In mammalian cells, activation of a Golgi-associated phospholipase D by ADP-ribosylation factor results in the hydrolysis of phosphatidylcholine to form phosphatidic acid (PA). This reaction stimulates the release of nascent secretory vesicles from the trans-Golgi network of endocrine cells. To understand the role of PA in mediating secretion, we have exploited the transphosphatidylation activity of phospholipase D. Rat anterior pituitary GH3 cells, which secrete growth hormone and prolactin, were treated with 1-butanol resulting in the synthesis of phosphatidylylbutanol rather than PA. Under these conditions transport from the ER through the Golgi apparatus and secretion of polypeptide hormones were inhibited quantitatively. Furthermore, the in vitro synthesis of phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P2) by Golgi membranes was inhibited quantitatively. Most significantly, in the presence of 1-butanol the architecture of the Golgi apparatus was disrupted, resulting in its disassembly and fragmentation. Removal of the alcohol resulted in the rapid restoration of Golgi structure and secretion of growth hormone and prolactin. Our results suggest that PA stimulation of PtdIns(4,5)P2 synthesis is required for maintaining the structural integrity and function of the Golgi apparatus.

Phospholipid metabolism plays a key role in regulating intracellular vesicular transport particularly in distal steps of the secretory pathway (1–4). Initially, studies using the yeast Saccharomyces cerevisiae (5) demonstrated that the VPS34 gene encodes a phosphatidylinositol (PtdIns) 3-kinase that is required for protein transport from the late Golgi apparatus to the vacuole (2, 6). In mammalian cells multiple isoforms of PtdIns 3-kinases mediate signal transduction events (7), and based on their differential sensitivity to the drug wortmannin, PtdIns 3- and 4-kinases were subsequently implicated in protein trafficking to lysosomes (8–10). The human homologue of PtdBtCho to phosphatidic acid (PA) (and it is itself regulated by PtdIns(4,5)P2 (27–29). Although PLD1 is cytosolic (23), ARF activated PLD1a activity can be detected in association with Golgi membranes (20, 30), and the product of this reaction PA, enhances coat protein-I binding to Golgi membranes in vitro (19, 31). Using permeabilized rat anterior pituitary GH3 cells, which secrete high levels of growth hormone (GH) and prolactin (PRL), our laboratory demonstrated that ARF-1 activates Golgi associated PLD1a and enhances release of nascent secretory vesicles from the TGN (20).

PLDs possess transphosphatidylation activity, and in the presence of primary alcohols a phosphatidyl alcohol is synthesized rather than PA. Several lines of evidence suggest that PA is required for vesicle trafficking. First, pretreatment of cells with PA partially protected them from alcohol-induced inhibition of vesicle transport (32). Second, incubation of permeabilized GH3 cells with 1-BtOH inhibited nascent secretory vesicle budding from the TGN (20). Finally, incubation of permeabilized GH3 cells with enzymes that generate PA independently of PLD showed that the synthesis of PA resulted in efficient vesicle budding from the TGN (33).

To understand the function of PLD activity and PA in the
late secretary pathway, we treated GH3 cells with 1-BtOH. Here we demonstrate that intra-Golgi transport as well as growth hormone and prolactin secretion were inhibited by alcohol treatment. Most significantly, in the presence of 1-BtOH, the morphology of the Golgi apparatus was rapidly disrupted, resulting in its complete fragmentation.

**MATERIALS AND METHODS**

Monoclonal antibody to mannosidase-II (33F3C) was a generous gift of Dr. Brian Burke (University of Calgary, Calgary, Canada), mouse anti-rat GH was a gift of Dr. Carter Bancroft (Mt. Sinai School of Medicine, New York, NY), and mouse monoclonal antibody to human GH was from Sigma.

**Cell Culture and Viral Infection**—Rat anterior pituitary GH3 cells were grown as described previously (20). VSV (Indiana serotype) was propagated in BHK cells, and GH3 cells were infected with VSV as previously (34). The cells were pulse-labeled with 35S-Easytag 3.5–5 h after viral infection.

