Phospholipase D1 Regulates Secretagogue-stimulated Insulin Release in Pancreatic β-Cells

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Phospholipase D (PLD) has been strongly implicated in the regulation of Golgi trafficking as well as endocytosis and exocytosis. Our aim was to investigate the role of PLD in regulating the biphasic exocytosis of insulin from pancreatic β-cells that is essential for mammalian glucose homeostasis. We observed that PLD activity in MIN6 pancreatic β-cells is closely coupled to secretion. Cellular PLD activity was increased in response to a variety of secretagogues including the nutrient glucose and the cholinergic receptor agonist carbachol. Conversely, pharmacological or hormonal inhibition of stimulated secretion reduced PLD activity. Most importantly, blockade of PLD-catalyzed phosphatidic acid formation using butan-1-ol inhibited insulin secretion in both MIN6 cells and isolated pancreatic islets. It was further established that PLD activity was required for both the first and the second phase of glucose-stimulated insulin release, suggesting a role in the very distal steps of exocytosis, beyond granule recruitment into a readily releasable pool. Visualization of granules using green fluorescent protein-phogrin confirmed a requirement for PLD prior to granule fusion with the plasma membrane. PLD1 was shown to be the predominant isoform in MIN6 cells, and it was located at least partially on insulin granules. Overexpression of wild-type or a dominant negative catalytically inactive mutant of PLD1 augmented or inhibited secretagogue-stimulated secretion, respectively. The results suggest that phosphatidic acid formation on the granule membrane by PLD1 is essential for the regulated secretion of insulin from pancreatic β-cells.

The ubiquitously expressed enzyme phospholipase D (PLD) catalyzes the hydrolysis of the major membrane phospholipid phosphatidylcholine to produce metabolically active phosphatidic acid (PtdOH). The cellular activity of the enzyme in mammalian cells is increased by a variety of growth factors, cytokines, or hormones (1), and it is now clear from extensive characterization that the two mammalian PLD species, PLD1 and PLD2, and their splice variants are subject to numerous regulatory inputs. PLD1 and PLD2 have an almost absolute requirement for phosphatidylinositol 4,5-bisphosphate, and the activity of PLD1, in particular, is also subject to synergistic regulation by small GTPases, including ADP-ribosylation factor and Rho, and also classical protein kinase C (PKC) isoforms such as PKCa and β (2–5). PLDs are membrane-bound and have been shown by various methodologies to occupy a variety of cellular locations dependent on cell type. PLD1 isoforms in general appear to be located in the Golgi apparatus and on intracellular vesicles; PLD2 isoforms are found predominantly on the plasma membrane (2, 4). Consistent with these diverse locations and inputs, PLD exerts multiple cellular functions in different cell types; it has been implicated in numerous signal transduction cascades, in modulating vesicle trafficking, and also in regulating cytoskeletal organization (2).

A role for PLD in modulating vesicular trafficking is particularly compelling because its cellular location and regulation are consistent with such a role, as is its control of cytoskeletal reorganization, which often accompanies trafficking events (6, 7). Moreover, the PLD activators phosphatidylinositol 4,5-bisphosphate (7), ADP-ribosylation factor (8), Rho (9), and classical PKCs (10) have all been implicated in the control of trafficking and/or exocytosis. PLD itself has been attributed roles in vesicular trafficking within the Golgi (11–13) and in the process of endocytosis (14–16). PLD has also been associated with exocytosis; PLD activity correlates with and, in some instances appears necessary for, secretion from neuroendocrine cells (17, 18), mast cells (19–21), adipocytes (22), rat parotid acinar cells (23), human bronchial epithelial cells (24), and neutrophils (25).

Exocytosis of insulin from the β-cells of the pancreatic islets of Langerhans controls glucose homeostasis in mammals. The processes that are involved in this stimulus-secretion coupling and that maintain exquisite control of biphasic insulin release are incompletely understood. Metabolism of glucose results in a resultant rise in the cytoplasmic free Ca2+ concentration that is both necessary and sufficient for triggering an initial phase of insulin release that is mediated by fusion of preocked insulin granules with the plasma membrane. A second, long lasting phase of insulin secretion, involving recruitment of a reserve pool of granules, requires Ca2+ as well as a poorly defined signal generated from mitochondrial metabolism of glucose (27–29). Insulin secretion can also be regulated via inputs from hormones and neurotransmitters (30) through classical signaling cascades involving transmembrane receptors, heterotrimERIC G-proteins, and second messengers such as diacylglycerol and cyclic AMP.

* This work was supported by funding from the National Health and Medical Research Council (Australia). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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‡ The abbreviations used are: PLD, phospholipase D; PtdOH, phosphatidic acid; KRB, Krebs-Ringer bicarbonate buffer; RIA, radioimmunoassay; PBS, phosphate-buffered saline; PKC, protein kinase C; carbachol, carbachol; GFP, green fluorescent protein; TRITC, tetramethylrhodamine isothiocyanate; WT, wild type.

This paper is available online at http://www.jbc.org
PLD1 in Insulin Secretion

The molecular mechanisms underlying the translocation, priming, docking, and fusion of insulin secretory vesicles are poorly understood. Because of the evidence of a role for PLD in other examples of exocytosis, we sought here to investigate the role of this enzyme in stimulated insulin secretion from pancreatic β-cells. Work in the late 1980s identified an agonist-activated PLD-like activity in pancreatic β-cells (31), and further characterization established that PLD activation was an event downstream of PKC activation following addition of se-cretagogue (32). In other studies, PLD activity has been indirectly implicated in both receptor- and PKC-mediated insulin secretion from mouse pancreatic β-cells (33). Interestingly, insulin secretion from β-cells had also been shown to be promoted by the addition of exogenous PLD (34). However, all of these early studies focused on PLD and PtdOH as signaling intermediates rather than addressing their role in exocytosis. More recently, PLD1 cDNA expression was seen to increase in intermediates rather than addressing their role in exocytosis. Therefore, we have undertaken biochemical and cell biological analyses to fully assess the role of PLD genes in regulating insulin secretion in pancreatic β-cells and define for the first time a requirement for PLD activity during insulin granule exocytosis.

EXPERIMENTAL PROCEDURES

Materials—All of the chemicals were obtained from Sigma-Aldrich except as detailed below. Culture plasticware was obtained from Bec-ton-Dickinson (Franklin Lakes, NJ). Cell media and transfection reagents were from Invitrogen, except fetal calf serum was from Thermo-Trace (Noble Park, Victoria Australia) and bovine serum albumin was from ICN Biomedicals (Aurora, OH). Kits for radioimmunoassay (RIA) of rat insulin were obtained from Linco Research (St. Louis, MO), and radiolabeled myristic acid (19,20-“H(N)/myristic acid) and En”Hance spray were from PerkinElmer Life Sciences. Chromatography plates were obtained from Whatman (Maidstone, UK); liquid scintillant and UltimaGold were from Packard Biosciences (Meriden, CT); autoradiography film (BioMaxMR) was from Kodak; and Fuji Super RX was from FujiFilm (Tokyo, Japan). The antibodies for Western blot or microscopic analysis were anti-PLD2, anti-PLD1, and anti-insulin (P6618, P5743, and L2018, respectively) from Sigma-Aldrich; anti-GFP (AB18901) from Chemicon International (Temecula, CA); anti-PLD (sc1784, sc17848) from Santa Cruz Biotechnology (Santa Cruz, CA); anti-PLD1 (3832) from Cell Signaling Technology (Beverly, MA); and anti-PLD (65-608) from Upstate Cell Signaling Solutions (Waltham, MA). Antibodies were labeled with appropriate Cy2-, Cy3-, Cy5-, or horse rabbit peroxidase-conjugated anti-mouse, -goat, and -rabbit antibo-dies from Jackson ImmunoResearch (West Grove, PA). DNA was visualized for microscopy using ToPro3 from Molecular Probes (Eugene, OR) and actin with fluorescein isothiocyanate- or TRITC-conjugated phalloidin from Sigma-Aldrich. The coverslips were mounted in Immuno-fluore. The slides were examined using a Leica laser scanning confocal microscopes (Leica Microsystems, Wetzlar, Germany), DM IRE2 TCS SP2 AOBS with HCX PL APO 63/1.4—0.8 oil immersion objectives and an argon laser providing 488-nm excitation lines, a HeNe laser providing 543-nm excitation lines, and a HeNe laser providing 633-nm excitation lines. The fluorochromes and fluoroproteins, Cy2, fluorescein isothiocyanate, Cy3, TRITC, Cy5, ToPro3, and GFP were excited with the appropriate excitation wavelengths, and individual channels were scanned in se ries. Each image represents a single 0.8-μm Z optical section. The images were processed using Adobe Photoshop version 7 (San José, CA) for an Apple computer.

Cellular Fractionation—Eight 15-cm dishes of MIN6 cells were washed in cold (4 °C) PBS and resuspended in cold (4 °C) homogeniza tion buffer (70 mM sucrose, 10 mM HEPES-KOH, pH 7.2, 1 mM EDTA, 200 μg/ml leupeptin, 2 mM benzamidine, and 2 mM phenylmethylsulfonyl fluoride) before disruption by Dounce homogenization. Post-nuclear supernatants were prepared by centrifugation in a Beckman JA-6 cen trifuge at 3200 rpm for 10 min at 4 °C. This was separated through a 14-ml equilibrium sucrose step gradient (1 ml; 20–0.6 ml in 0.2 ml steps sucrose, 10 ml HEPES-KOH, pH 7.2, 1 mM EDTA). The fractions were then processed for Western blot analysis, insulin RIA, or phospholipid assay, and then used for extraction using chloroform:methanol:aqueous (8:4:3 by volume) and TLC as described above.

Western Blot Analysis—The extracts were resuspended in urea sample buffer (8% urea, 1% SDS, 100 mM Tris (unbuffered), 150 mM NaCl, 50 mM EDTA, 1% β-mercaptoethanol) before separation overnight by SDS-PAGE. The proteins were transferred “wet” to nitrocellulose mem branes and incubated in Dulbecco’s Modified Eagle’s medium containing 25 mM glucose, 10 mM HEPES, 10% (v/v) fetal calf serum, 50 IU/ml penicillin, and 50 μg/ml streptomycin in 24- and 6-well plates or 15-cm dishes. Before experimentation the cells, at a density between 2.5 × 10^6/ml and 5.0 × 10^6/ml, were switched to medium containing low glucose (6 m M) for 48 h followed by modified Krebs-Ringer bicarbonate buffer (KRB) containing 5 mM NaHCO3, 1 mM CaCl2, 0.5% bovine serum albumin (w/v), 10 mM HEPES (pH 7.4), 2.8 mM glucose for 30 min.

Inulin Secretion Assays—Batches of 15 isolated islets or cells grown in 24-well dishes were washed into KRB and then incubated in 0.5 ml (or 1 ml for islets) of prewarmed KRB containing additions as indicated for 1 h at 37 °C. An aliquot of KRB was then removed and diluted appropriately for analysis of insulin content by RIA. For perfusion MIN6 cells grown on 4.5-cm glass coverslips were washed with KRB and then placed in a POC (perfusion, open or closed) perfusion chamber, in a heated microscope stage (both supplied by PeCon, Erbach-Bach, Germany) at 37 °C. Prewarmed KRB was pumped using a peristaltic pump at 0.5 ml/min for 15 min before experimentation to allow the cells to equilibrate. The fractions (0.5 ml) were collected using a fraction collector for analysis of insulin content by RIA.

PLD Assay—PLD activity was assessed using the transphosphadit-
tagged PLD1 cDNAs were subcloned into pShuttle-cytomegalovirus, and the PLD1 cDNAs and cytomegalovirus promoter were transferred into the adenovirus genome by homologous recombination in an adenovirus-packaging cell line. The adenoviruses were purified using cesium chloride ultracentrifugation and titration with Adeno-X Rapid Titer kit (according to the manufacturer’s instructions). MIN6 cells were infected in low glucose medium overnight with virus at 50 plaque forming units/cell before transferring to fresh medium for insulin secretion or PLD assay.

Data Presentation—All data are presented as the means ± S.E., unless otherwise indicated. Statistical significance was by analysis of variance.

RESULTS

Cellular PLD Activity Increases on Stimulation of Secretion in Pancreatic β-Cells—To establish whether PLD plays a regulatory role in the release of insulin from pancreatic β-cells, we sought to assess PLD activity under a variety of conditions known to promote insulin secretion from a pancreatic β-cell line, MIN6, which has a well documented response to metabolic and hormonal secretory stimuli (36, 42). Under basal conditions (2.8 mM glucose) MIN6 cells exhibited low levels of insulin secretion (~0.85 ng/ml/min/10^6 cells). When treated with 25 mM glucose, a depolarizing concentration of KCl (30 mM) or with the muscarinic cholinergerg agent, carbachol, insulin secretion was 5–7-fold higher (Fig. 1A). Secretion can also be augmented by combinations of treatments such that combinations with carbachol elicited responses greater than 10-fold above basal (Fig. 1A). Cellular PLD activity was assessed under these conditions by measuring changes in the levels of radiolabeled phospholipids. The changes in PtdOH (data not shown) suggested significant increases in PLD activity in response to the secretory stimuli. However, cellular PLD activity cannot be accurately assessed by measuring PtdOH levels because this phospholipid is rapidly metabolized and has numerous sources alternate to PLD-mediated hydrolysis. Cellular PLD activity is optimally quantified by taking advantage of the unique ability of PLD to “transphosphatidylate” primary alcohols (38). In the presence of a primary alcohol (such as ethanol or butan-1-ol), PLD hydrolysis of phosphatidylcholine predominantly results in the production of metabolically stable phosphatidyalcohol, measurement of which accurately reflects cellular PLD activity. In the presence of ethanol (not shown) or butan-1-ol, the secretory stimuli glucose, carbachol, and KCl increased production of phosphatidyalcohol 2–4-fold above basal, and combinations with carbachol induced PLD activity by more than 10-fold (Fig. 1B).

Inhibitors of Secretion Also Inhibit PLD Activity—The above data indicate that secretory stimuli can activate PLD in pancreatic β-cells. To further test this relationship, we assessed secretory competence and PLD activity under stimulatory conditions in the presence of diazoxide, which opens K⁺ channels to prevent gating of voltage-dependent Ca²⁺ channels (28), or clonidine, an α₂-adrenoreceptor agonist that inhibits secretion at a variety of levels (43). As expected, both inhibitors have profound effects on insulin secretion from MIN6 cells irrespective of the secretory stimulus (Fig. 2A). Under the same conditions cellular PLD activity was reduced, notably more than 50% in the presence of glucose and carbachol (Fig. 2B). These data confirm a close relationship between insulin secretion and PLD activity in pancreatic β-cells.

Phospholipase D1 Is Located on Vesicles in Pancreatic β-Cells—To further assess the potential role of PLD in insulin secretion, we first sought to identify PLD isoforms and their cellular location in pancreatic β-cells. MIN6 extracts were analyzed by Western blot for PLD using a variety of antisera generated to PLD proteins. The analysis revealed a single antigen of ~120 kDa corresponding to the predicted size of the PLD1 gene product (Fig. 3A). No antigen corresponding to PLD2 (~108 kDa) could be detected using antiserum against generic PLDs or against PLD2 specifically. However, PLD2 is expressed in RBL-2HT mast cells (44), and antigen corresponding to PLD2 could be detected in extracts prepared from these cells (Fig. 3A). This finding is consistent with our gene transcript analysis (37) and with a reverse transcriptase-PCR analysis of primary β-cells (35) where cDNA for PLD1 was detected but not PLD2.

We also assessed the intracellular distribution of PLD1 antigen by indirect immunofluorescence microscopy. Using various PLD1 antisera, generally a punctate, vesicle-like, distribution of antigen, largely excluded from the nucleus, was seen in MIN6 cells (Fig. 3B). This distribution was seen for endogenous PLD1 (Fig. 3B, top panels) and overexpressed PLD1 (Fig. 3B, middle panels). Using cells transiently transfected with GFP-PLD1, this vesicle-like distribution was also evident, and it was clear that the various antisera used accurately label PLD1 protein (not shown). Therefore, it is likely that the pattern seen microscopically represents the intracellular distribution of endogenous PLD1. In addition, using antisera to insulin it was clear that some, but by no means all, of the punctate structures identified with the PLD1 antisera also stained for insulin (Fig. 3B, bottom panels). A partial colocalization is perhaps not unexpected given that not all cells within the population would be expected to be secretion-competent. These data indicate that
PLD1 is likely to represent the predominant PLD species in pancreatic β-cells and that the protein is located on small intracellular vesicles, many of which also contain insulin.

