Regulated vesicular trafficking is essential for the transport of proteins and lipids between subcellular compartments and for the maintenance of organelles in eukaryotic cells. These organelles help to establish an ordered structure within which the complex biochemical reactions and signaling processes are executed. The molecular mechanism underlying vesicular trafficking from the endoplasmic reticulum (ER) to the cell surface through the Golgi apparatus has been investigated extensively. Malhotra and coworkers (1) have reported that the marine sponge metabolite ilimaquinone (IQ) inhibits protein trafficking events by reversibly breaking the Golgi apparatus into small vesicles. Several attempts were made to identify downstream molecules necessary for IQ-induced Golgi membrane vesiculation. Using a reconstitution assay system, protein kinase D (PKD) was identified as a key enzyme responsible for IQ-induced Golgi membrane vesiculation (2). However, the exact mechanism underlying IQ-induced Golgi apparatus breakdown, especially a relationship between PKD and lipid remodeling, remains unclear.

Membrane trafficking is energetically unfavorable and does not occur spontaneously in vivo, but occurs under strict control of specialized proteins. Evidence is accumulating that these proteins do not act alone but in concert with particular membrane lipids such as phosphoinositides (3), diacylglycerol (DAG) (4), and phosphatidic acid (PA) (5–7). Phospholipases hydrolyze phospholipids, the backbone of biological membranes. Phospholipase activity not only has a profound impact on the structure and stability of cellular membranes but also plays a pivotal role in regulating many critical cellular functions. Phospholipase D (PLD) generates PA, a multifunctional lipid that has been proposed variously to alter membrane curvature, to serve as a protein attachment site, to activate selected enzymes, or to represent the starting material for the production of additional signaling lipids, particularly in the context of membrane vesicle trafficking and cytoskeletal dynamics (8–12).

In this work, we found that IQ causes robust activation of PLD in various cell types. The purpose of this study was to clarify the roles of PLD-catalyzed formation of PA in IQ-induced Golgi apparatus fragmentation. We provide evidence that the generation of PA catalyzed by PLD and the subsequent conversion of PA to DAG catalyzed by PA phosphohydrolase are prerequisites for both PKD activation and IQ-induced Golgi membrane vesiculation.

EXPERIMENTAL PROCEDURES

Materials—Dulbecco’s modified Eagle’s medium and RPMI 1640 medium were purchased from Sigma. Ilimaquinone from sea sponge was purchased from BIOMOL Research Laboratories Inc. (Plymouth Meeting, PA). 1-Butanol, 2-butanol, and trichloroacetic acid were purchased from Nacalai Tesque (Kyoto, Japan). [γ-32P]ATP, [1-14C]oleic acid, and [1-14C]dipalmitoylphosphatidylcholine were purchased from GE Healthcare (Buckinghamshire, UK). Syntide-2 was purchased...
from AnaSpec (San Jose, CA). Silica Gel 60 TLC plates were purchased from Merck (Darmstadt, Germany). Phosphocellulose-P-81 paper was purchased from Whatman. Dipalmitoylphosphatidylcholine was purchased from Avanti Polar Lipids Inc. (Alabaster, AL). Other chemicals were purchased from Wako Pure Chemical Industries (Osaka, Japan).

Cell Culture—HeLa and normal rat kidney (NRK) cells were cultured in Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum, 100 units/ml penicillin, and 100 μg/ml streptomycin in a humidified atmosphere of 5% CO₂ at 37 °C. DT40 cells were cultured in RPMI 1640 medium containing 10% fetal calf serum, 100 units/ml penicillin, and 100 μg/ml streptomycin in a humidified atmosphere of 5% CO₂ at 37 °C.

ts045 Vesicular Stomatitis Virus Glycoprotein (VSVG) Transport Assay—The expression plasmid for temperature-sensitive (ts045) vesicular stomatitis virus glycoprotein (VSVG)-green fluorescent protein (GFP) was kindly donated by Dr. J. Lippincott-Schwartz (National Institutes of Health). HeLa cells were grown in 35-mm glass-bottomed dishes (Matsunami Glass Industries, Ltd., Osaka) and transfected with plasmid encoding VSVG-GFP protein using FuGENE 6 reagent (Roche Applied Science) according to the manufacturer’s instructions. After transfection, cells were maintained at 37 °C for 36 h, transferred to 40 °C for 2 h, and then transferred to 20 °C for 30 min. At the end of the 30-min incubation at 20 °C, VSVG-GFP protein was concentrated mainly in the Golgi. These cells were incubated further at 20 °C in medium containing 30 μM IQ and monitored by fluorescence microscopy.