**Post-Golgi Secretion**—GH3 cells were pulse-labeled with 35S-Easytag for 12 min followed by chase for 2 h at 19 °C (35), after which the cells were treated with or without 1-BtOH, t-BtOH, or medium alone at 37 °C and chased for varying times. At each time point the medium and cells were processed (20, 35) and incubated with antibodies to GH or PRL. Immunoprecipitation of 1-BtOH-treated cells was performed as described previously (35). Fractions (1 ml) were collected from the top of the gradient, and aliquots were precipitated with an equal volume of ice-cold 20% (w/v) trichloroacetic acid, dissolved in SDS gel buffer, resolved by SDS-polyacrylamide gel electrophoresis, transferred to polyvinylidene difluoride membranes, and probed for TGN38 or the ER marker BiP by immunoblotting.

**Endoglycosidase H Digestion**—VSV-infected GH3 cells were pulse-labeled with 35S-Easytag for 5 min and chased for the indicated times in the presence or absence of 1- or t-butanol. Cells were harvested directly into 1% SDS (100 μl/35-mm dish), sonicated, and boiled. Equal aliquots of each lysate were adjusted to a final concentration of 0.2% SDS, 5% β-mercaptoethanol, 0.1 M sodium citrate, pH 5.5, and 20 milliunits/ml of endoglycosidase H (endo H; Oxford Glycosystems, UK) were added to one sample, whereas the control received an equal volume of buffer. Incubation was at 37 °C for 16 h, after which samples were analyzed in a 5–15% SDS-polyacrylamide gel electrophoresis followed by fluorography.

**Detection of Phosphatidylbutanol**—The assay was performed according to Wakelam et al. (37) with modifications as described (20).

**Measurement of PtdIns(4)P and PtdIns(4,5)P2**—Approximately 107 GH3 cells were treated with or without 1-BtOH or t-BtOH, after which they were grown as described previously (20). VSV-infected GH3 cells were pulse-labeled with 35S-Easytag for 10 min at 37 °C and chased at 19 °C for 2 h followed by an additional 30-min incubation in serum-free medium containing 1.2-dilauroyl PA, -PtdCho, or -DAG at 19 °C. At the end of 30 min, the phospholipid containing medium was replaced with fresh serum-free medium containing half the original lipid concentration with or without 1-BtOH or t-BtOH as indicated. The samples were then incubated for an additional 45 min at 37 °C, following which the cell lysates and media were analyzed for the presence of immunoreactive GH or PRL.

**Electron Microscopy**—Samples were treated with or without 1-BtOH or t-BtOH, fixed with 2.5% glutaraldehyde in 0.1 M cacodylate buffer, and postfixed with 1% osmium tetroxide followed by 1% uranyl acetate. The samples were then dehydrated through a series of graded ethanol concentrations and embedded in LX112 resin (LADD Research Industries, Burlington, VT). Ultrathin sections were cut on a Reichert Ultracut E, stained with uranyl acetate followed by lead citrate, and viewed on a JEOL 1200EX transmission electron microscope at 80 kV.

**Morphometry**—To quantitate the extent of Golgi apparatus fragmentation, electron micrographs (at a final magnification of 40,500) taken from control GH3 cells and cells treated with 1-BtOH or t-BtOH or following 1-BtOH removal were analyzed for the presence of Golgi cisternae defined as consisting of at least two cisternae; cisternae were membrane images with a length greater than four times the width, in which the width was not greater than 80 nm (39). 16–34 randomly selected micrographs of each sample from each treatment were analyzed by determining the number of Golgi structures that intersected the lines of a 7.5 × 7.5-cm grid placed on different areas of each micrograph. The number of structures corresponding to Golgi cisternae and stacks were counted; very rarely dilated Golgi cisternae greater than 80 nm in width were detected in response to alcohol treatment.

**RESULTS**

**Exit from the TGN and Secretion Are Inhibited by Primary Alcohols**—To determine whether PA synthesis was required for sorting of polypeptide hormones into post-Golgi vesicles and secretion in vivo, we exploited the transphosphatidylation activity of PLD. GH3 cells were pulse labeled with 35S-Easytag, followed by 2 h of incubation at 19 °C to accumulate radiolabeled cargo molecules (GH and PRL) in the TGN (36). Cells were then shifted to 37 °C for 45 min in the absence or presence of 1-BtOH, t-BtOH, or buffer alone, and hormone secretion was determined (Fig. 1). GH3 cells store their endogenous GH and PRL poorly, and in control cells approximately 80% of the pulse labeled hormones are rapidly secreted into the medium (Fig. 1, A, lanes 3 and 4, and B). However, in the presence of 1-BtOH, all the pulse labeled GH and PRL were retained within the cells, and no radiolabeled hormones were secreted (Fig. 1, A, lanes 5 and 6, and B). In contrast, when t-BtOH was present during incubation at 37 °C, GH and PRL were secreted with the same efficiency and kinetics as control untreated GH3 cells (Fig. 1, A, lanes 7 and 8, and B). The inhibition of secretion induced by 1-BtOH could be reversed by wash-out of the BtOH-containing medium; under these conditions, GH and PRL were then secreted efficiently (Fig. 1, A, lanes 9–11, and B).

If PA accumulation were required for secretory vesicle budding, then preloading cells with PA should abrogate the 1-BtOH-mediated inhibition of secretion. To test this idea, GH3 were treated with or without 1-BtOH or t-BtOH, fixed with 2.5% glutaraldehyde in 0.1 M cacodylate buffer, and postfixed with 1% osmium tetroxide followed by 1% uranyl acetate.
Fig. 1. 1-Butanol inhibits exit of nascent secretory vesicles from the TGN. A, GH3 cells were pulse labeled for 10 min with \textsuperscript{35}S-Easytag and chased for 120 min at 19 °C to accumulate radiolabeled GH and PRL in the TGN (lanes 1 and 2). Cells were then incubated at 37 °C for 45 min in the absence (lanes 3 and 4) or presence of 1.5% 1-butanol (lanes 5 and 6) or t-butanol (lanes 7 and 8). For wash-out, following 2 h of incubation at 19 °C, samples (lanes 9–11) were incubated at 37 °C for 45 min with 1.5%-butanol, after which the medium was removed and the cells were incubated for 2 h in chase medium. Following the chase, cells and medium were incubated sequentially with rabbit anti-GH followed by rabbit anti-PRL antisera, and the immunoprecipitable material was resolved by SDS-polyacrylamide gel electrophoresis and fluorography. C, intracellular material; M, secreted material; M1, medium from cells treated with 1.5% BtOH for 45 min prior to wash-out; M2, medium from the same cells followed by 2 h of wash-out of BtOH. B, quantitation of secretion. The band intensities (A) were quantified by densitometry. The percentage of secretion (total secreted GH or PRL = (intracellular + secreted GH or PRL) × 100) corresponds to the secretion of GH (filled bars) and PRL (hatched bars) in the presence of the indicated concentrations of 1-BtOH or t-BtOH compared with untreated control cells. Values are the averages of duplicate experiments. C, cells were pulse-labeled as above, transferred to serum-free media, and incubated in the absence (lanes 1–4) or presence (lanes 5–14) of 1,2-dilauroyl PA or 250 μM 1,2-dilauroyl DAG (lanes 15 and 16) for an additional 30 min at 19 °C (see "Materials and Methods"). Following incubation, the cells were chased for 45 min at 37 °C with (lanes 3, 4, and 11–16) or without (lanes 1, 2, and 5–10) 1.5% 1-BtOH. Initial PA concentrations were as follows: lanes 5 and 6, 100 μM; lanes 7 and 8, 250 μM; lanes 9 and 10, 500 μM; lanes 11 and 12, 100 μM; lanes 13 and 14, 250 μM. Following the chase, the cells (C) and medium (M) were incubated with rabbit anti-GH antibodies, and the immunoprecipitable material was resolved by SDS-polyacrylamide gel electrophoresis and fluorography. Note the low levels of basal GH secretion in the absence of serum; compare lanes 1 and 2 with lanes 3 and 4. D, quantitation of secretion. The band intensities (C) were quantified by densitometry. The fold stimulation corresponds to the secretion of GH (filled bars) and PRL (hatched bars) in the presence of the indicated concentrations of 1-BtOH or t-BtOH compared with untreated control cells. Values are the averages of duplicate experiments.

Fig. 2. 1-BtOH inhibits transport of VSV-G protein through the Golgi apparatus. GH3 cells were infected with VSV for 5 h, after which they were pulse-labeled with \textsuperscript{35}S-Easytag for 5 min (lanes 1 and 2) and chased with t-BtOH (lanes 7–12) or 1-BtOH (lanes 15–22) for the indicated times. For wash-out (lanes 23–26), following treatment with 1-BtOH for 60 min, the medium was removed, and the cells were incubated in fresh medium for an additional 30 or 60 min. Following incubation, cell lysates were prepared (34) and divided into two equal aliquots, one of which was digested with endo H for 16 h at 37 °C (+), whereas the other was incubated with buffer alone (−). The resulting material was analyzed by 5–15% SDS-polyacrylamide gel electrophoresis. The arrow indicates the migration of mature VSV-G protein (~70 kDa).

