Protein Traffic from the Secretory Pathway to the Endosomal System in Pancreatic β-Cells*

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Constitutive-like secretion involves vesicular trafficking corresponding kinetically and biochemically with a post-trans-Golgi network (TGN) origin. In pancreatic β-cells, the budding of AP-1/clathrin-coated vesicles, a portion of which is derived from immature secretory granules, has been hypothesized to initiate constitutive-like trafficking. However, ~30 min after release of a 20 °C intracellular transport block in pancreatic β-cells (to synchronize protein egress from the TGN), addition of brefeldin A (BFA) (which inhibits AP-1 recruitment) was reported not to block subsequent constitutive-like secretion. To further explore post-TGN trafficking in pancreatic β-cell lines, we have followed the fate of pulse-labeled procathepsin B (ProB, a lysosomal protease) after postpulse wortmannin treatment or the BFA treatment described above. We find that continuous wortmannin treatment allows ProB to reach immature secretory granules but inhibits its egress from maturing granules. Remarkably, BFA treatment causes augmented unstimulated secretion of newly synthesized ProB that is not paralleled by insulin. This effect requires a delay of 25–35 min after release from the 20 °C block. Further, when ProB delivery to endosomes is inhibited, its BFA-augmented secretion is eliminated. We hypothesize that the constitutive-like pathway involves an endosomal intermediate.

Regulated secretory cells package selected proteins within storage granules, which first form as immature secretory granules (ISGs) that bud from the TGN (1, 2). Sorting of a subpopulation of secretory proteins at the TGN contributes to the distinct composition of constitutive secretory vesicles (which rapidly convey their contents from the TGN directly to the cell surface) versus ISGs (3–7). This does not work the same way in all regulated secretory cells (8). In an additional mechanism, sorting of a subpopulation of proteins during granule maturation leads to vesicular exit of unstored proteins from ISGs, via the constitutive-like pathway (9–12). During this process, selective condensation of luminal proteins (13), possibly in conjunction with proteins associated with the luminal side of the granule membrane (14–17), contributes to sorting by retention within maturing granules (18).

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‡§ The abbreviations used are: ISG, immature secretory granule; CCV, clathrin-coated vesicle; M6P, mannose 6-phosphate; PAGE, polyacrylamide gel electrophoresis; ProB, procathepsin B; TGN, trans-Golgi network; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine.

The only vesicles thus far established to bud from ISGs are clathrin-coated (19–21). Indeed, in pancreatic β-cells treated with canavanine, ISGs are unable to shed their clathrin coats (22), and this correlates strongly with a blockade of constitutive-like secretion but leaves unaltered the regulated exocytosis of new granules (23). Although multiple different adaptor components have now been described (24–27), to date, the AP-1 adaptor complex primarily is thought to coat the buds emerging from ISGs (28), resulting in the selective removal from ISGs of furin (29), mannose 6-phosphate receptors (30), and probably other transmembrane proteins (31) that exit from maturing granules. These same AP-1/clathrin-coated vesicles (AP-1/CCVs) are thought to convey lysosomal proenzymes with high efficiency from ISGs to endosomes (32). We have hypothesized that with low efficiency, AP-1/CCVs also convey the “first limb” of the constitutive-like pathway, using the fluid phase to capture soluble proteins such as proinsulin, as well as C-peptide and possible conversion intermediates (23), whereas a “second limb” might involve subsequent trafficking from endosomes to the cell surface (18).

By exploiting release from the 20 °C TGN exit block, we previously investigated the effects of brefeldin A (BFA) on proinsulin trafficking through the distal secretory pathway in pancreatic islet β-cells; with this protocol, we found, aside from the well recognized BFA blockade of export from the TGN (33–36), no significant additional BFA-mediated inhibition of constitutive-like secretion (37). This result has been a long-standing puzzle to us, not only because of the known ARF-mediated, BFA-sensitive block of AP-1/clathrin recruitment to membranes (38–41), including ISGs (28), but because results from other groups (using protein sulfation with 35SO4 to synchronize pulse labeling in the TGN) have suggested that BFA significantly impairs constitutive-like coated vesicle budding in two independent secretory cell types (42, 43). Thus, in conjunction with our experimental protocol, our previous results in β-cells might reflect operation of a BFA-insensitive class of AP-1/CCVs, such as those utilizing γ2-adaptin (25, 26), or alternatively, other unsuspected effects of BFA (e.g. on the second limb of the constitutive-like pathway) might offset or obscure detection of effects on AP-1/CCV budding. Recently we have developed assays indicating that newly synthesized procathepsin B (ProB) is a favorable (i.e. sensitive and specific) marker of AP-1/CCV traffic from the secretory pathway to endosomes in pancreatic β-cells (32). Using this marker, we now present results that show that once newly synthesized luminal proteins have reached the endosomal system, BFA enhances their trafficking to the cell surface, effectively stimulating the second limb of the constitutive-like pathway.

