Resting secretion of salivary proteins by the parotid gland is sustained in situ between periods of eating by parasympathetic stimulation and has been assumed to involve low level granule exocytosis. By using parotid lobules from ad libitum fed rats stimulated with low doses of carbachol as an in vitro analog of resting secretion, we deduce from the composition of discharged proteins that secretion does not involve granule exocytosis. Rather, it derives from two other acinar export routes, the constitutive-like (stimulus-independent) pathway and the minor regulated pathway, which responds to low doses of cholinergic or \( \beta \)-adrenergic agonists (Castle, J. D., and Castle, A. M. (1996) J. Cell Sci. 109, 2591–2599). The protein composition collected in vitro mimics that collected from cannulated ducts of glands given low level stimulation in situ. Analysis of secretory trafficking along the two pathways of resting secretion has indicated that the constitutive-like pathway may pass through endosomes after diverging from the minor regulated pathway at a brefeldin A-sensitive branch point. The branch point is deduced to be distal to a common vesicular budding event by which both pathways originate from immature granules. Detectable perturbation of neither pathway in lobules was observed by wortmannin addition, and neither serves as a significant export route for lysosomal procathepsin B. These findings show that parotid acinar cells use low capacity, high sensitivity secretory pathways for resting secretion and reserve granule exocytosis, a high capacity, low sensitivity pathway, for massive salivary protein export during meals. An analogous strategy may be employed in other secretory cell types.

Salivary acinar cells are highly polarized epithelial cells that are specialized for the secretion of the macromolecular, electrolyte, and fluid components of saliva. Macromolecular secretion is achieved by the intracellular transport pathway, culminating in exocytosis of post-Golgi carriers at the apical plasma membrane. In contrast, export of electrolytes and fluid is achieved by transepithelial flux of ions and passive flow of water mediated by specific membrane-associated pumps and channels. Secretion is mostly neurally regulated with the discharge of macromolecules and of electrolytes and fluid being controlled differentially (1). In the parotid gland, the rate of macromolecular (mostly protein) secretion is mainly controlled by sympathetic stimulation through \( \beta \)-adrenergic receptors. Rates of fluid and electrolyte secretion are mainly controlled by parasympathetic stimulation through muscarinic acetylcholine receptors and, to a lesser extent, sympathetic stimulation through \( \alpha \)-adrenergic receptors (see Refs. 2–4 and reviewed in Refs. 5 and 6). Although these are the primary divisions in secretory signaling, they are not absolute. Indeed, the dilute secretion elicited by parasympathetic stimulation in situ (or muscarinic cholinergic agonists in vitro) contains a protein content, whereas the protein-rich, low volume secretion produced by \( \beta \)-adrenergic agonists contains fluid and electrolytes. Furthermore, parasympathetically and sympathetically controlled signaling pathways are synergistic in enhancing the rates of protein export and fluid and electrolyte transport (4, 7–10). Thus alone and in combination, the signaling pathways enable acinar cells to vary the amount and composition of saliva over a wide range.

The versatile secretory response of acinar cells enables them to play two critical physiological roles that are distinguished by the quantity and quality of secretion. Between periods of eating, an ongoing “resting” or basal secretion aids in maintaining the homeostasis (hydration, mineralization, and microbial population) of the oral cavity, while during eating, an acutely amplified discharge of saliva facilitates lubrication and initiates digestion of ingested food. Resting secretion is maintained at least in part by low frequency parasympathetic stimulation (6). We have been interested in the pathways and mechanisms of protein secretion used by acinar cells of the parotid gland as they relate to the differing physiological states of resting and eating. By using in vitro biosynthetic labeling of parotid tissue in a pulse-chase protocol coupled with secretagogue stimulation, we have identified up to four pathways that export the principal salivary proteins. Two pathways occur without stimulation, and two others are secretagogue-dependent (1).