Secretagoguestimulated PLD Activity Is Located on Insulin Granules—To confirm that vesicular PLD1 is responsible for secretagogue-activated PLD activity, we carried out Western blot and phospholipid analyses of fractions generated from sucrose gradient centrifugation of post-nuclear supernatants, rich in cellular vesicles, from MIN6 cells. Examination by Western blot identified that PLD1 antigen was concentrated in fractions 6–12 (Fig. 4). Fractionation and subsequent phospholipid analysis of postnuclear extracts from MIN6 cells labeled with [3H]myristic acid and treated with 0.4% butan-1-ol identified that the PLD-specific product, phosphatidylbutanol, was predominantly seen in fractions 8–12, overlapping significantly with PLD1 antigen (Fig. 4). The fractions were also assayed for insulin content by RIA identifying that insulin content peaked in fraction 8 and distributed in a manner overlapping with that of PLD1 and PLD products. These data show that fractions displaying PLD1 antigen also contain PLD-specific products. As has been seen before in different cell systems (45), this suggests that PLD1 may be the enzyme responsible for the increase in PLD products. These fractions also contain insulin consistent with our microscopic observations that PLD1-positive vesicles also contain insulin.

Inhibition of Phosphatidic Acid Production by Cellular PLD

Inhibits Secretion—We next sought to establish whether PLD activity was required for secretion by assessing stimulated insulin secretion under conditions where PLD activity is reduced. PtdOH is thought to be the “active” product of PLD hydrolysis, and because transphosphatidylation in the presence of primary alcohols reduces the production of PtdOH, primary alcohols can be used to “inhibit” PLD activity, whereas secondary alcohols (such as butan-2-ol) that are unable to be transphosphatidylated, serve as an ideal experimental control. We therefore measured stimulated insulin secretion from MIN6 cells in response to varying concentrations of both primary and secondary alcohols. Primary alcohol, ethanol (not shown), or butan-1-ol but not butan-2-ol profoundly inhibited glucose- and carbachol-stimulated insulin secretion and the augmented secretion seen with both stimuli used in combination. No significant effect was seen on basal secretion (Fig. 5A).

To further investigate this phenomenon and confirm these data, we also investigated the effect of alcohols on first and second phase secretion in response to glucose. MIN6 cells grown on large coverslips in a sealed chamber were perfused in buffer with or without glucose and butan-1-ol. The results (Fig. 5B) indicate that the dramatic increase in insulin release seen almost immediately on switching to high glucose buffer can be completely blocked in the presence of butan-1-ol. In addition, the second phase of insulin release commencing ~10 min after the switch to high glucose is also inhibited in the presence of butan-1-ol (Fig. 5B). Similar effects are seen with a combined glucose and carbachol stimulus that at 2 min post-stimulus produces a peak of 38.2 ng (±0.6 ng), whereas in the presence of butan-1-ol this peak is reduced to 4.6 ng (±3.9 ng).

We also investigated the effect of alcohols on the localization of insulin granules within individual cells by direct fluorescence microscopy. MIN6 cells were transiently transfected with DNA encoding GFP-phogrin, a membrane protein residing on
vesicular granules that has been used as a marker for insulin granules in MIN6 cells (39). In basal conditions, the majority of GFP seen in transfected cells is distributed in punctate vesicular structures excluded from the nucleus (Fig. 5C), the majority of which colocalize with insulin (data not shown). After secretagogue stimulation a significant redistribution of the GFP-phogrin to the plasma membrane occurs, consistent with fusion of insulin granules with the plasma membrane. In the presence of butan-1-ol, but not butan-2-ol, this redistribution is significantly reduced in the majority of transfected cells (Fig. 5C).