Construction of PLD-deficient DT40 Cells—Chicken spleen cDNA and genomic DNA libraries were obtained from Clontech. The chicken cDNA library was screened using the human PLD1 cDNA. Several chicken cDNA isolates were sequenced to confirm the identification. The genomic library was screened using the chicken PLD1 cDNA. After subcloning the genomic clone of chicken PLD1, the targeting construct was made as described previously (13), and the results are shown in supplemental Fig. 1. For pPLD-neo or pPLD-hisD, the neo cassette (2 kilobase pairs) or hisD cassette (3 kilobase pairs) was introduced into the BamHI site in the exon 19 genomic sequence. pPLD-neo was linearized and subcloned in PFGE on poly-L-lysine-coated coverglasses were stimulated with 30 μM IQ for 2 h at 37 °C. The cells were fixed with 3% paraformaldehyde, blocked with phosphate-buffered saline containing 0.1 M glycine, and permeabilized with 0.1% Triton X-100 in phosphate-buffered saline. After incubation with anti-GOSR1 monoclonal antibody followed by incubation with goat anti-mouse IgG conjugated with Alexa Fluor 594 (Molecular Probes), the bound antibody was detected with a fluorescence microscope.

Small Interfering RNAs (siRNAs) for Human PLD1 and PLD2—siRNAs for human PLD1 site 1 (siRNA-1, 5’-GUU AAG AGG AAA UUC AAG CTT-3’ and 5’-GCU UGA AUU UCC UCU UAA CTT-3’) and site 2 (siRNA-2, 5’-GGU CCA UCC GUA GUU UAC AGA-3’ and 5’-UGG AAA CUA CGG AUC GAC CGG-3’) and human PLD2 (5’-GAC ACA AAG UCU UGA UGA GTT-3’ and 5’-CUC AUC AAG ACU UUG UGU CTT-3’) and the control siRNA (5’-UUC UCC GAA CGU GUC ACG UdTdT-3’ and 5’-ACG UGA CAC GUU CGG AGA AdTdT-3’) were synthesized at Japan Bio Services (Saitama, Japan). HeLa cells were transfected with siRNA using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions.

Quantitative Real-time PCR for Human PLD1 and PLD2—Total RNA was isolated from siRNA-treated HeLa cells using the Nucleospin RNA II kit (Machery-Nagel, Duven, Germany). cDNA synthesis was performed with 1 μg of total RNA using murine leukemia virus reverse transcriptase (Applied Biosystems, Foster City, CA) priming with random hexamers. Quantitative PCR was performed by applying the real-time SYBR Green PCR technology with the use of an ABI PRISM 7000 sequence detection system (Applied Biosystems). The human PLD1- and PLD2-specific primers were designed using Primer Express software (Applied Biosystems), and their sequences were as follows: human PLD1, 5’-GGA AAT CGT TGG AGG TTG GA-3’ and 5’-TGT AGA CGC ATC AAA GTC CTC TTG-3’; and human PLD2, 5’-GCC AGC ACT TCC TCT ACA TTG A-3’ and 5’-CAA AAG CAC GTA GAC TCG GTA A-3’. The primer sequences for glyceraldehyde-3-phosphate dehydrogenase were 5’-GCC ATC AAT GAC CCC TTC ATT-3’ and 5’-TCT CGC TCC TTG AAG ATG G-3’. The amplification reaction was performed with SYBR Premix Ex Taq (Takara Bio Inc., Otsu, Japan), and the thermal cycling conditions were as follows: 10 s at 95 °C, 40 cycles for 5 s at 95 °C and 31 s at 60 °C. The expression of PLD1 and PLD2 mRNAs was normalized to that of glyceraldehyde-3-phosphate dehydrogenase mRNA.