The unstimulated pathways are not inhibited by secretory antagonists, atropine, propranolol, and phentolamine, and thus appear to be truly secretagogue-independent. Together, they account for 10–15% of the newly synthesized secretory protein over a 4–6-h time course. The best characterized unstimulated pathway is the constitutive-like pathway, which has been observed in various exocrine and endocrine cells and has been distinguished from the constitutive secretory pathway. It preferentially exports newly synthesized proteins, beginning soon after transit through the Golgi, and has been deduced to originate by vesicular budding that is linked to the maturation of newly formed secretory granules (11). The secretory composition of the constitutive-like pathway is enriched in proteins that are inefficiently sorted by condensation within
the cores of maturing granules (12, 13). A second unstimulated pathway occurring at >3-h chase times (after the bulk of constitutive-like secretion) exports proteins having a radiochemical composition resembling the content stored in mature secretory granules, and it may represent unstimulated granule exocytosis. Evidently, the magnitude of this type of release, which has been variable in previous studies (12–14), is of significant interest with respect to its potential contribution to resting secretion.

Of the two stimulated pathways, the principal one is secretory granule exocytosis, which we refer to as the major regulated pathway and which accounts for most secretory protein export. The other is the minor regulated pathway, which accounts for release of only a few percent of stored secretory proteins. It is distinguished from granule exocytosis by preferentially releasing newly synthesized secretory proteins rather than those that have been in prolonged storage and by its secretory composition, which closely resembles that of the constitutive-like pathway. Notably, the minor regulated pathway is much more sensitive to secretagogue stimulation than is granule exocytosis and is mobilized by both cholinergic and β-adrenergic stimuli (14).

Since resting secretion in situ is maintained by low level parasympathetic stimulation, we were interested in whether the minor regulated pathway serves as its stimulus-responsive component. We now present evidence that this is the case, and we show that granule exocytosis contributes to resting secretion only in tissue from fasted animals where intracellular storage of salivary proteins is near maximal capacity. In tracing the minor regulated pathway in acinar cells, we provide experimental support for its common origin with the constitutive-like pathway by vesicular budding from immature granules and for its divergence from the constitutive-like pathway at a brefeldin A-sensitive branch point. Subsequent trafficking of the minor regulated pathway to the cell surface appears to be direct, albeit stimulus-controlled, whereas the constitutive-like pathway appears to be an indirect route involving passage through a distal compartment postulated to be endosomal.

**EXPERIMENTAL PROCEDURES**

**Materials**

Male Sprague-Dawley rats (100–124 g) were obtained from Hilltop (Scottsdale, PA), maintained 1 week in the vivarium, and were used either after ad libitum feeding or fasting overnight from 4:00 pm as specified. Expired35S35S used for biosynthetic labeling of parotid lobules was obtained from PerkinElmer Life Sciences. Secretagogues, Dl-isoproterenol, carbachol (carbachol; CCh), and pilocarpine were obtained from Sigma. Brefeldin A (BFA) was obtained from Eppendorf Technologies (Madison, WI), and wortmannin was obtained from Sigma. Both were dissolved in dimethyl sulfoxide and stored at −20 °C. The activity of the specific batch of wortmannin used was confirmed by demonstrating its ability to elicit endosomal vesiculation in HeLa cells (15). Antibodies against amylase and proline-rich protein used in the present studies have been described previously (16, 17). A rabbit antibody against parotid secretory protein was generously provided by Dr. William Ball (Howard University). Rabbit antibody against rat procathepsin B, which recognizes both precursor and mature forms of the enzyme, was from Upstate Biotechnologies, Inc. (Lake Placid, NY).

**Methods**

**Incubations**—Tissue from one rat was used in each experiment. Rats were sacrificed by carbon dioxide asphyxiation, and lobules were dissected from excised parotid glands in chilled incubation medium (Dulbecco’s modified Eagle’s medium equilibrated with 95% O2, 5% CO2). Preincubations (3 times for 10 min) and incubations including pulse labeling and subsequent chases (identified below) were performed in stoppered Erlenmeyer flasks as described previously (14). Although most experiments were conducted entirely at 37 °C, one set of experiments involved incubation post-pulse at 19 °C for 2 h to accumulate biosynthetically labeled proteins in the trans-Golgi network (18) prior to warming to 37 °C for subsequent incubation and analysis (see “Results”). At designated time intervals, all medium in each sample (1 ml) was removed and replaced with fresh medium including secretagogues or perturbants of transport as specified. At the end of incubation, the tissue in each sample was washed with chilled phosphate-buffered saline and homogenized. