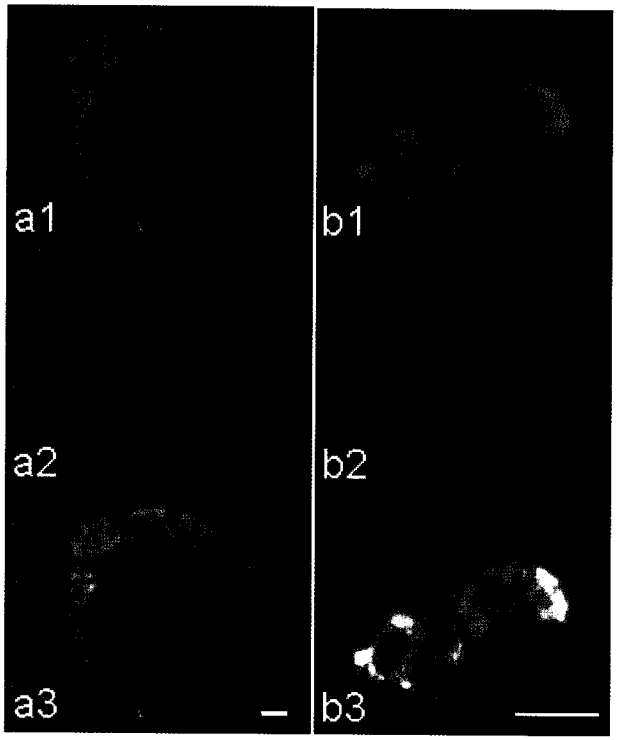

Fig. 3. Immunofluorescent colocalization of PLD2 with glucagon in rat (a1-a3) and guinea pig (b1-b3) pancreatic islet. Colocalization of glucagon (FITC, a1, b1) and PLD2 (Texas Red, a2, b2) is observed in the same cells of the islet, demonstrated by yellow color (a3, b3). The experiment was performed at least three times with similar results: one representative example is shown. Figs 3a1-a3 on left panel were previously published (Ryu et al, 2003), however, for comparison with guinea pig, we again presented them. Scale bars: 20 μm.

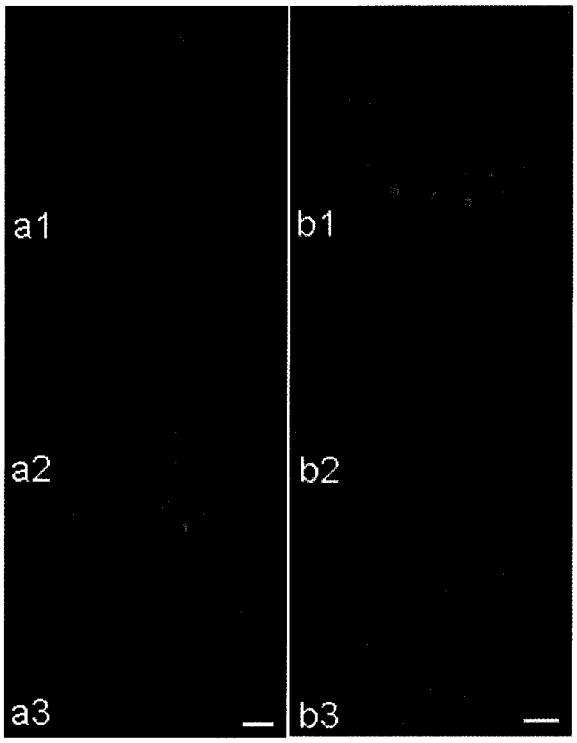

Fig. 4. Immunofluorescent colocalization of PLD2 with somatostatin in rat (a1-a3) and guinea pig (b1-b3) pancreatic islet. Somatostatin (FITC, a1, b1) immunoreactive cells are localized in the islet periphery, similar to the pattern of PLD2 (Texas Red, a2, b2). However, somatostatin and PLD2 are hardly colocalized in the same cells after simultaneous dual exposure (a3, b3). The experiment was performed at least three times with similar results: one representative example is shown. Figs 4a1-a3 on left panel were previously published (Ryu et al, 2003), however, to compare with guinea pig, we again presented them. Scale bars: 20 μm.

in PP cells of the guinea pig pancreas (Fig. 5). These findings indicate that the colocalization pattern of PLD2 and PP varies depending on species.

DISCUSSION

PLD plays an important role in the membrane lipid-mediated signal transduction of many cell types. PLD catalyzes the hydrolysis of phosphatidylcholine (PC) to choline and phosphatidic acid (PA) (Exton, 1994), and PA has the ability to act directly as a signaling molecule and also to be converted into other signaling molecules such as diacylglycerol (DAG) and lysophosphatidic acid (LPA). DAG, which is formed from PA by phosphatidic acid phosphatase, regulates certain protein kinase C isozymes. LPA, produced from PA through the action of phospholipase A_2, is now recognized as an important extracellular signaling molecule (Exton, 1997). Although the precise role of PLD is not fully understood, receptor-mediated PLD activation has been implicated in a number of physiological processes including cell proliferation (Boarder, 1994), differentiation (Min et al, 1999), cytoskeletal reorganization (Colley et al, 1997) and the control of protein trafficking and secretion (Cockcroft, 1996; Roth & Sternweis, 1997).

Recently, in an initial attempt to characterize the role of PLD in the rat pancreas, we examined the site-specific distribution of PLD1 and PLD2 (Ryu et al, 2003). Unlike even distribution of PLD1 throughout the islets, PLD2 was mainly enriched in A and PP cells which secrete glucagon and pancreatic polypeptide, respectively. This PLD2 distribution pattern is in good agreement with the observations made recently by Lainé et al, (2000). In the present study, we confirmed this PLD2 localization pattern in the guinea pig pancreatic islets. Interestingly, PLD2 was exclusively enriched in A cells, similar to that of rat, and not in PP cells.

The results presented above do not reveal the functional role of PLD2 in A and PP cells. However, the association of PLD with hormone secretion including insulin has been reported. In the rat pancreatic islets, endogenous PA generated by PLD activation promotes insulin release (Metz & Dunlop, 1990), and glucose stimulates both GPI-PLD and insulin secretion from TC3 cells, a mouse insulinoma cell line (Deeg & Verchere, 1997). Furthermore, in immortalized gonadotropin-releasing hormone (GnRH) neurons, PLD serves as a common intracellular effector for PLC- and voltage-gated signaling pathways, and also participates in GnRH secretion (Zheng et al, 1997). In glucagonoma cell line, PLD is involved in bradykinin- induced glucagon release (Yibchok-anun et al, 2002). From the above observations, it is highly likely that our results provide a clue

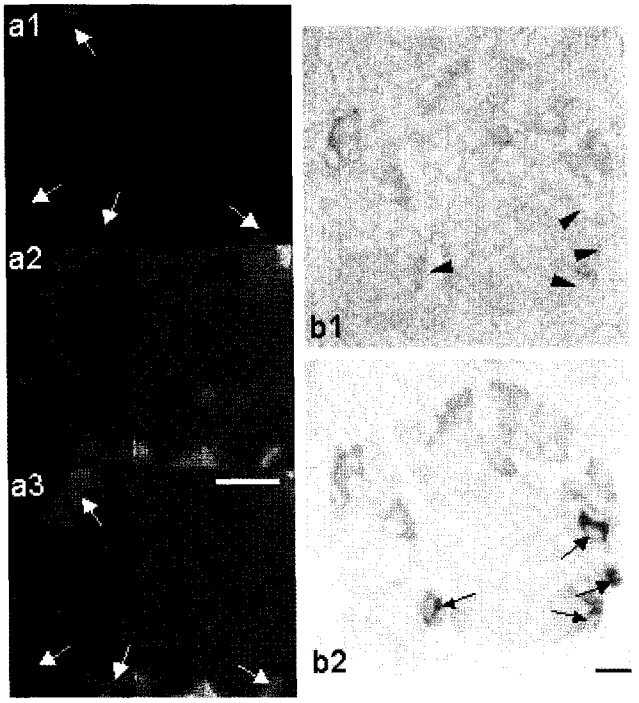

Fig. 5. Immunofluorescent colocalization of PLD2 with pancreatic polypeptide in rat (a1-a3) and guinea pig (b1, b2) pancreatic islet. In the rat, colocalization of pancreatic polypeptide (FITC, a1) and PLD2 (Texas Red, a2) is observed in the same cells of the islet periphery, demonstrated by yellow color (a3). On the other hand, in the guinea pig, PLD2 immunoreactivity (light brown, b1) was never detected in the same cells (arrowheads in b1), in which pancreatic polypeptide immunoreactivity (dark brown, arrows in b2) was observed. The experiment was performed at least three times with similar results: one representative example is shown. Figs 5a1-a3 on left panel has been published in our previous study (Ryu et al, 2003), however, to compare with guinea pig, we again presented them. Scale bars: 20 μm.

to understand the role of PLD2 in glucagon secretion.

In conclusion, we comparatively analyzed PLD2 localization between rat and guinea pig pancreatic islets, and these findings suggest that PLD2 plays a regulatory role in the secretion of glucagon and/ or pancreatic polypeptide, depending on species.

ACKNOWLEDGMENT

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

REFERENCES

Boarder MR. A role of phospholipase D in control of mitogenesis. *Trends Pharmacol Sci* 15: 57−62, 1994

Chen M-C, Paez-Espinosa V, Welsh N, Eizirik DL. Interleukin-1 β regulates phospholipase D-1 expression in rat pancreatic β-cells. *Endocrinology* 141: 2822−2828, 2000

Cockcroft S. ARF-regulated phospholipase D: a potential role in membrane traffic. *Chem Phys Lipids* 80: 59−80, 1996

Colley WC, Sung TC, Roll R, Jenco J, Hammond SM, Altshuller Y, Bar-Sagi D, Morris AJ, Frohman MA. Phospholipase D2, a distinct phospholipase D isoform with novel regulatory properties that provokes cytoskeletal reorganization. *Curr Biol* 7: 191−201, 1997

Deeg MA, Verchere B. Regulation of glycosylphosphatidylinositol-specific phospholipase D secretion from βTC3 cells. *Endocrinology* 138: 819−826, 1997

Dunlop M, Metz SA. Activation of phospholipase D by glyceraldehydes in isolated islet cells follows protein kinase C activation. *Endocrinology* 130: 93−101, 1992

Exton JH. Phosphatidylcholine breakdown and signal transduction. *Biochim Biophys Acta* 1212: 26−42, 1994

Exton JH. Phospholipase D: enzymology, mechanisms of regulation, and function. *Physiol Rev* 77: 303−320, 1997

Hammond SM, Jenco JM, Nakashima S, Cadwallader K, Gu Q, Cook S, Nozawa Y, Prestwich GD, Frohman MA, Morris AJ. Characterization of two alternately spliced forms of phospholipase D1. *J Biol Chem* 272: 3860−3868, 1997

Hsu S, Raine L, Fanger H. The use of avidin-biotin-peroxidase complex (ABC) in immunoperoxidase techniques: a comparison between ABC and unlabelled antibody (PAP) procedures. *J Histochem Cytochem* 29: 577−580, 1981

Kodaki T, Yamashita S. Cloning, expression, and characterization of a novel phospholipase D complementary DNA from rat brain. *J Biol Chem* 272: 11408−11413, 1997

Lainé J, Bourgoin S, Bourassa J, Morisset J. Subcellular distribution and characterization of rat pancreatic phospholipase D isoforms. *Pancreas* 20: 323−326, 2000

Lee MY, Kim SY, Min DS, Choi YS, Shin SL, Chun MH, Lee SB, Kim M-S, Jo Y-H. Upregulation of phospholipase D in astrocytes in response to transient forebrain ischemia. *Glia* 30: 311−317, 2000

Metz SA, Dunlop M. Stimulation of insulin release by phospholipase D. *Biochem J* 270: 427−435, 1990

Metz CN, Zhang Y, Guo Y, Tsang TC, Kochan JP, Altszuler N, Davitz MA. Production of the glycosylphosphatidylinositol-specific phospholipase D by the islets of Langerhans. *J Biol Chem* 266: 17733−17736, 1991

Min B-H, Jeong S-Y, Kang S-W, Crabo BG, Foster DN, Chun B-G, Bendayan M, Park I-S. Transient expression of clusterin (sulfated glycoprotein-2) during development of rat pancreas. *J Endocrinol* 158: 43−52, 1998a

Min DS, Park SK, Exton JH. Characterization of a rat brain phospholipase D isozyme. *J Biol Chem* 272: 7044−7051, 1998b

Min DS, Shin KS, Kim E-G, Kim SR, Yoon SH, Kim M-S, Jo Y-H. Down-regulation of phospholipase D during differentiation of mouse F9 teratocarcinoma cells. *FEBS Lett* 454: 197−200, 1999

Min DS, Cho NJ, Yoon S-H, Lee YH, Hahn SJ, Lee KH, Kim M-S, Jo Y-H. Phsopholipase C, protein kinase C, Ca^{2+}/calmodulin-dependent protein kinase II, and tyrosine phosphorylation are involved in carbachol-induced phospholipase D activation in Chinese hamster ovary cells expressing muscarinic acetylcholine receptor of *Caenorhabditis elegans*. *J Neurochem* 75: 274−281, 2000

Orci L, Unger R. Functional subdivision of islets of Langerhans and possible role of D-cells. *Lancet* 2: 1243−1244, 1975

Roth MG, Sternweis PC. The role of lipid signaling in constitutive membrane traffic. *Curr Opin Cell Biol* 9: 519−526, 1997

Ryu GR, Kim M-J, Song C-H, Sim SS, Min DS, Rhie D-J, Yoon SH, Hahn SJ, Kim M-S, Jo Y-H. Site-specific distribution of phospholipase D isoforms in the rat pancreas. *Acta Histochem Cytochem* 36: 51−60, 2003

Yibchok-anun, S, Cheng, H, Abu-Basha, E. A, Ding, J, Ioudina, M. and Hsu, W. H. Mechanisms of bradykinin-induced glucagon release in clonal α-cells In-R1-G9: involvement of Ca^{2+}-dependent and independent pathways. *Mol Cell Endocrinol* 192; 27−36, 2002

Zheng L, Krsmanovic LZ, Vergara LA, Catt KJ, Stojilkovic SS. Dependence of intracellular signaling and neurosecretion on phospholipase D activation in immortalized gonadotropin-releasing hormone neurons. *Proc Natl Acad Sci USA* 94: 1573−1578, 1997