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Stimulation of Golgi Membrane Tubulation and Retrograde Trafficking to the ER by Phospholipase A_2 Activating Protein (PLAP) Peptide

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Abstract Recent pharmacological studies using specific antagonists of phospholipase A_2 (PLA_2) activity have suggested that the formation of Golgi membrane tubules, 60–80 nm in diameter and up to several microns long, both in vivo and in a cell-free cytosol-dependent reconstitution system, requires the activity of a cytoplasmic Ca^{2+}-independent PLA_2. We confirm and extend these studies by demonstrating that the stimulators of PLA_2, melittin and PLA_2 activating protein peptide (PLAPp), enhance cytosol-dependent Golgi membrane tubulation. Starting with preparations of bovine brain cytosol (BBC), or a fraction of BBC that is highly enriched in tubulation activity, called the gel filtration (GF) fraction, that are at subsaturating concentrations for inducing tubulation in vitro, we found that increasing concentrations of melittin or PLAPp produced a linear and saturable stimulation of Golgi membrane tubulation. This stimulation was inhibited by cytosolic PLA_2 antagonists, including the Ca^{2+}-independent PLA_2-specific antagonist, bromoenol lactone. The stimulatory effect of PLAPp, and its inhibition by PLA_2 antagonists, was reproduced using a permeabilized cell system, which reconstitutes both cytosol-dependent Golgi membrane tubulation and retrograde trafficking to the endoplasmic reticulum (ER). Taken together, these results are consistent with the idea that cytosolic PLA_2 activity is involved in the formation of Golgi membrane tubules, which can serve as trafficking intermediates in Golgi-to-ER retrograde movement. J. Cell. Biochem. 74:670–683, 1999.

Key words: PLAPp; Golgi membrane tubules; endoplasmic reticulum

The existence of thin membrane tubules (60–80 nm in diameter) that emanate from various regions of the Golgi complex has been recognized for many years [Cunningham et al., 1966; Morré et al., 1970; Novikoff et al., 1971; Rambourg and Clermont, 1990; Rambourg et al., 1979]. However, only relatively recently have we begun to appreciate the extent and dynamic nature of these tubules, and to investigate their functional roles in membrane trafficking and their mechanisms of formation. For example, fluorescence imaging studies on live cells have shown that Golgi membrane tubules are continuously forming, sometimes establishing bridges between spatially separate Golgi elements [Cooper et al., 1990], and other times extending into the cytoplasm for many microns only to detach completely from the main body of the Golgi complex [Presley et al., 1998; Sciaky et al., 1997]. Similar tubules can be induced to form in the presence of the fungal metabolite, brefeldin A (BFA), and to mediate Golgi-to-endoplasmic reticulum (ER) retrograde [Lippincott-Schwartz et al., 1989] and *trans*-Golgi network (TGN)-to-endosome trafficking [Lippincott-Schwartz et al., 1991; Wood et al., 1991]. Finally, membrane tubules can also be seen to connect separate cisternae within a single stack [Weidman et al., 1993; Happe and Weidman, 1998].

The mechanisms by which these membrane tubules form are beginning to be addressed. In vivo, BFA-stimulated tubulation of Golgi and TGN membranes is clearly facilitated by microtubules and microtubule-associated motor pro-

Abbreviations used: $AACOCF_3$, arachidonyl trifluoromethyl ketone; BBC, bovine brain cytosol; BEL, bromoenol lactone; BSA, bovine serum albumin; GF, gel filtration; ONO, ONO-RS-082; $PACOCF_3$, palmitoyltrifluoromethylketone; PLAPp, phospholipase A_2 activating protein peptide.

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teins [Lippincott-Schwartz et al., 1991, 1995; Wood et al., 1991]. However, short membrane tubules and slowed retrograde trafficking induced by BFA still occur even in cells treated with nocodazole to depolymerize microtubules [Klausner et al., 1992; Wood et al., 1991]. These observations led us to propose that other factors must exist to stimulate the initial events in tubule formation.

To identify these factors, we have established a cell-free system in which the formation of membrane tubules from isolated Golgi complexes has been reconstituted [Cluett et al., 1993]. The membrane tubules formed in this system require an activity present in an organelle-free extract of bovine brain cytosol (BBC) [Banta et al., 1995], and they are morphologically similar to those normally seen, and following BFA stimulation in vivo. Extensive fractionation of BBC, culminating in a gel filtration (GF) column, resulted in a fraction that was highly enriched in tubulation activity and a handful of proteins having molecular weights of ~80, 66, 40, 30, and 18 kD [Banta et al., 1995] (P. de Figueiredo and E. Racoosin, unpublished results). We do not know which of these proteins, or combination of proteins, is directly responsible for the tubulation activity.

Previous studies using the in vitro tubulation assay showed that very small amounts of BBC (relative to Golgi membrane protein) were required for maximal tubulation, suggesting that the tubulation activity may work enzymatically. These results led us to screen for pharmacological agents that inhibit various classes of enzymatic activities that have been associated with alterations in membrane structure. We found that a broad spectrum of phospholipase A_2 (PLA_2) antagonists, but not antagonists of other phospholipases, profoundly inhibited BFA-stimulated Golgi tubulation and retrograde trafficking in vivo [de Figueiredo et al., 1998], tubule-mediated events that occur late in the reassembly of the Golgi complex, and BBC-dependent Golgi tubulation in vitro [de Figueiredo et al., 1999]. Results from these inhibitor studies, including those using the irreversible, mechanism-based suicide substrate, bromoenol lactone (BEL) [Hazen et al., 1991; Street et al., 1993; Ackermann et al., 1995], suggested that the PLA_2 activity required for tubulation was likely a cytoplasmic Ca^{2+}-independent enzyme.

To obtain independent evidence for the involvement of a cytoplasmic PLA_2 in membrane tubulation, the PLA_2 stimulators melittin and PLA_2 activating protein peptide (PLAPp) were used here to determine whether they had any effect on in vitro Golgi membrane tubulation, as well as on resident Golgi protein redistribution to the ER in a permeabilized cell system. Melittin is a well-characterized 2,800-Da peptide PLA_2 stimulator found in bee, wasp, and hornet venoms [Blackwell et al., 1980; Mollay et al., 1974], and it is known to stimulate a variety of mammalian PLA_2 enzymes [Arigolas and Paisano, 1983]. PLAP was originally isolated using anti-melittin antibodies to screen a cDNA expression library in the hope of finding a mammalian protein with melittin-like regulatory activity [Clark et al., 1987]. The results identified a cDNA predicting an ~35-kD protein containing a short, 17-amino acid region with homology to melittin [Clark et al., 1987]. More recent studies have identified other cDNAs predicting a larger molecular weight, PLAP-related protein of ~71 kD [Wang et al., 1995]. Although the precise function of any PLAP is unknown, synthetic peptides (PLAPp) identical to the original melittin-homology region have been shown to stimulate several cytosolic PLA_2 activities [Clark et al., 1991]. Thus, it appears that PLAP may be involved in several physiologically relevant pathways. Here we show that PLA_2 stimulators melittin and PLAPp enhance both BBC-dependent Golgi membrane tubulation in vitro and tubule-mediated trafficking to the ER in permeabilized cells.

MATERIALS AND METHODS

Chromatography reagents were obtained from phenyl-Sepharose (Pharmacia, Piscataway, NJ), Affi-Gel Blue, and Affi-Gel Protein A-agarose (Bio-Rad, Hercules, CA), Sephadex G100–125 (Sigma Chemical Co., St. Louis, MO). Cell culture media and calf supreme supplement (CSS) were purchased from Gibco-BRL (Grand Island, NY). Nitrocellulose (pore size 0.45 μm) was from Schleicher and Schuell (Keene, NH), and E-6-(bromomethylene)tetrahydro-3-(1-naphthalenyl)-2H-pyran-2-one (bromoenol lactone, also known as haloenol lactone suicide substrate), 2-(p-amylcinnamoyl)amino-4-cholorbenzoic acid (ONO-RS-082), arachidonyl trifluoromethyl ketone ($AACOCF_3$), palmitoyl trifluoromethyl ketone ($PACOCF_3$), and PLAP peptide were purchased from Biomol (Ply-

mouth Meeting, PA). Melittin and most of the other reagents were obtained from Sigma.

Antibodies

Rabbit anti-α-mannosidase II (Man II) serum was from Marilyn G. Farquhar (University of California at San Diego) and Kelly Moreman (University of Georgia). Mouse anti-α-tubulin antibody was purchased from Amersham Life Sciences (Arlington Heights, IL). Donkey anti-rabbit IgG conjugated to rhodamine (TRITC) and donkey anti-mouse IgG conjugated to fluorescein (FITC) were obtained from Jackson ImmunoResearch Laboratories (West Grove, PA). Polyclonal anti-40k antibodies were raised against the 40-kD protein of the GF fraction enriched in tubulation activity and described by Banta et al. [1995].

Cell Culture

Clone 9 rat hepatocytes were grown in Eagle's minimal essential medium (MEM) with 10% CSS in an atmosphere of 5% CO_2, 95% air at 37°C.

Preparation of Fractionated Cytosol and In Vitro Tubulation Assay

BBC and a fraction enriched in intact Golgi complexes from rat liver were prepared essentially as described in Banta et al. [1995] with some minor modifications. The original protocol loaded the dialysate from the phenyl-Sepharose column onto a DEAE-sepharose column; however, the DEAE column did not remove many contaminants; thus, the dialysate was loaded directly onto an Affi-Gel blue column and eluted as described. Tubulation of isolated Golgi complexes was visualized by a whole-mount electron microscopy (EM) procedure on negatively stained organelle preparations as previously described by Cluett et al. [1993], except that BBC or the enriched GF fraction in tubulation assay buffer was incubated at 37°C for 15 min with the PLA_2 agonists or antagonists before being added 1:1 to the Golgi-enriched fraction. Immuno-depletion of BBC and quantitation of the results of tubulation were performed as described by Banta et al. [1995]. Briefly, tubulation was quantified by determining the percentage tubulation, which was the percentage of total Golgi stacks with one or more tubule extensions that were 60–80 nm in diameter and at least three times as long (see Fig. 1, for examples). As we have previously described, this analysis is semiquantitative because it does not take into account the length of tubules or the number of tubules/Golgi stack because obtaining this information is virtually impossible in most cases (e.g., see Fig. 1). In some experiments, we wished to measure the extent to which increasing amounts of PLAPp or melittin could stimulate tubulation at different concentrations of BBC. These data were expressed as the fold-stimulation of tubulation, determined by dividing the percentage tubulation in the presence of PLAPp or melittin at a given BBC concentration by the percentage tubulation in the absence of PLAPp or melittin at that same BBC concentration.

Permeabilized Cell Assay and Immunofluorescence

Clone 9 cells were seeded onto coverslips 3 days before the experiment and used at 85–95% confluency. The cells were permeabilized using a filter-stripping protocol adapted from Donaldson et al. [1991]. Briefly, coverslips were drained of excess moisture and placed cell side up on a glass plate. A piece of nitrocellulose filter paper, presoaked in incubation buffer (125 mM K-acetate, 25 mM Mg-acetate, 1 mM DTT, 25 mM Hepes, pH 7.0) and blotted, was laid on top of the coverslips for 1 min at room temperature (RT). The filter was carefully removed and the coverslips were placed in 5 ml of incubation buffer for 30 min at RT to "wash out" the cytosol. After washout, the cells were placed in 200 µl of assay mix (incubation buffer plus 5 mM EDTA, 1.8 mM $CaCl_2$, ATP regenerating mix, BBC or enriched GF fraction) and incubated at 37°C for up to 1 h. Treatment with PLAPp was as follows: 5 µM PLAPp was added to the assay mix with 1.0 mg/ml BBC or 0.75 µg/ml-enriched GF fraction, added to the cells, and incubated at 37°C for up to 1 h. Treatment with PLA_2 antagonists was as follows: cells were incubated in assay mix containing 1.0 mg/ml BBC, 10 µM inhibitor, 2.5 µM PLAPp for 1 h at 37°C. After all experimental treatments, the cells were fixed in 3.7% formalin/phosphate-buffered saline (PBS) for 10 min, washed in PBS, treated with 0.1% Triton X-100 in PBS to ensure that internal organelles were permeabilized, and then processed for immunofluorescence using antibodies against the medial Golgi protein, α-mannosidase II (Man II).

To quantify the results of these experiments, we assigned the Man II immunofluorescence staining pattern of control and treated cells to

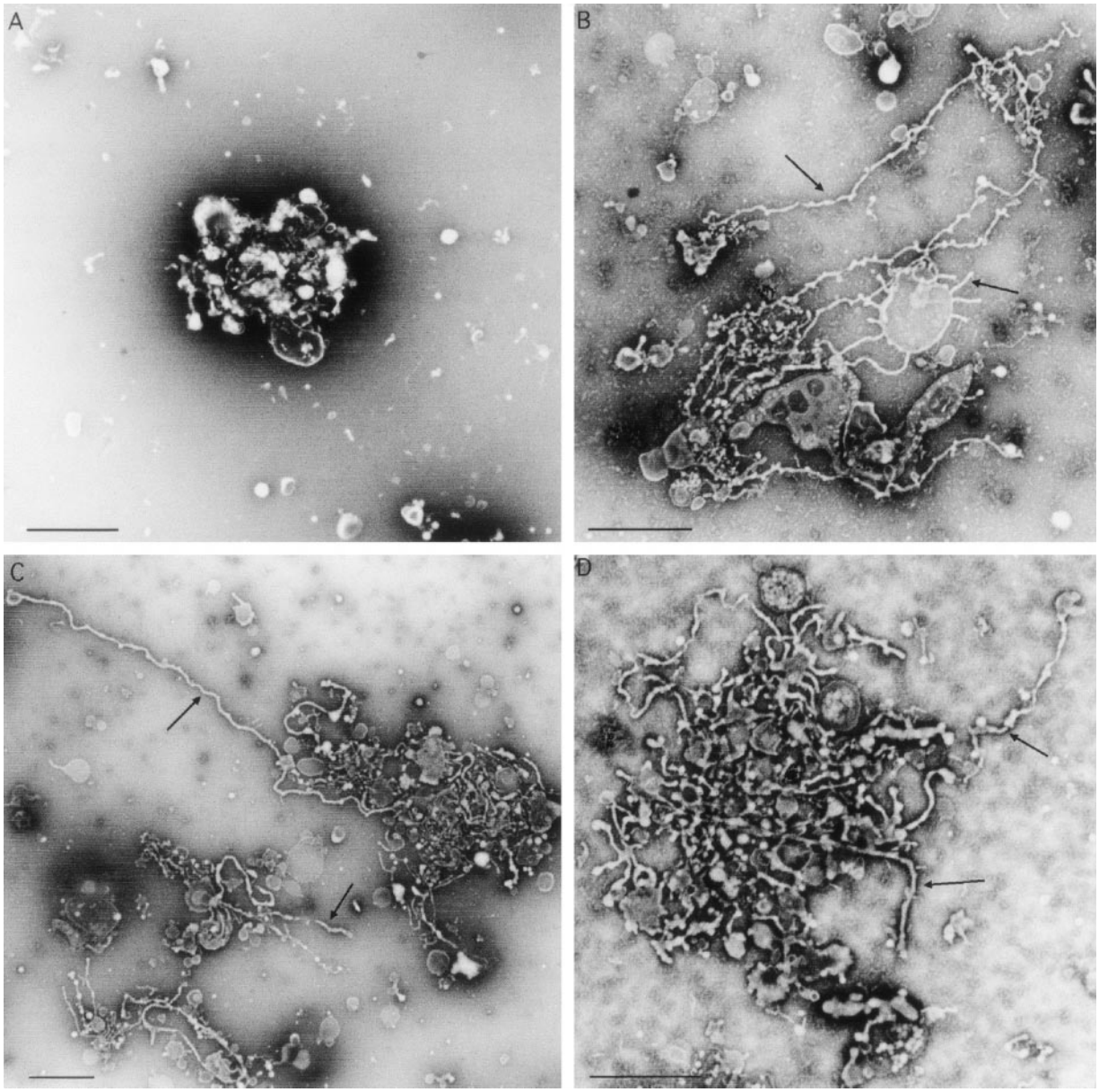

Fig. 1. Visualization of membrane tubule formation from isolated Golgi complexes in vitro by negative stain electron microscopy. **A:** Golgi membranes treated with 1.5 mg/ml bovine serum albumin (BSA) have associated vesicles but few or no tubules. **B:** Golgi membranes treated with 1.5 mg/ml unfractionated bovine brain cytosol (BBC). A significant number of long membrane tubules (arrows) were found in **(C)** 5 μg/ml-enriched gel filtration (GF) fraction, or **(D)** BBC (0.75 mg/ml) plus phospholipase A_2 activating protein peptide (PLAP) (3 μM). All incubations were for 15 min at 37°C and contained 50 μM ATP and 0.5 mM $MgCl_2$. Scale bars = 1 μm.

one of three categories: "juxtanuclear" indicates cells that had a typical Golgi ribbon staining pattern that is found adjacent to the nucleus; "tubular" indicates cells that contained at least one Man II-stained membrane tubule whether or not the cells were stained in the juxtanuclear Golgi region, or diffusely throughout the cytoplasm; "diffuse" indicates cells that had staining throughout the cytoplasm and surrounding the nucleus which is indicative of an ER/nuclear envelope distribution [Wood et al., 1991]. The results were expressed as the percentage total cells found in each category.

RESULTS

Melittin and PLAPp Stimulate In Vitro Tubulation

As we have previously shown [Cluett et al., 1993], the addition of BBC to isolated, intact Golgi complexes in a cell-free system stimu-

lates the formation of long, membranous tubules of 60–80 nm in diameter and up to several microns as seen by EM negative staining (Fig. 1). Extensive fractionation of this BBC preparation, culminating in a gel filtration (GF) column, resulted in a fraction with small subset of proteins that were highly enriched in tubulation activity (Fig. 1C) [Banta et al., 1995]. Half-maximal tubulation occurs with 1.5 mg/ml BBC [Banta et al., 1995] and 1 μg/ml enriched GF fraction [de Figueiredo et al., 1999].

Because PLA_2 antagonists inhibit Golgi membrane tubule formation both in vivo and in vitro [de Figueiredo et al., 1998] [de Figueiredo et al., 1999], we asked whether PLA_2 agonists might conversely stimulate Golgi tubulation. Indeed, addition of melittin, a secreted peptide stimulator of PLA_2s, to isolated Golgi complexes in vitro in the presence of BBC (at subsaturating concentrations for tubulation) resulted in an ~2.5-fold stimulation of BBC-dependent Golgi tubulation in a dose-dependent manner (Fig. 2A). In fact, melittin-stimulated tubules showed a noticeable increase in the length and number of tubules per Golgi stack as compared to BBC-stimulated tubulation (data not shown). Because melittin preparations often contain some Ca^{2+}-dependent phospholipase activity, an alternative PLA_2 stimulator, synthetic PLAPp, was examined for BBC-dependent stimulation of Golgi tubule formation.

To determine whether PLAPp, when added to BBC, could stimulate the formation of Golgi tubules in vitro, various concentrations of PLAPp were incubated with BBC (at subsaturating concentrations for inducing tubulation) for 15 min at 37°C, and then used in the standard in vitro tubulation assay. PLAPp behaved like melittin in that tubules were induced to form (Fig. 1D), resulting in ~2.5-fold stimulation (Fig. 2B, closed circles). Similar results were seen when PLAPp was incubated with subsaturating concentrations of the enriched GF fraction (Fig. 2B, open circles). Stimulation of tubule formation by PLAPp was also investigated using increasing concentrations of BBC to determine whether (1) PLAPp alone could stimulate Golgi tubulation and (2) the stimulation was dependent on the amount of cytosolic protein present. As can be seen in Figure 2C, the addition of PLAPp alone in the absence of BBC did not stimulate tubulation, demonstrating that it acts on a cytosolic factor and not directly on Golgi membranes. The fold stimulation produced by PLAPp was dependent on the

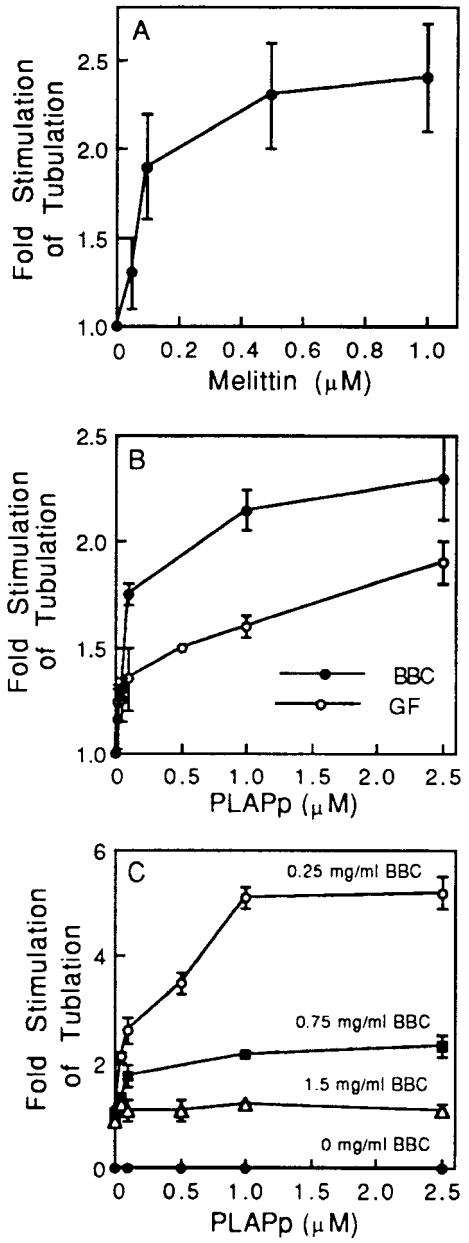

Fig. 2. Phospholipase A_2 (PLA_2) agonists stimulate cytosol-dependent Golgi tubulation in vitro. Various concentrations of **(A)** melittin or **(B)** phospholipase A_2 activating protein peptide (PLAP) were added to 0.75 mg/ml bovine brain cytosol (BBC) (A and B, closed circles) or 0.01 μg/ml-enriched gel filtration (GF) fraction (B, open circles) and incubated for 15 min at 37°C and used in the standard in vitro tubulation assay. **C:** Phospholipase A_2 activating protein peptide ($PLAP_p$) was incubated with increasing concentrations of BBC, incubated for 15 min at 37°C and used in the standard in vitro tubulation assay. The greater the BBC concentration, the smaller the fold stimulation due to a saturation of the tubulation factor(s) at the highest concentration of BBC. The greatest stimulation was found with a concentration of BBC (0.25 mg/ml) that was just above the threshold for stimulating tubulation (open circles). Isolated Golgi complexes were counted for tubule formation and results were represented as the fold stimulation over BBC alone. All samples contained 50 μM ATP and 0.5 mM $MgCl_2$. The data points are averages of duplicate experiments with the associated range in the data.

concentration of BBC such that the greatest fold stimulation occurred at BBC concentrations (0.25 mg/ml) just above the threshhold for inducing tubulation, and the lowest fold stimulation, essentially none, was produced when BBC was at saturating concentrations (1.5 mg/ml). A comparison of the absolute numbers used to derive the fold stimulation under different conditions as shown in Figure 2 is revealing. For example, with saturating concentrations of BBC (1.5 mg/ml), regardless of the concentration of PLAPp added, the percentage tubulation ranged within ~63–67%, which is equal to the maximal amount of tubulation normally achieved in this assay [Banta et al., 1995]. On the other hand, the absolute percentage tubulation achieved with 0.25 mg/ml BBC in the absence of PLAPp was just above background levels at ~12%. However, addition of PLAPp at ≥1 μM to 0.25 mg/ml BBC stimulated the percentage tubulation to 61–62% (or ~5-fold stimulation, as shown in Fig. 2C). Therefore, at concentrations of BBC just above threshhold (0.25 mg/ml), increasing amounts of PLAPp produced a linear and saturable increase in the percentage tubulation, which reached nearly the same levels achieved by saturating amounts of BBC alone. A similar PLAPp-dependent saturation in the fold stimulation of tubule formation was obtained using 0.85 μg/ml of the enriched GF fraction (data not shown).

Reconstitution of Golgi Tubulation in a Permeabilized Cell System

To study the process of Golgi membrane tubulation in a more physiological setting, a permeabilized cell assay was developed that appears to be as sensitive as the EM assay for detecting and quantifying Golgi membrane tubules. Because this assay uses permeabilized cells, the involvement of Golgi tubules in intracellular trafficking events could also be investigated. In this assay, cells were permeabilized by filter stripping with a nitrocellulose membrane, washed to remove soluble cytoplasmic proteins but not cytoskeletal elements or membranous organelles, and then incubated in the presence or absence of various added-back factors, e.g., BBC, GF fraction, and PLAPp. The morphology of the Golgi complex was then visualized by immunofluorescence using an antibody raised against Man II, a medial Golgi enzyme.

When permeabilized cells were incubated with BBC (Fig. 3B) or the enriched GF fraction (Fig. 3C,D) in the presence of an ATP regenerating system and a Ca^{2+}/EDTA buffer, Golgi membranes were seen to tubulate and, in some cells, Man II appeared to redistribute to the ER, as evidenced by a diffuse cytoplasmic and nuclear envelope staining (Fig. 3D). These cells were shown to be permeable by staining of microfilaments when fluorescent phalloidin was included in the incubation mix (data not shown). Tubulation was BBC dependent because cells incubated with assay buffer alone retained the juxtanuclear Golgi staining of control, intact cells (Fig. 3A).

Both BBC and the enriched GF fraction induced Golgi tubulation in permeabilized cells in a time- and dose-dependent manner (Fig. 4). Increasing amounts of BBC (data not shown) or the enriched GF fraction (Fig. 4B) resulted in a

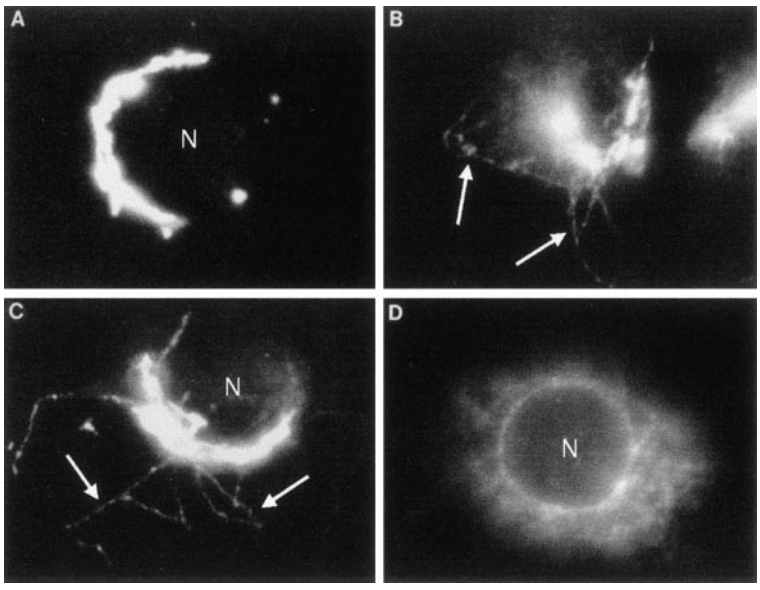

Fig. 3. Tubulation and redistribution of the Golgi complex in a permeabilized cell system as detected by Man II immunofluorescence. Clone 9 rat hepatocytes were filter-stripped, incubated in assay buffer for 30 min at room temperature to "wash out" the soluble cytosolic components, and incubated in **(A)** assay buffer alone showing typical juxtanuclear staining of the Golgi complex, **(B)** bovine brain cytosol (BBC) (1.5 mg/ml) showing extensive Golgi tubulation, **(C)** enriched gel filtration (GF) fraction (5 μg/ml) showing Golgi tubulation, and **(D)** the same as C, but showing nuclear envelope/diffuse endoplasmic reticulum (ER) staining. All samples were incubated at 37°C for 30 min and contained 1.8 mM $CaCl_2$, 5 mM EDTA, and an ATP regenerating system. Arrows, point to Man II-stained tubules. N, nucleus.

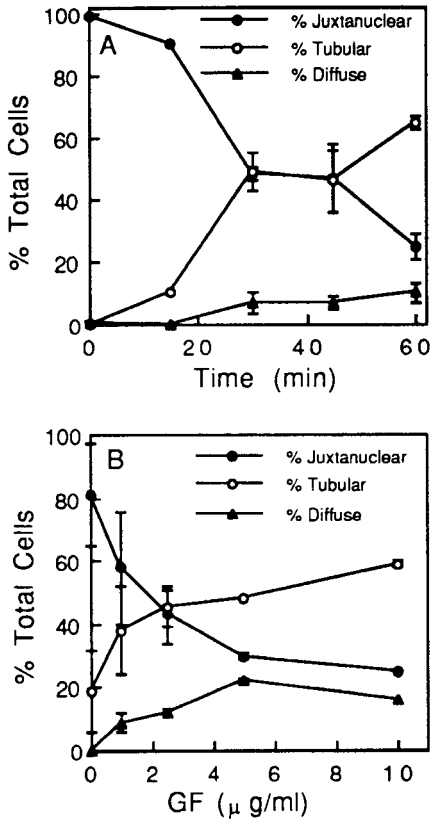

Fig. 4. Time- and cytosolic protein-dependent Golgi complex tubulation and redistribution to the endoplasmic reticulum (ER) in the permeabilized cell system. Clone 9 cells were filter stripped and incubated in assay mix plus **(A)** 1.0 mg/ml bovine brain cytosol (BBC) for up to 1 h or **(B)** increasing amounts of the enriched gel filtration (GF) fraction for 30 min at 37°C after a 30-min washout at room tmeperature. Cells were then stained by immunofluorescence with anti-Man II antibodies and counted to determine the percentage with a typical "juxtanuclear"Golgi pattern as in Fig. 3A (closed circles), a "tubular" pattern as in Fig. 3B, C (open circles), or a "diffuse" pattern with clear nuclear envelope staining as in Fig. 3D (triangles). The data points are averages of duplicate experiments.

loss of juxtanuclear Golgi staining and an increase in the percentage of cells with tubulated Golgi membranes or diffuse/nuclear envelope staining. Maximally, about 20% of the cells showed nuclear envelope staining, whereas >50% of cells after 30 min had distinct Man II-stained tubules. Also, the results showed that the increase in the percentage of cells with tubules preceeded that of ER/nuclear envelope-stained cells, consistent with the idea that tubules serve as intermediates for retrograde trafficking in this assay.

Previously, we described antibodies raised against the major 40-kD protein of the enriched GF fraction that can specifically immuno-deplete tubulation activity from BBC [Banta et al., 1995]. Recent studies show that these anti-40-kD antibodies actually immunoprecipitate a set of proteins with molecular weights similar to those proteins found in the enriched GF fraction, i.e., ~80, 66, 40, 30, and 18 kD (P. de Figueiredo and E. Racoosin, unpublished results). Immuno-depletion experiments with the anti-40-kD antibodies were done here to determine if Golgi tubulation in the permeabilized cell assay had similar requirements. Mock-depletion of BBC with pre-immune IgG did not inhibit Golgi tubulation and redistribution of Golgi enzymes to the ER (Fig. 5B,C). However, when BBC was immuno-depleted with the anti-40-kD antibody (Fig. 5D), cells retained the typical juxtanuclear Golgi staining of control cells (Fig. 5A). Thus, the anti-40-kD antibody removed proteins from BBC that are involved in Golgi membrane tubulation in permeabilized cells. The addition of the enriched GF fraction (at 0.75 μg/ml) back to immuno-depleted BBC restored Golgi tubulation (Fig. 5E), and some retrograde transport activity as seen by diffuse/nuclear envelope staining (Fig. 5F), suggesting that the antibodies did not remove other factors from BBC that might also be required for regulating the tubulation activity in the GF fraction.

PLAPp Stimulates Cytosol-Dependent Tubulation in Permeabilized Cells

Because PLAPp stimulates the formation of BBC-dependent Golgi membrane tubules in vitro, we asked whether the same effect was exhibited in the permeabilized cell assay. After a 30-min washout, filter-stripped cells were incubated in the assay mix containing various combinations of BBC and PLAPp for up to 1 h. Neither PLAPp alone (Fig. 6A) nor BBC alone, at the concentrations used in this study (Fig. 6B), induced tubule formation to a significant degree after 30 min. However, incubation in BBC plus PLAPp induced significant Golgi tubulation by 25 min (Fig. 6D) and ER/nuclear envelope staining by 45–60 min (Fig. 6E,F). Quantitation of these results revealed that ~60% of cells had ER/nuclear envelope staining by 45 min (Fig. 7).

We next investigated whether PLAPp-stimulation in permeabilized cells was dependent on those same cytosolic proteins that were removed by immuno-depletion with the anti-40-kD proteins in the in vitro assay. BBC or the enriched GF fraction was incubated with either pre-immune IgG (mock-depleted) or immune

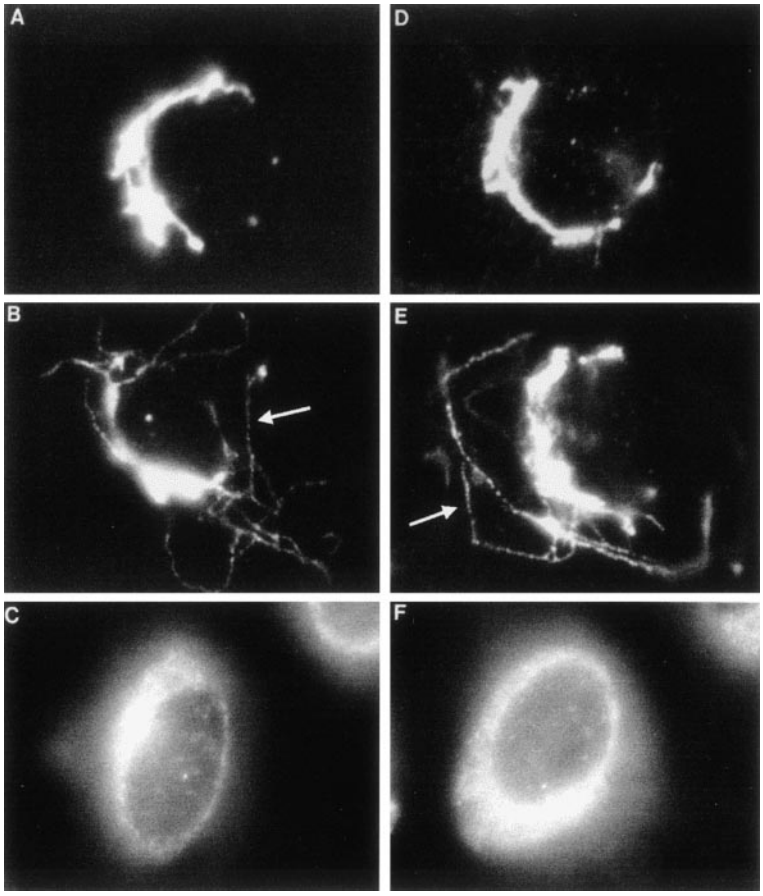

Fig. 5. The anti-40-kD antibody immunodepletes Golgi tubulation activity from bovine brain cytosol (BBC) as detected by the permeabilized cell assay. Clone 9 cells were filter-stripped and incubated in **(A)** buffer alone, which had no effect on the morphology of the Golgi complex; **(B,C)** BBC mock-depleted using pre-immune IgG, which had no effect on the ability of BBC to induce tubules (B) or the redistribution of Man II to an endoplasmic reticulum (ER)-like pattern (C); **(D)** BBC immunodepleted with the anti-40-kD antibody, which abolished the ability of BBC to induce tubules or redistribution to the ER; **(E,F)** BBC immunodepleted as above followed by the addition of enriched GF fraction (0.75 μg/ml) added back, which restored the ability of BBC to tubulate Golgi membranes (E) and redistribute Man II to the ER (F). Arrows, point to Man II-stained tubules. N, nucleus.

IgG (anti-40-kD) and immune complexes were precipitated. The resulting supernatants were incubated with PLAPp for 15 min at 37°C and then used in the permeabilized cell assay (Fig. 8). Mock-depleted samples displayed typical PLAPp-stimulated Golgi tubulation (Fig. 8B) and redistribution to the ER/nuclear envelope (Fig. 8C). However, permeabilized cells incubated with immuno-depleted BBC did not display PLAPp-stimulated tubulation (Fig. 8D), as they maintained a typical juxtanuclear Golgi pattern that resembled control cells (Fig. 8A). This loss of tubulation activity could be stored by adding back the enriched GF fraction to immuno-depleted samples, which resulted in the formation of membrane tubules (Fig. 8E), and in some cells, a diffuse ER-like staining (Fig. 8F). These results suggest that PLAPp was exerting its effects on a cytosolic protein(s) that was removed by the anti-40-kD antibody and required for Golgi membrane tubulation.

PLA_2 Antagonists Inhibit PLAPp-Stimulated Golgi Tubulation

To further support the conclusion that a cytosolic PLA_2 is involved in Golgi membrane tubulation, and to investigate whether PLAPp was apt to be operating via a PLA_2 activity, BBC was treated with a variety of PLA_2 antagonists followed by PLAPp treatment (Fig. 9). These PLA_2 antagonists have previously been shown to inhibit BFA-stimulated tubulation in vivo [de Figueiredo et al., 1998]. Indeed, we found that ONO-RS-082 (10 μM), a potent antagonist of cytosolic PLA_2s [Banga et al., 1989] inhibited both BBC-dependent (Fig. 9A,B) and PLAPp-stimulated (Fig. 9C,D) tubulation of Golgi membranes in permeabilized cells.

These observations were extended to compare the effects of a variety of PLA_2 inhibitors on PLAPp-stimulated tubulation in both the permeabilized cell and in vitro assays. For these studies, several highly specific inhibitors of cytoplasmic PLA_2s were used, including the trifluoromethyl ketone derivatives of arachidonic ($AACOCF_3$) and palmitic acid ($PACOCF_3$)[Gelb et al., 1994; Hazen et al., 1991; Street et al., 1993], ONO-RS-082, and BEL, an inhibitor specific for Ca^{2+}-independent cytoplasmic PLA_2s [Ackermann et al., 1995; Hazen et al., 1991]. The results showed that these antagonists inhibited BBC-depentent, PLAPp-stimulated

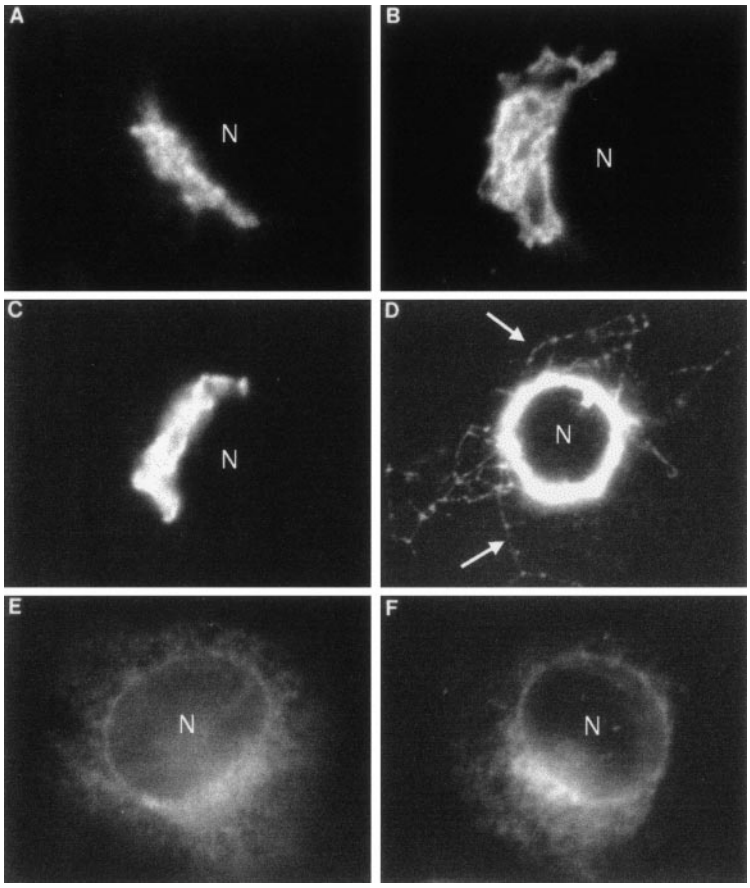

Fig. 6. Morphology of phospholipase A_2 activating protein peptide (PLAPp)-stimulated Golgi complex tubulation and redistribution over time in permeabilized cells. Clone 9 cells were filter-stripped and incubated in assay buffer in the presence or absence of various combinations of bovine brain cytosol (BBC) and PLAPp (5 μM) for up to 1 h at 37°C after a 30-min washout at room temperature. **A:** PLAPp alone, 1 h. **B:** BBC alone, 1 h. **C:** BBC + PLAPp for 10 min. **D:** 25 min. **E:** 45 min. **F:** 1 h. Arrows, point to Man II-stained tubules. N, nucleus.

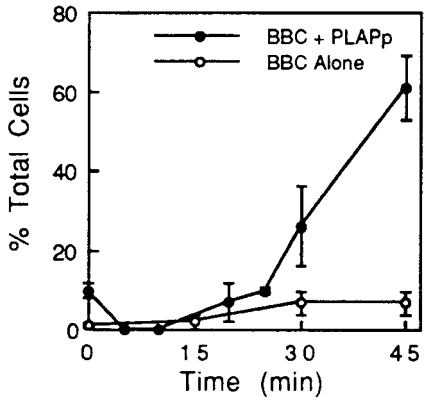

Fig. 7. Time course of phospholipase A_2 activating protein peptide (PLAPp)-stimulated Golgi complex redistribution in permeabilized cells. Clone 9 cells were permeabilized and incubated in assay buffer containing bovine brain cytosol (BBC) (open circles) or BBC + 5 μM PLAPp (closed circles) for up to 1 h at 37°C after a 30-min washout at room temperature. Cells were counted to determine the percentage with a diffuse endoplasmic reticulum (ER)/nuclear envelope distribution. All data points are averages of duplicate experiments.

Golgi tubulation to approximately the same extent, both in vitro and in permeabilized cells (Fig. 10A,B).

Finally, we examined whether PLAPp could stimulate a PLA_2 activity in our BBC preparation. Using phosphatidic acid with ^{3}H-oleate in the sn-2 position as a substrate and a vesicle-based assay as previously described [de Figueiredo et al., 1998], we found that BBC (at a concentration that was subsaturating for tubulation) increased the amount of ^{3}H-oleate released to 185% (±1.01) that of controls. The addition of PLAPp (1 μg/ml final) to BBC further increased the amount of ^{3}H-oleate released to 271% (±8.99) of control, an enhanced release that was completely abolished by addition of BEL (25 μM). These results show that PLAPp stimulated a BEL-sensitive PLA_2 activity in BBC.