MATERIALS AND METHODS
Antibodies and Other Materials—Immunoprecipitating antisera against rat ProB (44) was graciously provided by Dr. J. Mort (Shriners
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Hospital, Montreal, Quebec, Canada). Guinea pig anti-insulin and anti-C-peptide were from Linco (St. Louis, MO). Tunicamycin was from Sigma, calf serum and antibiotic-antimycotic solution were from Life Technologies, Inc., [35S]methionine/cysteine (Expre[S]S) was from NEN Life Science Products, and zysorbin-protein A was from Zymed Laboratories Inc. (San Francisco, CA).

Preparation and labeling of cell lines—The INS-1 (rat β cell line was cultured exactly as described (45) except that in some experiments, plasticware was precoated with poly-n-lysine. The β-T3C (mouse cell line was cultured as described previously (46, 47). Both cell lines were grown in Dulbecco’s modified Eagle’s medium, prior to pulse-labeling for 30 min at 37 °C in the same medium containing ~100 μCi of [35S]methionine and [35S]cysteine, both cell lines were cultured as described previously (46, 47). Both cell lines were grown in Dulbecco’s modified Eagle’s medium, containing 10 mM glucose, 1 mM isobutyl-methylxanthine, 1 mM tolbutamide, 10 mM acetate, 1 mM isosubtlxythanol, 1 mM tsubtalamide, 10 mM leucine, and 10 mM glutamine. Cells were lysed in boiling 0.3% SDS. Samples were then diluted 1:3 in immunoprecipitation buffer yielding final concentrations of 100 mM NaCl, 1% Triton X-100, 0.2% sodium deoxycholate, 0.1% SDS, 10 mM EDTA, and 25 mM Tris, pH 7.4. An antiproteasome mixture of aprotinin (1 milliunits/ml), leupeptin (0.1 mM), pepstatin (10 μM), E64 (10 μM), EDTA (5 μM), and diisopropylfluorophosphatase (1 mM) was added to the cell lysates and collected media. Both cells and media were precleared by a brief incubation with Zyorbin prior to immunoprecipitation.

SDS-PAGE, Fluorography, and Phosphorimaging—Immuno-precipitated cathepsin B was resolved by SDS 12%-PAGE, and immunoprecipitated insulin was analyzed by SDS 15%-PAGE plus urea using the Tricine buffer system (48). The products of the two insulin genes expressed in rodents were not distinguished electrophoretically. The insulin gels were fixed initially in 20% trichloroacetic acid (20 min), then in 12.5% trichloroacetic acid plus 50% methanol (15 min), then bathed in water (10 min), and finally incubated with 1 N sodium salicylate for 15 min. Dried gels were exposed to XAR film at ~70 °C. Scanned x-ray films were finally analyzed using the ImageQuant program (Molecular Dynamics).

Endosome to lysosome delivery assay—The assay performed was a modification of that described in Ref. 49. Briefly, purified [35S]methionine-labeled Semliki Forest virus particles (50) were applied to subconfluent INS-1 cells for 1.5 h at 4 °C. The cells were washed extensively to remove unbound virus and then warmed to 37 °C to permit endocytosis to take place. In preliminary experiments, we estimated the kinetics of endocytic uptake as measured by resistance of virus digestion to extracellularly added subtilisin at 4 °C (51).

Results

Effect of Wortmannin on the Trafficking of Newly Synthesized Insulin and Lysosomal Enzymes in ISGs—It is generally agreed that the AP-1/CCV-mediated trafficking of lysosomal proenzymes to endosomes is sensitive to inhibition by the PI 3-kinase inhibitor wortmannin (52, 53) (although the underlying mechanism for this sensitivity is debated (54–56)). In pancreatic β-cells, a significant portion of lysosomal proenzymes fail to exit the secretory pathway at the level of the TGN and proceed to enter ISGs. We therefore examined the effects of 100 nM wortmannin, a dose that does not inhibit secretory granule exocytosis from β-cells (57). In pulse-chase experiments, 100 nM wortmannin had no effect on the amount of newly synthesized secreted protein immunoprecipitable with anti-insulin antibodies in the first 3 h of chase or in the subsequent 4 h of chase under unstimulated or stimulated conditions (although there was a very modest inhibitory effect on intragranular proinsulin conversion; Fig. 1). In addition, recovery of intracellular insulin at the end of the chase was indistinguishable between wortmannin treated and untreated INS cells (Fig. 1).