Inhibition of PA Synthesis Alters Golgi Structure

To determine whether 1-BtOH treatment affected earlier steps in the secretory pathway, GH3 cells were infected with VSV, and the transit of the viral membrane G glycoprotein to the late Golgi apparatus was determined by measuring its acquisition of resistance to endo H (Fig. 2). Early work from our laboratory (34) demonstrated that VSV-G protein rapidly exits the ER in these cells and becomes largely endo H-resistant (>60%) by 10 min of chase. GH3 cells were infected with VSV, radiolabeled for 5 min with \textsuperscript{35}S-Easytag, and chased in the presence of t- or 1-BtOH (Fig. 2). In agreement with our previous results, by 10

cells were pulse labeled and chased at 19 °C followed by incubation with 1,2-dilauroyl-PA for an additional 30 min at 19 °C to facilitate lipid endocytosis. Following this step, the cells were treated with and without 1-BtOH at 37 °C, and GH secretion was determined (Fig. 1, C and D). To ensure PA uptake from the medium, cells were incubated in the absence of serum, and consequently basal GH secretion was diminished considerably (Fig. 1C, lanes 1 and 2). Strikingly, addition of PA itself stimulated GH secretion approximately 2–5-fold (Fig. 1, C, lanes 5–10, and D), whereas 1,2-dilauroyl-PtdCho or -DAG were without effect (not shown). Most significantly, addition of 250 μM PA enhanced hormone secretion about 3-fold even in the presence of 1-BtOH (Fig. 1, C, lanes 13 and 14, and D); in contrast, DAG had no effect (Fig. 1C, lanes 15 and 16). It might be argued that PA behaved as a detergent to render the membranes leaky. However, this was not the case because pretreating the cells with PA at 4 °C (which allows uptake of PA; Ref. 40) did not enhance secretion or reverse the BtOH effect, and no abundant cytosolic proteins (e.g. ARFs) were detected in the medium following incubation at 37 °C (data not shown).

Inhibition of PA Synthesis Blocks ER to Golgi Transport—To determine whether 1-BtOH treatment affected earlier steps in the secretory pathway, GH3 cells were infected with VSV, and the transit of the viral membrane G glycoprotein to the late Golgi apparatus was determined by measuring its acquisition of resistance to endo H (Fig. 2). Early work from our laboratory (34) demonstrated that VSV-G protein rapidly exits the ER in these cells and becomes largely endo H-resistant (>60%) by 10 min of chase. GH3 cells were infected with VSV, radiolabeled for 5 min with \textsuperscript{35}S-Easytag, and chased in the presence of t- or 1-BtOH (Fig. 2). In agreement with our previous results, by 10
cells were incubated with 10 μCi/ml [3H]oleic acid for 24 h prior to treatment with 1.5% 1-BtOH for 45 min. Following incubation, the cells were washed with complete F-10 media, chased for the indicated times, and lysed, and the total phospholipids were extracted. The radiolabeled phospholipids were separated by TLC followed by fluorography, and the band intensities corresponding to PtdBtOH were quantitated by Image Quant. Results are the averages of three experiments; error bars represent the standard error.

min of chase approximately 60% of pulse labeled G protein was endo H-resistant in control or t-BtOH-treated cells and was completely resistant by 20–40 min of chase (Fig. 2, lanes 5, 6, 9, and 10). In contrast, in cells treated with 1-BtOH, the G protein was endo H-sensitive at all time points and remained endo H-sensitive up to 60 min of chase, the longest time examined (lanes 13–22). Upon wash-out of the 1-BtOH, the G protein became endo H-resistant by 20–30 min following removal of the alcohol (lanes 23–26).

**PLD and Intracellular Transport**—We argued that the inhibition of VSV-G transport and hormone secretion by 1-BtOH resulted from PLD-mediated PtdBtOH synthesis. To test this idea, cells were incubated with [3H]oleate to radiolabel phospholipids and treated with 1-BtOH, and the phospholipids were analyzed by thin layer chromatography. Consistent with our hypothesis, PtdBtOH accumulated only in cells treated with 1-BtOH (data not shown). These results and our previous observation that PA promotes vesicle budding from the TGN (33) provides further evidence that PLD activity is a prerequisite for secretory vesicle release from the TGN.