DISCUSSION

We have recently provided evidence, based on the use of broad spectrum of inhibitors, that a cytoplasmic PLA_2 is intimately involved in

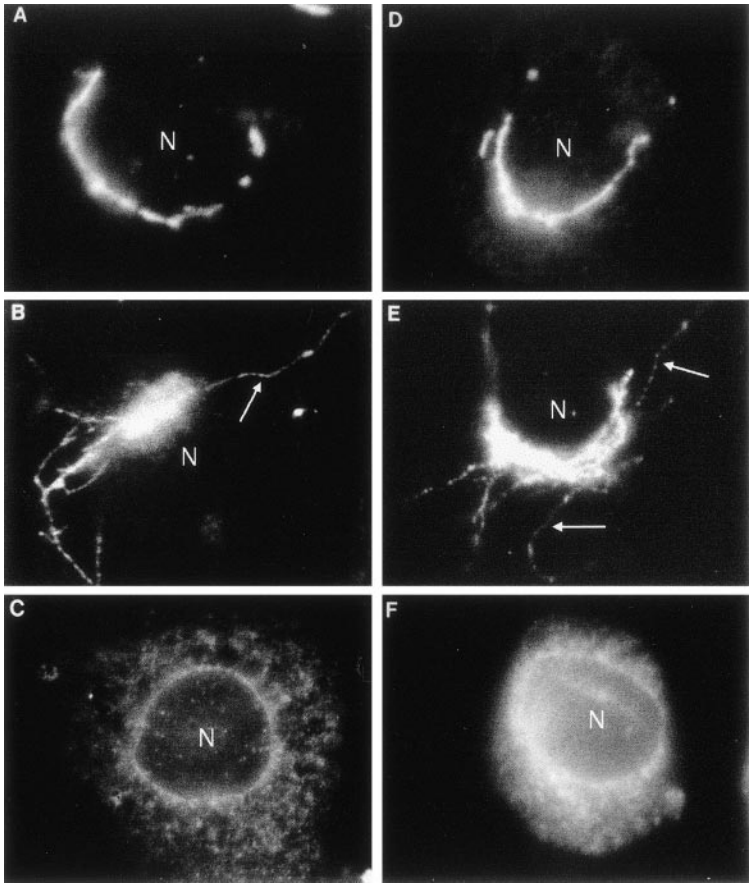

Fig. 8. Phospholipase A_2 activating protein peptide (PLAPp)-stimulated Golgi tubulation and redistribution of Man II to the endoplasmic reticulum (ER) in permeabilized cells is inhibited by immuno-depleting bovine brain cytosol (BBC) with the anti-40-kD antibody, which can be restored by the GF fraction. Clone 9 cells were permeabilized and incubated in **(A)** buffer control; **(B,C)** mock-depleted BBC using pre-immune IgG + PLAPp; **(D)** BBC immuno-depleted with the anti-40-kD antibody + PLAPp; **(E,F)** immuno-depleted BBC + PLAPp followed by addition of 0.1 µg/ml-enriched gel filtration (GF) fraction. Arrows, point to Man II-stained tubules. N, nucleus.

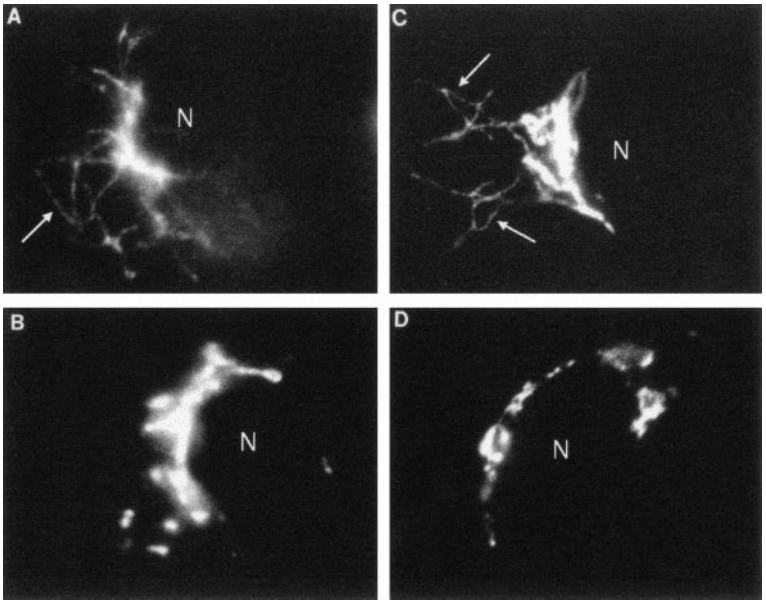

Fig. 9. The cytosolic phospholipase A_2 (PLA_2) antagonist ONO-RS-082 inhibits bovine brain cytosol (BBC)-dependent and phospholipase A_2 activating protein peptide (PLAPp)-stimulated Golgi tubulation in permeabilized cells. Clone 9 cells were filter-stripped, washed for 30 min at room temperature to remove cytosolic proteins, and incubated in assay buffer containing BBC and various combinations of ONO-RS-082 (10 µM) or PLAPp (5 µM) for various periods of time at 37°C. **A:** Stimulatory amounts of BBC alone for 15 min. **B:** BBC + 10 µM ONO-RS-082 for 15 min. **C:** BBC + 2.5 µM PLAPp for 1 h. **D:** BBC + 10 µM ONO-RS-082 + 2.5 µM PLAPp for 1 h. Arrows, point to Man II-stained tubules. N, nucleus.

the process of membrane tubule formation from the Golgi complex, both in vivo and in an in vitro reconstitution assay [de Figueiredo et al., 1998] [de Figueiredo et al., 1999]. In this study we provide complementary evidence for the role of a PLA_2 in Golgi membrane tubulation by showing that known stimulators of PLA_2 activity, melittin and PLAPp, enhance cytosol-dependent Golgi tubulation in the in vitro assay, and in a newly developed permeabilized cell system.

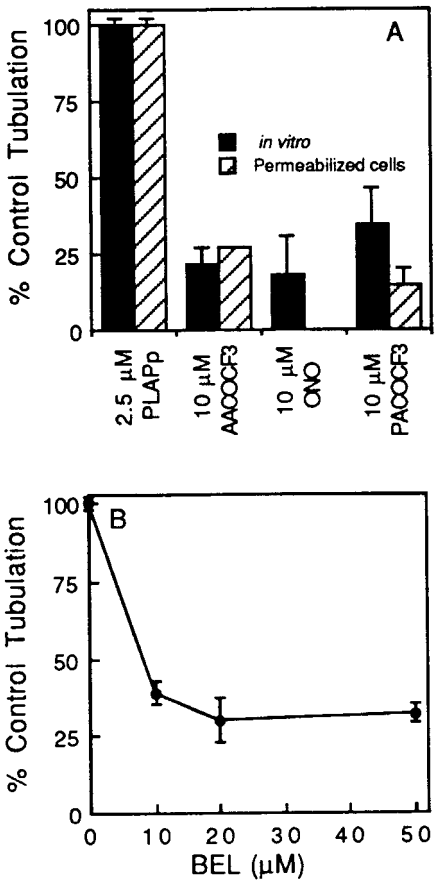

Fig. 10. Cytosolic phospholipase A_2 (PLA_2) antagonists inhibit PLAPp-stimulated Golgi tubulation both in vitro and in permeabilized cells. **A:** Bovine brain cytosol (BBC) (0.75 mg/ml) was incubated with 10 µM inhibitor for 15 min at 37°C followed by 1 h at 37°C with 2.5 µM PLAPp and then used in the standard in vitro tubulation (solid bars) or permeabilized cell (striped bars) assay. **B:** BBC was incubated with increasing concentrations of BEL at 37°C for 15 min and then dialyzed against BBC dialysis buffer for 45 min at 4°C to remove unbound BEL. The dialyzed BBC (0.5 mg/ml) was incubated with 2.5 µM PLAPp for 15 min at 37°C and used in the standard in vitro tubulation assay. In order to compare samples from different conditions and experiments, the results were expressed as the percentage control tubulation, which was the absolute percentage tubulation without PLA_2 antagonists divided by the percentage tubulation with antagonists times 100. The data points are averages of duplicate experiments; error bars show the range in the data.