Finally, to validate the significance of these observations, we investigated the role of PLD in insulin secretion from other pancreatic β-cells. We tested INS1, a rat insulinoma β-cell line, and primary islets isolated from rat pancreata. Carbachol-stimulated insulin release from INS1 cells was blocked in the presence of primary alcohols but not secondary alcohols (data not shown) as was seen in MIN6 cells. Using isolated pancreatic islets primary alcohol also inhibited glucose-stimulated insulin secretion in a dose-dependent manner with a maximal inhibition of ~75% occurring at 0.65% (v/v) butan-1-ol (Fig. 5D). In contrast, the secondary alcohol had limited effect on secretion of insulin that was only statistically significant at the highest concentration (0.8%, v/v). Neither alcohol affected basal secretion (not shown).

Thus, by using primary alcohols in cell biological and biochemical analyses to reduce the production of PtdOH via PLD, it is clear that PLD activity is required for stimulated secretion of insulin from clonal pancreatic β-cells and those isolated from primary tissue. The analyses also indicate that the enzyme is required in both the initial and second phase of insulin release.

PLD1 Activity Is a Key Regulator of Insulin Secretion in Pancreatic β-Cells—To confirm that PLD1 is the key cellular PLD required for efficient secretagogue-stimulated insulin release, we investigated the effects on insulin secretion of overexpressing wild-type and catalytically inactive PLD1 proteins. To achieve this efficiently, we overexpressed protein by infecting MIN6 cells with adenoviral constructs engineered to express wild-type and a catalytically inactive PLD1 mutant at amino acid 898, an essential part of the catalytic domain (46), which when overexpressed has been demonstrated to confer dominant inhibitory effects on cellular PLD activity (47).

MIN6 cells were either uninfected or infected with control “empty” adenovirus or the constructs expressing wild-type or inactive PLD1. PLD expression detected by Western blot was unchanged in cells infected with control virus in contrast to the significant overexpression (~10-fold) seen with adenovirus encoding PLD1-WT or PLD-K898R mutant (Fig. 6A). MIN6 cells infected with PLD1-WT or PLD1-K898R virus showed no significant changes in PLD activity under basal, unstimulated conditions, whereas with a glucose and carbachol stimulus cells infected with PLD1-WT virus showed significant additional PLD activity (Fig. 6A). In contrast, the PLD-K898R virus-infected cells showed reduced PLD activity. We also assessed insulin secretion in virally infected cells. MIN6 cells infected with PLD1-WT or PLD1-K898R virus showed no changes in secretion under basal, unstimulated conditions (data not shown). However, as was seen with PLD activity, secretagogue-stimulated cells infected with PLD1-WT virus showed increased stimulated secretory activity (Fig. 6B), whereas the cells infected with the PLD1-K898R mutant exhibited reduced stimulated secretion (Fig. 6B). These data confirm that the activity of the PLD1 isoform is required for stimulated secretion from pancreatic β-cells.

DISCUSSION

Nutrient-stimulated insulin secretion differs from other examples of stimulus-secretion coupling in at least two important aspects. Firstly, the agonist (glucose) does not bind to a cell surface receptor but initiates signaling cascades as a result of its intracellular metabolism (26–29). Secondly, glucose-stimulated insulin secretion is characterized by two distinct temporal phases; there is an initial 5–10 min-spike followed by decline toward the base line and a second rising phase that is maintained for the duration of the stimulus (27–29, 48). The two phases are initiated by differing (although incompletely understood) upstream signaling pathways (28) and are characterized by recruitment of functionally distinct populations of secretory granules. It has been long hypothesized (49, 50), and now experimentally supported (51), that first phase secretion represents fusion of pre-docked secretory granules (a readily releasable pool), whereas second phase necessitates recruit-
ment of additional granules from a reserve pool. These processes are in a dynamic equilibrium because it has been shown that some predocked granules detach from the plasma membrane and retreat to the reserve pool (51).

Although several earlier studies have addressed the function of PLD in pancreatic \(\beta\)-cells, these predated an appreciation of the involvement of the enzyme in exocytosis and therefore focused predominantly on proximal signaling pathways (31, 34). Our study greatly extends this earlier work with a number of novel findings. First, we show that glucose actually stimulates PLD activity. Previous investigations had focused on receptor-binding agonists and pharmacological agents, although the nutrient glyceraldehyde was shown to stimulate PLD in glucose-insensitive (neonatal) \(\beta\)-cells (32). Second, PLD1 accounts for this activity because we found PLD2 to be poorly expressed in \(\beta\)-cells, if at all. Third, and most importantly, ablation of PLD activity also inhibited secretion in response to a variety of stimuli, including glucose. This was demonstrated both pharmacologically using butan-1-ol and genetically by overexpression of a dominant negative PLD1 construct. Fourth,
our work defines PLD as a component of the exocytotic pathway rather than of proximal signaling cascades. This is apparent both from the localization of PLD1 to secretory granules and the demonstration that the enzyme was required for first, as well as second, phase secretion. Because first phase secretion in response to glucose and indeed the full response to KCl, is mediated by preloaded vesicles (51), the requirement for PLD would appear to be distal to priming and docking processes and is probably at the level of fusion of granules with the plasma membrane. Such a conclusion would be in keeping with the physicochemical properties of the PLD product, PtdOH, which favors negative membrane curvature and could thereby facilitate fusion of closedly apposed membranes (52, 53). A fusogenic role of PLD is also consistent with earlier studies using other secretory cells, although it has not previously been suggested for β-cells.

Our results demonstrating a requirement for PLD in exocytosis confirm similar investigations using butanol or overexpression of catalytically inactive PLDs in mast cells (19), chromaffin cells (18), and neurons (17), and yet there do appear to be differences in PLD function between the various secretory cell types. In chromaffin cells and synaptosomes, in which a role in fusion is probably best supported, PLD1 is not present on secretory vesicles but rather the plasma membrane. The enzyme, at least when overexpressed, is located on secretory vesicles in mast cells (19), but its role is suggested to relate to granule translocation rather than fusion, which, in an added complexity, appears to be mediated by PLD2 on the plasma membrane. Our results place PLD1 on β-cell granules but implicate its function in fusion. There may also be a requirement in recruiting granules from the reserve pool, because butan-1-ol appeared to block granule translocation as evidenced using phorbin-GFP. However, this assay requires transfer of phorbin-GFP from secretory granules to the plasma membrane and therefore relies on complete fusion of the two membranes. Blockade of fusion, coupled to an ongoing cycling of secretory granules between the readily releasable and reserve pools, might also explain the apparent inhibition of translocation. In any event, the secretory granule membrane would appear to be an ideal location for PLD1 to exercise a key role in exocytosis. The fact that it did not appear to be expressed on all insulin-containing vesicles is not inconsistent with this view, because not all granules would be expected to be secretory-competent, although this could also be due to the limitations of the antisera used in this study. A similar partial overlap of expression has been reported in a study using RBL cells, in which it was suggested that presence of PLD1 was actually a critical determinant of secretory competence (19). However, it should also be mentioned that the earlier work investigating expression of PLD isoforms in secretory cells relied on overexpression of tagged PLD constructs for visualization. To our knowledge this is the first study showing the localization of endogenous (as opposed to overexpressed) PLD1 on dense core secretory vesicles. This was demonstrated using immunofluorescence of fixed cells, activity assays, and immunoblotting of cell fractions.

In summary, we have shown that PLD1 is intimately involved and required for regulating β-cell exocytosis in response to a variety of stimuli. This PLD isoform is present on a subset of insulin granules and appears to act at a very distal step in exocytosis, potentially the fusion of the granule and plasma membranes. An emerging theme from this and previous studies is that, although PLD activity appears essential for secretion, there are probably subtle differences in the way it functions. This is apparent from the observed variations in expression, location, and role of PLD1 and PLD2 in mast cells, β-cells, and neurons (17, 19, 54). These features probably relate to the specialized functions of these particular cell types and potentially contribute to the observed differences in their secretory kinetics.

Acknowledgments—GFP-phorbin was provided by Guy Rutter (Bristol, UK). Microscopic equipment at the Garvan Institute of Medical Research was acquired with the generous assistance of many individuals and corporations and in particular Pieter Hueneveld and Lady Mary Fairfax. We thank Roger Doly, Ross Laybutt, and Ellen van Dam for critical review of the manuscript and Angela Hughes for help with the artwork.

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