The restoration of intra-Golgi transport and secretion following 1-BtOH removal may have resulted from the rapid metabolism of PtdBtOH, a nonphysiological lipid that might be toxic to cells. Alternatively, the absence of PA synthesis as a consequence of PtdBtOH formation may have lead to the inhibition of secretion. To distinguish between these possibilities, cells were radiolabeled with [3H]oleic acid and treated with 1-BtOH followed by removal of the alcohol. At various times (up to 2 h) after 1-BtOH wash-out, total phospholipids were extracted, analyzed by TLC, and the level of PtdBtOH was determined (Fig. 3). Approximately 50% of the initial amount of PtdBtOH was still present in the cells at 120 min after alcohol removal. Significantly, the restoration of intra-Golgi transport of VSV-G protein and hormone secretion occurred by 30 min after 1-BtOH wash-out (Fig. 2 and data not shown), i.e. when PtdBtOH was present at approximately 75% of its initial level. This result demonstrated that the accumulation of PtdBtOH per se was not toxic to GH3 cells; in agreement with the data of Fig. 1C, these results suggested that the absence of PA synthesis lead to inhibition of secretion. Because of its low abundance and rapid turnover in cells (37), we were unable to measure changes in PA levels directly in response to alcohol removal.

**PtdIns(4,5)P2 Levels in Golgi Membranes Are Regulated by PA Synthesis**—Phosphatidic acid exists transiently in cells and does not accumulate to significant steady state levels. We hypothesized that PA might be rapidly metabolized to another phospholipid in the Golgi apparatus or might affect the synthesis of other phospholipids. In this context, PA has previously been shown to stimulate Type I but not Type II phosphatidylinositol 4-phosphate (PtdIns(4)P)-5-kinase isoforms, the final enzymes involved in phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P2) biosynthesis (41). More recently Arneson et al. (42) demonstrated that PtdIns(4,5)P2 biosynthesis can occur on lysosomal membranes, and this was enhanced by PA. In addition, PtdIns(4,5)P2 is an essential co-factor for PLD activity (29, 43).

To determine whether PtdIns(4,5)P2 synthesis occurred on Golgi membranes and if it was responsive to PA or PtdBtOH, isolated Golgi membranes were incubated with [32P]ATP in the absence and presence of cytosol with and without 1-BtOH (Fig. 4). Although both types of PtdIns(4)P 5-kinase are thought to reside in the cytoplasm (7), a low level of PtdIns(4,5)P2 synthesis was detected on Golgi membranes in the absence of cytosol (Fig. 4A, lane 1). As expected PtdIns(4,5)P2 synthesis was enhanced (~3–4-fold) in the presence of cytosol, which is an abundant source of both Types I and II PtdIns(4,5)P2 5-kinases (Fig. 4A, lane 2, and C). Significantly, the level of PtdIns(4,5)P2 was reduced dramatically by inclusion of 1-BtOH (Fig. 4A, lanes 3 and 4, and C). In addition, 1-BtOH also decreased the level of PA synthesis on Golgi membranes (Fig. 4A, compare lanes 2 and 4). Most likely, the source of this [32P]PA was derived from de novo phosphorylation of DAG generated by the action of PA phosphatase. Presumably, PtdBtOH cannot be converted to DAG, and consequently, the substrate pool for DAG kinase was diminished resulting in decreased PA synthesis. As expected 1-BtOH did not affect the synthesis of either PA, PtdIns(4)P or PtdIns(4,5)P2 (Fig. 4A, lanes 5 and 6, and C), further demonstrating the specificity of the 1-BtOH effect. It is noteworthy that PtdIns(4)P synthesis (presumably also PtdIns(4,5)P2) was much less affected by 1-BtOH (~40% inhibition) than PtdIns(4,5)P2 (~90% inhibition; Fig. 4C, lane 3), suggesting that the alcohol does not disrupt all inositol phospholipid synthesis nonspecifically. To further characterize the inositol phospholipids synthesized by Golgi membranes, the spots corresponding to the major phosphoinositides were excised, deacylated, and analyzed by ion exchange HPLC (Ref. 72 and Fig. 4B). This analysis confirmed that the spot marked PIP corresponded to PtdIns(4)P (not shown) and the identity of the PIP2 species (Fig. 4A) as PtdIns(4,5)P2.

We presumed that the decrease in PtdIns(4,5)P2 resulted from an absence of PA because of the synthesis of PtdBtOH and hence lack of PtdIns(4)P 5-kinase stimulation. If this were correct, then addition of PLD to generate PA on the cytoplasmic leaflet of the Golgi membrane should stimulate the putative Type I PtdIns(4,5)P 5-kinase activity and enhance PtdIns(4,5)P2 synthesis. Conversely, in the absence of 1-BtOH and PLD, PtdBtOH would be synthesized resulting in diminished synthesis of PtdIns(4,5)P2. Consistent with this idea, addition of human PLD1a stimulated PtdIns(4,5)P2 synthesis 4–5-fold (Fig. 4A, compare lanes 1 and 7), whereas in the presence of 1-BtOH, PLD had a minimal effect on PtdIns(4,5)P2 levels (Fig. 4A, lanes 7 and 8). Most significantly, these data demonstrated that PtdIns(4,5)P2 synthesis occurred on Golgi membranes and was regulated by PA.

**Golgi Morphology Is Disrupted by 1-BtOH**—We assumed, because secretion was inhibited by 1-BtOH, that both GH and PRL were trapped in the TGN. To test this idea we used confocal microscopy employing antibodies to GH, mannosidase-II, and TGN38 (Figs. 5 and 6). In control untreated GH3 cells,
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and Sf-9 cells expressing human PLD1a (lanes 7) were incubated in the absence or presence of 1-BtOH with an extract of t-BtOH (lanes 5 and 4, lanes 3 and 2). Further, the distinct perinuclear Golgi staining was absent, and instead the Golgi marker proteins were located randomly throughout the cell and toward the periphery. Additionally, the 1-BtOH-treated cells underwent a change in their overall morphology, becoming rounded rather than polygonal in shape (data not shown). As expected, in cells treated with t-BtOH, the cell morphology and localization of Golgi marker proteins was virtually identical to that of control untreated cells (Fig. 5, G–I).

Our earlier work had shown that in GH3 cells GH is mostly localized to the perinuclear Golgi region and to a lesser extent small secretory vesicles subjacent to the plasma membrane (44). Thus in control cells, GH-immunoreactivity was seen in the cis- and trans-Golgi network regions but did not manifest the lacy ribbon-like morphology of the Golgi apparatus nor overlap completely with the marker proteins mannosidase-II and TGN38 (Fig. 6, A–C). In the presence of 1-BtOH, GH localization to the Golgi region was also disrupted (Fig. 6, D–F and J–L). Indeed, some of the GH-immunoreactive material had a similar distribution and morphology to the Golgi marker proteins in that it partially co-localized with the fragmented cis-Golgi and TGN-derived structures (Fig. 6, D–F and J–L). Significantly, there was no disruption of GH in the plasma membrane-localized secretory vesicles (Fig. 6D).

These observations suggested that 1-BtOH caused the Golgi apparatus to become disassembled and the organelle structure disrupted. This was confirmed by analyzing control and alcohol-treated cells using electron microscopy (Fig. 7). Control GH3 cells or those exposed to t-BtOH (Fig. 7, A and D) had an obvious, highly organized Golgi apparatus with its characteristic flattened cisternae; control cells had an average of 3.6 cisternae/Golgi stack (Table I). In contrast, cells treated with 1-BtOH lacked any discernible organized Golgi structure (Fig. 7, B and C, and Table I). Instead, the 1-BtOH-treated cells possessed a large number of heterogenously sized small vesicles (~50–150 nm in diameter) in the region of the cell which presumably corresponded to the Golgi apparatus (Fig. 7C, arrowhead); this was identified by localization of the centriole in adjacent sections (data not shown). In rare sections, images of slightly dilated individual Golgi sacsules were seen (Fig. 7C, arrowhead). Consistent with the transport and secretion data (Figs. 1 and 2), the fragmentation of the Golgi apparatus was rapidly and completely reversible (Fig. 7E). Following wash-out of 1-BtOH, the organization and structure of the Golgi was restored within ~40 min of alcohol removal. Significantly, although the structural organization of the Golgi apparatus was completely disrupted by exposure to 1-BtOH, other organelles were largely unaffected, e.g. the endoplasmic reticulum (Fig. 7F).