Purification of Recombinant Proteins—COS-7 cells were transfected with FLAG-tagged PLD1, influenza hemagglutinin (HA)-tagged PKD1, or HA-tagged constitutively active PKD1. Three days after transfection, recombinant proteins were purified using either anti-HA antibody (Roche Applied Science) or anti-FLAG antibody M2 beads (Sigma) and finally eluted with HA or FLAG peptide (Sigma), respectively, according to the manufacturer’s protocol.

PLD Assay in Intact Cells—NRK or HeLa cells were suspended at a density of 2 × 10⁶ cells/ml and cultured for 1 day. Twenty-four h later, cells were incubated for 12 h at 37 °C with [⁴⁰⁰C]oleic acid (0.1 μCi/5 × 10⁶ cells). The labeled cells (5 × 10⁶ cells/ml) were washed with phosphate-buffered saline, transferred to Dulbecco’s modified Eagle’s medium containing 0.5% 1-butanol, and then stimulated either with IQ or phorbol 12-myristate 13-acetate (PMA). The cells were incubated for an additional 60 min at 37 °C. The reaction was stopped by changing the medium to extraction buffer containing methanol, KCl, and EGTA. Phase separation, lipid extraction, and separation were carried out as
described previously (14). PLD activity is expressed as a percentage of the radioactive [14C]phosphatidylbutanol in the total radioactivity found in all spots in a particular lane.

**In Vitro PLD Assay**—The effect of either recombinant PKD (200 ng) or protein kinase Ca (PKCa; 200 ng) on ARF1 (ADP-ribosylation factor-1; 100 ng; a kind gift from Dr. R. A. Kahn, Emory University, Atlanta, GA)-supported PLD activity in the absence or presence of 100 nM PMA was determined by measuring the formation of [14C]phosphatidylbutanol from [14C]phosphatidylcholine in the presence of 0.5% 1-butanol as described previously (15).

**PKD Assay in Semi-intact Cells**—Evaluation of PKD activation was performed by measurement of syntide-2 phosphorylation. NRK or HeLa cells were treated with digitonin (30 μg/ml) in KHM buffer (125 mM potassium acetate, 25 mM HEPES (pH 7.2), and 2.5 mM magnesium acetate), and then the PKD substrate syntide-2 was added (0.2 μg/ml). The cells were incubated for 30 min at 37 °C after stimulation with IQ (30 μM) and addition of [γ-32P]ATP (100 μM, 0.1 μCi). The proteins were precipitated with 10% trichloroacetic acid. After centrifugation, the supernatant was spotted onto phosphocellulose P-81 paper. After washing the paper with 75 ml H₃PO₄, the radioactivity incorporated into peptides was determined by Cerenkov counting.

**RESULTS**

**Inhibition of IQ-induced Golgi Membrane Fragmentation by 1-Butanol**—Because PLD is involved in various steps of membrane vesicle trafficking, we assumed that PLD may be involved in IQ-induced Golgi vesiculation processes. To test this possibility, the effects of 1-butanol, which is known to inhibit PLD-mediated processes by facilitating the PLD-specific transphosphatidylation reaction at the expense of PA production, on IQ-induced Golgi vesiculation were studied. The ts045 temperature-sensitive mutant of VSVG fused to GFP (16) was utilized to study vesicular trafficking from the Golgi. ts045 VSVG is reversibly misfolded and retained in the ER at the restrictive temperature (40 °C) and is able to exit the ER and traffic to the plasma membrane following incubation at the permissive temperature (40 °C) and is able to exit the ER and traffic to the plasma membrane following incubation at the permissive temperature (32 °C). However, if the cells are shifted directly from the restrictive temperature to 20 °C, ts045 VSVG is transported from the ER and subsequently arrested in Golgi/trans-Golgi network (TGN) compartments. HeLa cells transiently expressing ts045 VSVG-GFP were monitored by a fluorescence microscope equipped with a time-lapse recording program. Images were taken every 3 min for 2 h.

**PLD Mediates IQ-induced Golgi Vesiculation**

**FIGURE 1. Effect of 1-butanol on IQ-induced Golgi fragmentation.** HeLa cells were transfected with plasmid encoding ts045 VSVG-GFP. Two days after transfection, cells were incubated for 2 h at 40 °C to trap the protein at the ER. The protein was accumulated in the Golgi/TGN by incubating the cells at 20 °C for 30 min. The cells were then treated with 30 μM IQ at 20 °C in the absence (vehicle; Control) or presence of either 0.5% 1-butanol or 2-butanol. VSVG-GFP was monitored by a fluorescence microscope equipped with a time-lapse recording program. Images were taken every 3 min for 2 h.

**FIGURE 2. IQ-induced activation of PLD.** HeLa or NRK cells were metabolically labeled with [14C]oleic acid for 12 h and assayed for PLD activity in the presence of 0.5% 1-butanol after a 1-h treatment without (0.03% Me2SO) or with various concentrations of IQ. As a positive control PLD was activated for 1 h with 100 nM PMA. PLD activity is expressed as a percentage of the radioactive [14C]phosphatidylbutanol (PtdBut) in the total radioactivity found in all spots in a particular lane. Data are the means ± S.E. of three independent experiments carried out in triplicate.
PLD Mediates IQ-induced Golgi Vesiculation

**FIGURE 3. Identification of a PLD isotype activated by IQ using an analysis sensitive to siRNA-dependent isotype-specific down-regulation of PLD.** HeLa cells transfected with control siRNA, two different PLD1 siRNAs (siRNA-1 and siRNA-2), or PLD2 siRNA were cultured for 2 days. Total RNA was isolated from the cells, reverse-transcribed, and analyzed by quantitative real-time PCR for PLD1 (A) or PLD2 (B) mRNA expression. Thirty-six h after PLD isotype-specific siRNA transfection, cells were labeled with [14C]oleic acid and assayed for PLD activity in the absence or presence of 30 μM IQ (C) or 100 nM PMA (D) as described in the legend to Fig. 2. Data are the means ± S.E. of three independent experiments carried out in triplicate. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

PLD activity in the absence or presence of 30 siRNAs (siRNA-1 and siRNA-2), or PLD2 siRNA were cultured for 2 days. Total RNA was isolated from the cells, reverse-transcribed, and analyzed by quantitative real-time PCR for PLD1 mRNA expression. IQ treatment in the presence of 1-butanol, a specific inhibitor of PLD hydrolytic activity, blocked IQ-induced Golgi vesiculation. This suggests that the 1-butanol effect was specifically PLD-mediated and not a cytotoxic effect. These results indicate the involvement of PLD activity in IQ-induced Golgi vesiculation.

**IQ Activation of PLD in an Intact Cell System**—Because 1-butanol, a specific inhibitor of PLD hydrolytic activity, blocked IQ-induced Golgi vesiculation, we supposed that IQ treatment may have an effect on PLD activity. To address this issue, we measured PLD activity in an intact cell system after IQ treatment. For this experiment, we used HeLa and NRK cells, which are known to undergo IQ-dependent Golgi membrane fragmentation. Cells were metabolically labeled with radioactive oleic acid and assayed for phosphatidylbutanol production after IQ treatment in the presence of 1-butanol. In both cell lines, PLD was strongly activated by IQ in a time- and dose-dependent manner (Fig. 2). The extent of PLD activation by 30 μM IQ was greater than that obtained by PMA, a well known potent PLD activator. On the other hand, PLD activity was unchanged upon addition of IQ when measured in a cell-free system (data not shown). These findings strengthen our hypothesis that PLD is a downstream signaling molecule of IQ and that its product (PA) functions as a regulator of IQ-mediated Golgi vesiculation.

**PLD1 Is Responsible for IQ-induced Golgi Vesiculation**—To identify the PLD isform that is activated by IQ, the expression of each isorm was down-regulated by isorm-specific siRNA, and PLD activity was measured and compared with that in control siRNA-transfected cells after IQ treatment. First, the effect of siRNA was evaluated by quantification of mRNA for each PLD isorm in HeLa cells by quantitative real-time PCR. Two different site-specific PLD1 siRNAs, siRNA-1 and siRNA-2, inhibited the PLD1 mRNA by 66 and 71%, respectively, compared with the control siRNA (Fig. 3A). Similarly, PLD2 siRNA transfection caused 81% inhibition of PLD2 mRNA (Fig. 3B). Using these siRNA systems, transfection of either PLD1 siRNA-1 or siRNA-2 but not PLD2 siRNA almost completely abolished the ability of IQ to activate PLD (Fig. 3C), suggesting the involvement of PLD1 in these processes. In comparison, PMA-induced PLD activation was only mildly attenuated in PLD1 siRNA-treated cells (Fig. 3, C and D), suggesting that IQ caused more selective activation of PLD1, whereas PMA caused relatively nonselective activation of the PLD isoforms.

**Inhibition of IQ-induced Golgi Membrane Vesiculation in DT40-PLD−/− Cells**—We next tried to examine the effect of PLD knockdown by siRNA on IQ-induced Golgi vesiculation. However, we failed to observe accumulation of VSVG-GFP at the Golgi when the temperature was shifted from 40 to 20 °C presumably because of the toxic effect of Lipofectamine 2000 used for siRNA transfection (data not shown). Instead, we tried to obtain more direct evidence to show the importance of PLD in IQ-induced Golgi vesiculation using PLD knock-out cells. DT40 chicken B lymphocytes were used to prepare gene knock-out cells because DT40 cells are known to incorporate foreign DNA by targeted integration at frequencies similar to those for random integration (13, 17). Data base searches suggested that DT40 cells contain only one PLD isoenzyme that is close to mammalian PLD1 with 88% homology. Using gene targeting techniques, we generated homozygous DT40 mutants, DT40-
PLD Mediates IQ-induced Golgi Vesiculation

PKCα are known to activate PLD1 (18–20), whereas novel PKC isoenzymes such as PKCδ cause inhibition of PLD1 (21, 22), we tested the effect of PKD on PLD activity in a purified system. HA-tagged PKD1 and FLAG-tagged PLD1 expressed in COS-7 cells were separately purified by their respective antibody-conjugated beads and reconstituted for PLD assay. Reconstitution with either wild-type or constitutively active PKD1 had little or no effect on both basal and PMA-stimulated PLD activities compared with the control empty vector-expressing (mock) cells (Fig. 5). In contrast, reconstitution with PKCα caused a 2-fold increase in both basal and PMA-stimulated PLD activities, consistent with previous reports (18, 19). Similarly, the expression of wild-type or constitutively active PKD1 had little or no effect on PLD activity as measured in an intact cell system (Fig. 5). These results suggest that PLD activation may not be secondary to PKD activation. Conversely, we studied the effect of PLD stimulation on PKD activity. PKD activity was assessed by measuring the phosphorylation of syntide-2, an authentic peptide substrate for PKD, in digitonin-permeabilized NRK cells. IQ treatment caused stimulation of syntide-2 phosphorylation (Fig. 6), as suggested previously (23). Notably, this IQ-induced syntide-2 phosphorylation was specifically abrogated by 1-butanol but not by the same concentration of 2-butanol, suggesting that IQ-induced PLD activation is a prerequisite for the subsequent stimulation of PKD.

PA Phosphohydrolase-catalyzed Conversion of PA to DAG Is Necessary for the Subsequent Activation of PKD and IQ-induced Golgi Membrane Fragmentation—It has been shown recently that DAG formation from PA by sequential actions of PLD and PA phosphohydrolase is important in lysophosphatidic acid- or platelet-derived growth factor-induced PKD activation (24). To test whether this mechanism of PKD activation is true for IQ-induced phenomena, we tested the effect of propranolol, an inhibitor of PA phosphohydrolase, on IQ-induced PKD stimulation. As expected, IQ-induced PKD activation was nearly completely inhibited by propranolol (Fig. 7A). The effect of propranolol on IQ-induced Golgi membrane fragmentation was studied next. VSVG-GFP transiently expressed in HeLa cells was first accumulated in the Golgi by incubation at restrictive temperature (40 °C) for 2 h and then at 20 °C for 30 min as in Fig. 1. Cells were treated with IQ in the absence or presence of propranolol, and the dynamics of VSVG-GFP were monitored by time-lapse fluorescence microscopy. In contrast to the complete vesiculation of the Golgi throughout the cytoplasm (control), VSVG-GFP remained in a stacked form at the perinuclear regions in IQ-treated HeLa cells when the cells were pretreated with propranolol (Fig. 7, B and C). These results indicate that PA phosphohydrolase-catalyzed conversion of PA to DAG is necessary for both PKD activation and IQ-induced Golgi membrane fragmentation.