The establishment of a permeabilized cell system will be very useful for elucidating the mechanisms of tubule formation under more physiological conditions, and for identifying the trafficking events mediated by tubules. In this regard, we note that in permeabilized cells, extensive Golgi membrane tubulation preceeded the detection of Man II in the ER/nuclear envelope (Fig. 4), suggesting that tubules were the trafficking intermediates in Golgi-to-ER retrograde trafficking under these conditions. These results are consistent with previous studies using BFA to stimulate tubule-mediated retrograde trafficking [Lippincott-Schwartz et al., 1989, 1990], and more recent studies using GFP-labeled resident Golgi proteins to image tubule formation in untreated, living cells [Presley et al., 1998; Sciaky et al., 1997]. We note, however, that the apparent efficiency with which Man II appeared in the ER was much less than its appearance in tubules (maximally, only ~20% of cells exhibited ER staining after tubule formation), i.e., tubulation was more efficiently induced than retrograde transport. This result may be a consequence of the fact that the BBC, and especially the enriched GF fraction, were prepared so as to maximize tubulation activity and may be deficient in factors needed for recognition and fusion of Golgi-derived tubules with the ER.

The permeabilized cell assay for tubulation is also valuable in that it serves as an important check against results obtained by the in vitro tubulation assay. Significantly, tubulation induced and measured in both assays appeared to be dependent upon the same subset of cytosolic proteins found in the enriched GF fraction. Also, tubulation in both assays was potently inhibited by the same spectrum of PLA_2 antagonists, consistent with our previous in vivo studies from which we concluded that BFA-stimulated Golgi membrane tubulation requires a cytosolic PLA_2 activity [de Figueiredo et al., 1998]. We do not know which of the proteins found in the enriched GF fraction corresponds to the relevant PLA_2. It does not appear that a particular fatty acid is preferred by the enzyme because the trifluoromethylketone derivatives of arachidonyl and palmitoyl fatty acids, AACOCF$_3$ and PACOCF$_3$ [Ackermann et al., 1995; Hazen et al., 1991; Street et al., 1993] inhibited activity to the same extent. However, since BEL, which is 1,000-fold more selective for Ca^{2+}-independent versus Ca^{2+}-dependent cytoplasmic PLA_2s [Ackermann et al., 1995; Hazen et al., 1991], potently inhibited both BBC-dependent and PLAPp-stimulated tubulation, the PLA_2 may fall into the class of Ca^{2+}-independent enzymes [Dennis, 1997], consistent with previous studies indicating that Golgi membrane tubulation is Ca^{2+}-independent [Banta et al., 1995].

Our conclusion that Golgi membrane tubulation requires the action of a cytoplasmic PLA_2 is bolstered by finding that melittin and PLAPp stimulated tubulation both in vitro and in permeabilized cells. It appears likely that PLAPp was acting directly on the same cytoplasmic activity that is required for tubulation because PLAPp stimulated the tubulation activity in the enriched GF fraction and immuno-depletion of BBC by the anti-40-kD antibody, resulted in the loss of PLAPp-stimulated tubulation. Thus, one prediction from these results is that PLAPp should interact directly with the PLA_2 component of the GF fraction, an idea that we are pursuing. One potential source of concern when using melittin or PLAPp is that they may cause nonspecific perturbations to the phospholipid bilayer because both are enriched in hydrophobic amino acids; however, at the concentrations used here to stimulate cytosol-dependent tubulation, PLAPp alone had no effect.

Although the results showed that PLAPp stimulates Golgi membrane tubule formation, and PLA_2 activity in cytosolic extracts, we do not know whether endogenous PLAP, or PLAP-related proteins, carry out a such a regulatory function in vivo. In fact, the actual in vivo functions and PLA_2 targets of these proteins are unclear. Besides stimulating various PLA_2 activities, the PLAPp itself has been shown to influence a variety of activities both in vivo and in vitro including neutrophil aggregation and lysosomal enzyme release [Bomalski et al., 1989], Ca^{2+} oscillations and pancreatic amylase secretion via Gb protein and arachidonic acid cascades [Tsunoda and Owyang, 1994], contractions of smooth muscle cells via a MAP kinase-protein C cascade [Yamada and Bitar, 1995], the synthesis of interleukin-1 (IL-1) and tumor necrosis factor (TNF) production by human monocytes [Bomalaski et al., 1995], and cyclic addenosine monophophate (cAMP)-induced Ca^{2+} influx in *Dicytostelium* [Schaloske and Malchow, 1997]. Moreover, expression levels of PLAP and PLAP-related proteins have been shown to be influenced by a diverse array of conditions, including monsodiuim urate crystals in monocytes [Bomalaski et al., 1990], hypoxia in hepatocytes [Wang et al., 1996], response of yeast cells to volatile anesthetics [Keil et al., 1996], and IL-1 in murine T cells and rabbit colon [Bomalaski et al., 1992; Homaidan et al., 1997]. Thus, endogenous PLAP may be directly or indirectly involved in a variety of cellular responses, many of which may involve signaling via PLA_2-generated metabolites. Alternatively, the use of PLAPp in our studies, and many of the above, may simply reflect its value as a pharmacological tool to stimulate various PLA_2 activities, enzymes which, in fact, are not normally regulated by endogenous PLAP.

Even though these results strongly suggest the involvement of a cytosolic PLA_2 in the formation of Golgi membrane tubules, the mechanism by which the tubules form is unclear. A cytosolic PLA_2 could be involved both directly and indirectly in tubulation. Based on experiments using BFA to stimulate tubulation [de Figueiredo et al., 1998], we have suggested that the PLA_2 might act directly upon Golgi membranes to generate a local accumulation of hydrolysis products, primarily lysophospholipids, in the cytoplasmic leaflet of the bilayer. This accumulation could induce a conformational change of the local membrane environment from planar to convex resulting in the formation of a tubule, as has been demonstrated to occur in both biological membranes and artificial liposomes [Christiansson et al., 1985; Fujii and Tamura, 1979; Israelachvili et al., 1982; Mui et al., 1995]. This suggestion does not rule of the possibility that a PLA_2 also serves to generate lipid-based signaling molecules, e.g., eicosanoids from arachidonic acid; however, we found that a broad spectrum of antagonists of arachidonic acid metabolism did not inhibit BFA-stimulated tubulation [de Figueiredo et al., 1998]. In either case, these studies help to establish a novel PLA_2-dependent mechanism for inducing tubule formation from Golgi membranes, and, moreover, suggest a novel role for cytoplasmic PLA_2s in controlling organelle structure and in tubule-mediated intracellular trafficking events.

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