Further evidence that the Golgi apparatus became fragmented in response to 1-BtOH came from subcellular fraction-
**DISCUSSION**

**PA Is Required for Intra-Golgi Transport and Secretion**—Several recent observations have implicated PA synthesis and mediates coat protein-I recruitment to Golgi membranes (19) and budding of nascent secretory vesicles from the TGN (20, 33). However, the involvement of PA in coatamer recruitment to Golgi membranes has been questioned in light of data suggesting that its level declines during cell-free budding reactions (47, 48). In addition, studies (49) on the yeast temperature-sensitive SEC14 phosphatidylinositol transfer protein mutant have shown that although vesicle budding from the Golgi apparatus was inhibited, and this could be suppressed at the nonpermissive temperature by a “by-pass” mutant possessing increased levels of DAG. It is now apparent that PLD activity and PA are required both for suppression of the SEC14 mutation and secretion in yeast cells (50–52).

By exploiting the transphosphatidylation activity of PLD, Bi et al. (32) demonstrated that in Chinese hamster ovary cells, transport of VSV-G protein from the intermediate compartment to the late Golgi was inhibited by primary alcohols. Similarly, secretion of lysosomal hexosaminidase from RBL cells was inhibited by 1-BtOH (25). Consistent with these observations, our data demonstrate that hormone secretion from the TGN and VSV-G protein transport from the ER to late Golgi cisternae were inhibited by 1-BtOH (Figs. 1 and 2). Because phospholipid metabolism is involved in multiple steps of the late secretory pathway (3), it is also possible that 1-BtOH could have affected vesicle docking and fusion of the released vesicles with the plasma membrane resulting in diminished hormone secretion.

Our demonstration that addition of exogenous PA stimulates secretion and partially reversed the inhibitory effects of 1-BtOH (Fig. 1) further supports a role for PA in the late secretory pathway. Although the ability of PA to rescue secretion from the Golgi apparatus was inefficient, this was not entirely unexpected. Earlier work (40) has demonstrated that the majority of exogenously added short chain PA is rapidly transported to mitochondria and the ER. Consistent with this...
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At least two possibilities could explain how inhibition of PA synthesis by 1-BtOH lead to fragmentation of the Golgi apparatus. Firstly, PA might be required directly for coat protein recruitment, or, alternatively, it may be an intermediate in the biosynthesis of other lipids that function in promoting protein-lipid interactions. In agreement with the first model, acidic phospholipids are required for the efficient binding of coat protein-II and -I, clathrin, and dynamin to synthetic liposomes, implying a role for protein-lipid interactions in coat formation (31, 65, 66). In the experiments presented here, it is unlikely that negative charge per se plays a significant role in promoting vesicle release from the TGN because the net charge on PA and PtdBtOH is likely to be similar.

The second model is consistent with our data, demonstrating that PA regulates PtdIns(4,5)P₂ levels in the Golgi apparatus (Fig. 4). We suggest that in the TGN, PA synthesis is part of a feedback loop that stimulates the local production of PtdIns(4,5)P₂ via activation of PtdIns(4)P 5-kinase (Figs. 4 and 9), similar to that observed in isolated lysosomes (42). PtdIns(4,5)P₂ is an essential co-factor for PLD activity (29, 43);
it also stimulates the ARF guanine nucleotide exchange factor ARNO-1 (67) and ARF-1 binding to membranes (68). Our results showed that PtdBtOH was ineffective in stimulating PtdIns(4P) 5-kinase in vitro, and consequently the synthesis of PtdIns(4,5)P2 in the Golgi apparatus was inhibited (Fig. 4). We presume that diminished PtdIns(4,5)P2 levels in vivo would lead to decreased activities of ARF-GEF (e.g. ARNO-1 and-3) and PLD, further reducing local concentrations of PtdIns(4,5)P2.