DISCUSSION

We have demonstrated that IQ induces PLD activation in various cell lines, including HeLa cells (Fig. 2). The importance of PLD in IQ-induced Golgi fragmentation was demonstrated by several findings, i.e. IQ-induced Golgi fragmentation was specifically inhibited by 1-butanol (Fig. 1), and PLD knock-out DT40 cells were resistant to IQ for Golgi fragmentation (Fig. 4).
PLD Mediates IQ-induced Golgi Vesiculation

We have also provided evidence that PKD activation is secondary to PLD activation, as supported by the observation that PKD activation after IQ treatment was almost completely blocked by either 1-butanol (Fig. 6) or propranolol (Fig. 7) treatment.

It has been suggested previously that IQ is able to activate one or more heterotrimeric G-proteins located on the Golgi (25). The activated G-protein dissociates into Gα and Gβγ subunits, where the latter remains membrane-associated and causes the fragmentation of the Golgi through downstream mediators. Gβγ was further shown to activate PKD, and activated PKD was shown to mediate fragmentation of the Golgi (23). Gβγ subunits are considered to act directly upon PKD by associating with the pleckstrin homology domain and somehow bringing about PKD activation (23). Diaz Anel and Malhotra (26) have shown recently that the expression of Gβ1γ2 or Gβ2γ2 in cells induces Golgi fragmentation. Notably, this result in the phosphorylation of PKD in its activation loop, and the level of PKD activation loop phosphorylation correlates with the extent of Golgi fragmentation. They identified the kinase responsible for PKD phosphorylation as PKCζ. Indeed, the expression of constitutively active PKCζ results in Golgi fragmentation, and this effect is blocked by coexpression of kinase-dead PKD, again consistent with the idea that PKCζ-mediated phosphorylation of PKD regulates Golgi carrier formation. They also surmised that DAG production plays an important role in the activation of PKCζ during Gβγ subunit-induced Golgi fragmentation, although the exact mechanism of DAG production remains unclear. Our present results, including IQ-induced PLD activation and inhibition of IQ-induced PKD activation by either 1-butanol or propranolol, strongly suggest that the production of DAG from phosphatidylcholine through sequential actions of PLD and PA phosphohydrolase is a possible mechanism of the lipid production leading to subsequent PKD activation. The involvement of the Gβγ subunit in IQ-induced PLD activation remains to be clarified.

The PLD-catalyzed production of PA may have at least two physiological roles in IQ-induced Golgi fragmentation, i.e. PA is known to facilitate membrane bending and the formation of the highly curved intermediates (27). PA is also converted to DAG via enzymatic dephosphorylation by PA phosphohydrolase. In addition to a signaling role of DAG, i.e. activation of PKCζ as mentioned above, DAG has a central role in the constriction of Golgi membrane tubules stemming from the unique molecular shape of DAG characterized by an extremely large negative spontaneous curvature. PKD and lipid components such as PA and DAG may work in concert but in spatiotemporally different manners to cause Golgi membrane curvature and constriction and the formation of transport carriers. For example, when HeLa cells were transiently transfected with both catalytically inactive PKD1 and VSVG-GFP, VSVG-GFP, which accumulated in the ER at the nonpermissive temperature (40 °C), was transported to the Golgi at 20 °C at a speed similar to those cells expressing VSVG-GFP alone (data not shown). This suggests that PKD has