However, wortmannin-treated INS cells, even after a prolonged

FIG. 1. Wortmannin (WM) (100 nM) does not block the formation or regulated exocytosis of insulin secretory granules. INS-1 cells were pulse-labeled with [35S]Met/Cys for 30 min. After an initial 3-h chase period in the presence or absence of wortmannin, the cells were either stimulated (Stim+) or not stimulated (Stim−) with a secretagogue mixture for 4 h in the continued presence or absence of wortmannin. Media and cell lysates were then collected and analyzed by immunoprecipitation with anti-insulin antibodies as described under “Materials and Methods.” The mobilities of proinsulin (Pro) and mature insulin (Ins) are shown; proinsulin conversion intermediates (not specifically indicated in the figure) run above proinsulin in this gel system.

FIG. 2. Effect of 100 nM wortmannin (WM) on lysosomal enzyme trafficking. From the experiment described in Fig. 1, samples of media and cell lysates were examined for the presence of newly synthesized lysosomal cathepsin B by immunoprecipitation, SDS-PAGE, and fluorography. The mobilities of the cathepsin B precursor (ProB) and mature cathepsin B (B) are shown. Note that the presence of wortmannin during the chase causes a defect in intracellular cathepsin B maturation along with an increase in the secretion of ProB, primarily during the first 3 h of unstimulated chase, as well as during a subsequent period of stimulation with secretagogue.

FIG. 3. BFA-induced inhibition of procathepsin B maturation in post-TGN compartments. INS cells were pulse-labeled and chased for a pretreatment period of 2 h at 19.5 °C to accumulate newly synthesized proteins in the TGN (chase time = 0). Cells were then rewarmed to 37 °C, and BFA (10 μM)/b was added at either 0, 0.5, or 2 h of release chase from the temperature block. The cells were then incubated until 6 h of chase at 37 °C (in the absence or presence of a secretagogue mixture, indicated by Stim). Lysates were analyzed for cathepsin B by immunoprecipitation, SDS-PAGE, and fluorography. The positions of precursor (ProB) and mature form (B) are shown.
of cathepsin B approached completion (Fig. 3, right panel). As expected (52, 53), wortmannin caused an increase in the apparent constitutive secretion of newly synthesized ProB (Fig. 2, left panel). Importantly, however, after 3 h of chase (sufficient to allow for secretory granule maturation), wortmannin-treated INS cells showed abnormally persistent stimulus-dependent secretion of newly synthesized ProB (Fig. 2). These results are consistent with the idea that in addition to the TGN-derived route, newly synthesized lysosomal proenzymes in INS cells utilize an AP-1/CCV exit pathway from ISGs to endosomes.

Effect of BFA on the Delivery of Newly Synthesized ProB to Lysosomes—Because ProB appears to be a suitable marker for the ISG → endosome → lysosome pathway in β-cells, we next explored the effects of BFA on this process. As others have done, to avoid confounding inhibitory effects of BFA on transport through the ER/Golgi system, pulse-labeled cells were chased first for 2 h at 19.5 °C to accumulate ProB and proinsulin in the TGN, and BFA was then added at different times after release of the temperature block. If INS cells were allowed to warm for 2 h at 37 °C before the addition of BFA, maturation of cathepsin B approached completion (Fig. 3, right lanes). By contrast, if the TGN exit block was not released before BFA was added, intracellular ProB maturation failed to occur even after 6 h more at 37 °C (Fig. 3, left lanes), and there was no stimulus-dependent secretion of ProB, consistent with the known BFA blockade of export from the TGN (43). Based on published measurements of the half-time of TGN exit upon release from the 19.5 °C block in β-cells (23), warm-up for 25 min allows anterograde advance of ≥75% of labeled proinsulin from this compartment. However, even after a 30-min warm-up at 37 °C, BFA addition still prevented at least half of the intracellular ProB molecules from ever reaching lysosomes (Fig. 3, left panel, middle lane), suggesting the existence of one or more BFA-sensitive trafficking steps beyond the TGN in these cells.

After Exit from the TGN, BFA Selectively Augments Unstimulated Secretion of Newly Synthesized ProB—Reproducibly in our experiments, when the TGN block was first released for 7 h, chased, showed an obvious defect in the delivery of ProB to lysosomes, as measured by deficient maturation of the enzyme (Fig. 2, right panel). As expected (52, 53), wortmannin caused an increase in the apparent constitutive secretion of newly synthesized ProB (Fig. 2, left panel). Importantly, however, after 3 h of chase (sufficient to allow for secretory granule maturation), wortmannin-treated INS cells showed abnormally persistent stimulus-dependent secretion of newly synthesized ProB (Fig. 2). These results are consistent with the idea that in addition to the TGN-derived route, newly synthesized lysosomal proenzymes in INS cells utilize an AP-1/CCV exit pathway from ISGs to endosomes.

Effect of BFA on procathepsin B trafficking in the distal secretory pathway. Cells were incubated at 19.5 °C for 2 h to accumulate newly synthesized proteins in the TGN. Where indicated, BFA (10 μg/ml) was added after a 30-min warm-up to 37 °C, and this chase medium was collected after a further 10 min (+30–40 min, left panel). Subsequent to this period, the cells were incubated for 4 h at 37 °C in the continued presence or absence of BFA, in media lacking or possessing a secretagogue mixture (Stim). At the end of this period, cathepsin B in the media and cell lysates were analyzed by immunoprecipitation, SDS-PAGE, and fluorography.

Negligible effects of BFA on the maturation and secretion of proinsulin-derived peptides. From the experiment described in Fig. 4, samples of media and cell lysates were examined for the presence of newly synthesized insulin-containing peptides by immunoprecipitation, SDS-PAGE, and fluorography.

25–35 min at 37 °C, subsequent addition of BFA caused a clear increase in the quantity of unstimulated ProB secreted from INS cells during a 4-h interval (from 40 min to 4 h 40 min of chase; Fig. 4, middle panels), a time period expected to fully encompass constitutive-like secretion (as well as unstimulated granule exocytosis). From four independent experiments, this increased ProB secretion by BFA quantitatively appeared in the range of 15–20% of total immunoreactive cathepsin B. In the absence of BFA (32), secretagogue addition during this chase period stimulated the release of newly synthesized ProB (Fig. 4, right panels). Remarkably, in the presence of BFA, secretagogue addition caused an even greater release of newly synthesized ProB than from control INS cells, and the increment suggested that it was composed of the sum of the BFA-enhanced unstimulated secretion and regulated granule exocytosis (Fig. 4, right panels). Interestingly, in the presence of BFA, when measured at the identical chase times in the identical samples, there was no augmentation of labeled insulin secretion whatsoever, and there were only very subtle increases in the small amounts of unstimulated secretion of newly synthesized proinsulin and proinsulin conversion intermediates (Fig. 5, middle panels). These data suggest that newly synthesized ProB released between 40 min and 4 h 40 min after exit from the TGN derives from two pools, the first representing the exocytosis of ISGs (in parallel with insulin and exhibiting stimulus-dependent secretion) and the second from outside of the regulated secretory pathway (nonparallel with insulin and exhibiting augmented secretion by BFA even in the absence of secretagogue). The origin of this other compartment cannot be the TGN (because BFA blocks rather than stimulates protein export from the TGN (33, 34, 37, 43)), and it cannot be from lysosomes (because whereas lysosomes contain only mature cathepsin B, secretion is restricted to the proenzyme form (see Fig. 4)).

These data would appear to represent the first report of BFA-mediated enhancement (rather than blockade) of traffic of any protein through the anterograde secretory pathway. However, the effects of BFA may vary between cell types and species (58). To see whether the enhancement of unstimulated
ProB secretion was unique to INS-1 (rat β) cells, we performed similar experiments in the β-TC3 (mouse-derived) cell line. Initial experiments in β-TC3 cells confirmed that BFA treatment after release of the TGN exit block inhibited the intracellular maturation of cathepsin B (not shown). More importantly, when pulse-labeled β-TC3 cells arrested at 19.5 °C were shifted to 37 °C for 35 min before BFA addition, subsequent unstimulated ProB secretion was augmented, whereas proinsulin secretion was not (Fig. 6, right panels). However, when the initial warm-up period was only 20 min, the BFA-mediated increase of unstimulated ProB secretion could not be detected (Fig. 6, left panels). Similar negative results were obtained with a 15-min warm-up period in INS-1 cells (data not shown). These data suggest not only that our results in INS-1 cells (Figs. 3–5) represent a general finding in pancreatic β-cells but also that the BFA-mediated augmentation of unstimulated ProB release is a strictly time-dependent phenomenon, corresponding to a specific kinetic window after ProB exit from the TGN.

The Increased Unstimulated Secretion of Newly Synthesized Luminal Proteins by BFA Is Accounted for by Enhanced Endosome-to-Surface Trafficking—Based on the foregoing considerations, we reasoned that BFA is likely to enhance the endosome-to-surface trafficking route that has been postulated to represent the second limb of the constitutive-like pathway (18). If true, then manipulations that block ProB delivery to endosomes should eliminate the ability of BFA to enhance unstimulated ProB secretion. Indeed, it is well established that arrival of newly synthesized hydrolases in endosomes requires specific mannose 6-phosphate (M6P) receptor recognition (59). With this in mind, INS cells were pretreated with tunicamycin to inhibit N-glycosylation, thereby preventing newly synthesized lysosomal enzymes from acquiring the M6P targeting signal. As shown in Fig. 7, ProB synthesized in INS cells treated with 10 μg/ml tunicamycin exhibited a faster mobility and an insensitivity to digestion with PNGase F, consistent with the loss of N-linked glycans. Importantly, under pulse-chase conditions in tunicamycin-treated cells, BFA was no longer able to increase the unstimulated secretion of ProB (Fig. 8). Thus, using ProB as a sensitive marker whose trafficking into endosomes can be specifically blocked, the data establish that newly synthesized proteins originating in ISGs and the TGN can be secreted via an endosomal intermediate, at least in the presence of BFA.

To further examine the effects of BFA on endosomal function in INS cells, we examined endosome-to-lysosome delivery as reflected by degradation of 35S-labeled Semliki Forest virus, an endocytic marker. The appearance of trichloroacetic acid-soluble radioactivity, a measure of lysosomal degradation of viral proteins (54), first occurred within 30 min after endocytosis began and achieved a plateau by ~90 min after the endocytic pulse (Fig. 9, left panel). With these kinetics in mind, we tested the effects of BFA added 20 min after internalization of the labeled virus at 37 °C. As shown in Fig. 8, right panel, BFA caused a 50% decrease in degradation of viral proteins, consistent with a defect in endosome-to-lysosome delivery.
DISCUSSION

Although constitutive-like trafficking has gained increasing acceptance as a vesicular transport route (9–12), this pathway remains relatively poorly understood. We are working on the hypothesis that the constitutive-like pathway proceeds through an endosomal intermediate (see Fig. 10). Currently, we postulate that a single class of AP-1/CCVs conveys both lysosomal proenzymes bearing the M6P targeting signal (60) and proteins removed nonsel ectively from the lumen of the secretory pathway by fluid-phase capture (18).

The AP-1/CCVs that bud from the ISGs of regulated secretary cells (30) are thought to be quite similar if not identical to those that bud from the TGN (59). Given that a subset of secretory cargo normally transits through the endosomal system even in cells that lack secretory granules (61–64, 69), it follows that constitutive-like secretion probably does not require the co-existence of a regulated secretory pathway but is likely to be operative in all cells that direct AP-1/CCVs from the protein biosynthetic pathway to endosomes. Obviously, our model of AP-1/CCVs as the sole carrier initiating the constitutive-like pathway could change as more is learned about new members of the family of adaptor protein complexes (24–27, 65). However, as the γ1-AP-1, AP-3, and AP-4 adaptors are all recruited by ARF1-dependent mechanisms (27, 38–40, 65, 66), one might expect that BFA would block constitutive-like vesicle budding if any of these particular adaptors were involved.

We set out to clarify our previous finding that BFA did not significantly inhibit constitutive-like secretion from pancreatic β-cells (37), especially given the known susceptibility of AP-1/CCV budding to BFA (28, 42, 43). Although there are many possible explanations, an important technical issue is the fact that proinsulin cannot be metabolically labeled with $^{35}$S-sulfate, the method employed in demonstrating BFA inhibition of constitutive-like trafficking in other cell types (42, 43). Instead, we have attempted to synchronize ProB and proinsulin-derived protein exit from the TGN by warming to 37 °C for 25–35 min after a 19.5 °C temperature block. However, by this time of warm-up (which is required for efficient delivery of proinsulin to ISGs (23)), ProB was already divided into different pools (Fig. 3), one of which was an ISG pool from which ProB exhibited stimulus-dependent exocytosis (Fig. 4) in parallel with insulin (Fig. 5). A second pool was almost certainly endosomal, because BFA-mediated enhancement of unstimulated ProB secretion from this pool (Figs. 3 and 6) was blocked if ProB lacked the M6P recognition signal for delivery to endosomes (Fig. 8).

In the presence of a proper M6P recognition signal, the efficiency of capture and conveyance of ProB to endosomes is vastly more efficient than that for proinsulin, conversion intermediates, or C-peptide (23). Thus, these latter peptides are expected to show a far less dramatic BFA-mediated augmentation of unstimulated secretion from this compartment, consistent with the results of Fig. 5. Nevertheless, in addition to other possibilities, the present data are consistent with the idea that using our BFA protocol, significant inhibition of constitutive-like C-peptide secretion was not detected (37) because it was in part offset by increased BFA-mediated endosome-to-surface trafficking (see Fig. 10).

Our results are consistent with reports in other cell types that endocytosis and recycling to the cell surface are not blocked by BFA (33), and yet enhanced endosome to surface trafficking (67) may exist in conjunction with impaired traffic between endosomes and lysosomes (68). Indeed, a lysosomal delivery defect was clearly observed in INS cells treated with BFA (Fig. 9), and presumably, this inhibition is correlated with the enhancement of endosome to surface trafficking.

We tried to explore different warm-up periods after the

![Fig. 9. Effect of BFA on endosome-to-lysosome delivery. $^{35}$S-Labeled Semliki Forest virus was prebound to the surface of INS-1 cells for 90 min at 4 °C. The cells were then warmed to 37 °C to allow virus endocytosis to proceed. Arrival of labeled virus in the lysosome was followed by degradation of viral proteins with release of trichloroacetic acid-soluble radioactivity into the medium. A, time course of lysosomal degradation of labeled virus. B, after 20 min at 37 °C, the cells were further incubated for 100 min at 37 °C in the presence (hatched bar) or absence (black bar) of BFA (10 μg/ml). Release of trichloroacetic acid-soluble counts from the control cells (black bar) was set to 100%.

![Fig. 10. Hypothesis to account for effects of wortmannin and BFA (using the protocols described under “Materials and Methods”) on the constitutive-like secretory pathway, using lysosomal ProB (L) as a marker in INS cells. In this view, for clarity, not all membrane traffic routes are shown, and the AP-1/CCV route is the only route designated with coated vesicular intermediates. Wortmannin treatment decreases the efficiency of newly synthesized cathepsin B delivery to endosomes. BFA treatment may similarly impair ProB trafficking; however, using the protocol we employed, the most dramatic effects observed were persistence of intracellular ProB indicating diminished delivery to lysosomes (drawn as an endosome → lysosome block) accompanied by enhanced unstimulated secretion as indicated.]
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19.5 °C block to attempt to maximize kinetic resolution between the ISG and endosomal pools of ProB. Whereas warm-up times ≤15 and ≥45 min failed to result in subsequent BFA-mediated augmentation of newly synthesized ProB secretion in INS cells, significant kinetic overlap between labeled ISG and endosomal ProB pools cannot be avoided in our protocol because some endosomal ProB is probably derived directly from the TGN even in pancreatic β-cells (32). Thus, we cannot state with confidence exactly how much of the endosomal ProB secretion enhanced by BFA in our experiments has actually traversed the ISG compartment. Nevertheless, in INS-1 cells, a considerable fraction of the constitutive-like pathway is likely to be initiated from the ISG compartment (32); this is further supported by the observations in Fig. 2 that after wortmannin treatment (which inhibits prohylodase delivery via the AP-1/CCV route), there is abnormal intracellular accumulation of newly synthesized ProB at chase times ≥7 h, accompanied by persistent stimulus-dependent ProB secretion (Fig. 10).

In conclusion, the present data suggest that in pancreatic β-cells, in conjunction with an inhibition of protein delivery to lysosomes (Figs. 3 and 9), BFA enhances luminal protein secretion from endosomes (Figs. 4, 6, and 8). This evidence may help to resolve existing discrepancies between groups about BFA effects in regulated secretory cells and is consistent with a hypothesis of two limbs of the constitutive-like pathway: one from the biosynthetic pathway to the endosomal system, and a second from there to the cell surface (Fig. 10).

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