PtdIns(4,5)P2 regulates the function of the actin-based cytoskeleton and has been implicated in mediating synaptic vesicle docking with the plasma membrane (2, 3). Decreased PtdIns(4,5)P2 levels might therefore disrupt the interaction of putative cytoskeletal scaffolding proteins (e.g. giantin (69)) with Golgi membranes. Indeed, recent evidence suggests that ARF enhances the association of a specific form of β-spectrin to

TABLE 1
Quantitation of Golgi cisternae in the absence and presence of 1-BtOH
Random electron micrographs similar to those in Fig. 7 were chosen for quantitation of the number of Golgi stacks and cisternae (see “Materials and Methods”). The number of each sample analyzed (N) and the standard error of the mean are indicated.

| Treatment | Cisternae per Golgi Stack | N  |
|-----------|--------------------------|----|
| Control   | 3.6 ± 0.8                | 28 |
| 1-BtOH    | Not detected             | 34 |
| t-BtOH    | 3.5 ± 1.0                | 21 |
| Reversal  | 4.1 ± 1.3                | 17 |

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FIG. 8. Subcellular distribution of TGN and ER marker proteins in control and 1-BtOH-treated GH3 cells. Cells were either untreated (Control) or treated for 45 min with 1.5% 1-BtOH or 1.5% t-BtOH, or following 45 min treatment with 1-BtOH, the medium was replaced with control incubation medium (Wash-out). The cells were then homogenized, and the homogenate was fractionated on an equilibrium flotation gradient that separates TGN/Golgi membranes from the ER (35). An aliquot of each fraction was transferred to polyvinylidene difluoride membranes and immunoblotted with anti-TGN38 antiserum (α-TGN 38). The blots were stripped and reprobed with anti-BiP antiserum (α-BiP). In control and t-BtOH-treated cells, TGN38 migrates in fractions 2 and 3 (upper panel), whereas in cells incubated in 1-BtOH, TGN38 fractionates in the more dense region of the gradient (fractions 2–6). Mature, polysialated TGN38 appears as a smear at ∼85 kDa (35).

FIG. 7. Ultrastructure of GH3 cells treated with 1-BtOH and t-BtOH. GH3 cells were either untreated (A) or treated for 45 min with 1.5% 1-BtOH (B, C, and F) or 1.5% t-BtOH (D), or following 45 min 1-BtOH treatment, the alcohol-containing medium was replaced by control medium (E). The cells were then fixed and prepared for transmission electron microscopy. Open arrows indicate Golgi stacks. C, arrowhead indicates dilated saccules in a region of heterogeneously sized vesicles resulting from fragmentation of the Golgi apparatus. B and C, note the absence of a recognizable Golgi apparatus. F shows an area of the endoplasmic reticulum from the same cells as B; note that the ER is largely unaffected by treatment with 1-BtOH. E, wash-out of 1-BtOH; the structural organization of the Golgi apparatus is restored rapidly. Bar, 1 μm.

FIG. 9. Possible role of phosphatidic acid in mediating vesicle budding from the TGN. Based on our in vitro studies (20, 33) and the present data, we propose that activation of PLD1 in the TGN by ARF-1 results in the hydrolysis of phosphatidylcholine to generate PA. PA stimulates the final enzyme in the phosphatidyl 4,5-bisphosphate (PIP2) biosynthetic pathway, Type I phosphatidyl 4-phosphate 5-kinase (PIP5 K) to enhance production of phosphatidyl 4,5-bisphosphate. In the presence of 1-BtOH, PA synthesis is diverted to PtdBtOH production that leads to lowered phosphatidyl 4,5-bisphosphate levels because of diminished phosphatidyl 4-phosphate 5-kinase activity. Reduced levels of phosphatidyl 4,5-bisphosphate would decrease the activities of PLD and ARNO (ARF nucleotide-binding-site opener), diminish ARF recruitment (dotted arrow), and lead to disruption of the Golgi architecture by destabilizing interactions of cytoskeletal proteins with the membrane.
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Golgi membranes by increasing PtdIns(4,5)P₂ levels (70). Most recently, these investigators (71) demonstrated that ARF stimulates PtdIns(4,5)P₂ synthesis on Golgi membranes via recruitment of PtdIns₄-kinase-β. In contrast to our data, stimulation of PtdIns(4,5)P₂ synthesis was not mediated by PLD and PA but rather by ARF activation of both PtdIns 4-kinase and PtdIns 4,5-kinase activities (71). The reason for the discrepancy between our results and these studies is unclear at present. Nonetheless, overexpression of a mutant PtdIns₄-kinase devoid of enzyme activity resulted in a disorganized, irregular, punctate staining Golgi complex somewhat similar to that observed in the presence of 1-BtOH. We speculate that decreased PtdIns(4,5)P₂ synthesis disrupts interactions between cytoskeletal scaffolding proteins and the membrane leading to fragmentation of the Golgi apparatus. Experiments are currently in progress to test this idea.

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