FIGURE 5. Lack of effect of PKD1 on PLD activity measured in in vitro and in vivo systems. Recombinant FLAG-tagged PLD1 and HA-tagged PKD1 expressed in COS-7 cells were separately affinity-purified by their respective antibody-immobilized beads. The effects of PKD on ADP-ribosylation factor-supported PLD1 activity were measured in vitro in the absence or presence of 100 nM PMA. In some experiments, PKCα was added as a positive control for PLD1 activation (in vitro panel). HeLa cells were transfected with a vector alone (Mock) or with an expression vector encoding PKD1, constitutively active PKD1, or PKCα. Thirty-six h after transfection, cells were metabolically labeled with [14C]oleic acid for 12 h as described in the legend to Fig. 2. PLD activity was measured after treatment of cells without or with 30 μM IQ for 60 min (in vivo panel). Data are the means ± S.E. of three independent experiments carried out in triplicate. PtdBut, phosphatidylbutanol.

FIGURE 6. Requirement of PLD-catalyzed PA formation for IQ-induced PKD activation. After permeabilization of NRK cells with 30 μM digitonin, the cells were incubated for 30 min at 37 °C in KH buffer containing the PKD substrate syntide-2 and [γ-32P]ATP in the absence or presence of 30 μM IQ. In some experiments, PKCα was added to estimate the phosphorylation of syntide-2. IQ-induced syntide-2 phosphorylation was calculated by subtracting each value from that obtained in the absence of IQ. Data are the means ± S.E. of three independent experiments carried out in triplicate.

| IQ-induced syntide-2 phosphorylation (cpm) | None | IQ | IQ + 1-But. | IQ + 2-But. |
|-------------------------------------------|------|----|------------|------------|
| Number of experiments per group           |      |    |            |            |
| Mean ± S.E.                               |      |    |            |            |

For Figure 5 and Figure 6, the figure legends are not included as they are not necessary for understanding the text.
became tubulated by the expression of catalytically inactive PKD1, led to VSVG-GFP trafficking to the plasma membrane (supplemental Fig. 2). These results suggest that IQ-induced PLD activation may overcome the dominantly negative effects of catalytically inactive PKD1 on the Golgi trafficking. On the other hand, the expression of catalytically inactive PLD1 in HeLa cells resulted in the slow trafficking of VSVG-GFP from the Golgi to the plasma membranes (supplemental Fig. 3).

It has been reported that PLD is involved in vesicular trafficking from the ER to the Golgi complex (28). Our present results indicate that PLD is also involved in the regulation of membrane trafficking from the Golgi complex to the plasma membranes. In addition, when VSVG-GFP and constitutively active PKD were coexpressed in HeLa cells, VSVG-GFP accumulated at the Golgi/TGN upon a temperature shift from the restrictive temperature (40 °C) to 20 °C as in Fig. 1, remained stacked at the perinuclear regions, and did not undergo fragmentation as seen upon IQ treatment. This suggests that the activation of PKD alone is insufficient for the Golgi complex breakdown (data not shown).

In conclusion, PA and DAG are widely involved in the regulation of vesicular trafficking by effectively reducing the energy barriers to fusion and fusion, whereas protein factors such as PKD play more specialized roles, e.g. the constriction stage of vesicle formation during vesicular transport from the TGN to the plasma membrane (29). The concerted actions of lipid factors such as PA and DAG and protein factors such as PKD are needed for regulated vesicular trafficking.

REFERENCES

1. Takizawa, P. A., Yucel, J. K., Veit, B., Faulkner, D. I., Deerinck, T., Soto, G., Ellisman, M., and Malhotra, V. (1993) Cell 73, 1079–1090
2. Liljedahl, M., Maeda, Y., Colanzi, A., Ayala, I., Van Lint, J., and Malhotra, V. (2001) Cell 104, 409–420
3. Cremona, O., and De Camilli, P. (2001) J. Cell Sci. 114, 1041–1052
4. Baron, C. L., and Malhotra, V. (2002) Science 295, 325–328
5. Weigert, R., Silletta, M. G., Spano, S., Turacchio, G., Cericola, C., Colanzi, A., Senatore, S., Mancini, R., Polischuk, E. V., Salmona, M., Facchiano, F., Burger, K. N. J., Mironov, A., Luini, A., and Corda, D. (1999) Nature 402, 34091
PLD Mediates IQ-induced Golgi Vesiculation

6. Roth, M. G., Bi, K., Ktistakis, N. T., and Yu, S. (1999) Chem. Phys. Lipids 98, 141–152
7. Siddhanta, A., and Shields, D. (1998) J. Biol. Chem. 273, 17995–17998
8. Cross, M. J., Roberts, S., Ridley, A. J., Hodgkin, M. N., Stewart, A., Claesson-Welsh, L., and Wakelam, M. J. O. (1996) Curr. Biol. 7, 588–597
9. Ktistakis, N. T., Brown, H. A., Waters, M. G., Sternweis, P. C., and Roth, M. G. (1996) J. Cell Biol. 134, 295–306
10. Honda, A., Nogami, M., Yokozeki, H., Yamazaki, M., Nakamura, H., Watanabe, H., Kawamoto, K., Nakayama, K., Morris, A. J., Frohman, M. A., and Kanaho, Y. (1999) Cell 99, 521–532
11. Jones, D., Morgan, C., and Cockcroft, S. (1999) Biochim. Biophys. Acta 1439, 229–244
12. Liscovitch, M., Czarny, M., Fiucci, G., and Tang, X. (2000) Biochem. J. 345, 401–415
13. Buerstedde, J. M., and Takeda, S. (1991) Cell 67, 179–188
14. Nakamura, S., Shimooku, K., Akisue, T., Jinnai, H., Hitomi, T., Kiyojara, Y., Ogino, C., Yoshida, K., and Nishizuka, Y. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 12319–12322
15. Brown, H. A., Gutowski, S., Moomaw, C. R., Slaughter, C., and Sternweis, P. C. (1993) Cell 75, 1137–1144
16. Presley, J. F., Cole, N. B., Schroer, T. A., Hirschberg, K., Zaal, K. J., and Lippincott-Schwartz, J. (1997) Nature 389, 81–85
17. Takeda, S., Masteller, E. L., Thompson, C. B., and Buerstedde, J. M. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 4023–4027
18. Conricode, K. M., Brewer, K. A., and Exton, J. H. (1992) J. Biol. Chem. 267, 7199–7202
19. Singer, W. D., Brown, H. A., Jiang, X., and Sternweis, P. C. (1996) J. Biol. Chem. 271, 4504–4510
20. Oka, M., Hitomi, T., Okada, T., Nakamura, S., Nagai, H., Ohba, M., Kuroki, T., Kikkawa, U., and Ichihashi, M. (2002) Biochem. Biophys. Res. Commun. 294, 1109–1113
21. Hornia, A., Lu, Z., Sukezane, T., Zhong, M., Joseph, T., Frankel, P., and Foster, D. A. (1999) Mol. Cell. Biol. 19, 7672–7680
22. Oka, M., Okada, T., Nakamura, S., Ohba, M., Kuroki, T., Kikkawa, U., Nagai, H., Ichihashi, M., and Nishigori, C. (2003) FEBS Lett. 554, 179–183
23. Jamora, C., Yamanouye, N., Van Lint, J., Lautenslager, J., Vandenhedde, J. R., Faulkner, D. J., and Malhotra, V. (1999) Cell 98, 59–68
24. Kam, Y., and Exton, J. H. (2004) Biochem. Biophys. Res. Commun. 315, 139–143
25. Jamora, C., Takizawa, P. A., Zaarour, R. F., Denesvre, C., Faulkner, D. J., and Malhotra, V. (1997) Cell 91, 617–626
26. Diaz Anel, A. M., and Malhotra, V. (2005) J. Cell Biol. 169, 83–91
27. Kooijman, E. E., Chupin, V., de Kruijff, B., and Burger, K. N. J. (2003) Traffic 4, 162–174
28. Bi, K., Roth, M. G., and Ktistakis, N. T. (1997) Curr. Biol. 7, 301–307
29. Ponnambalam, S., and Baldwin, S. A. (2003) Mol. Membr. Biol. 20, 129–139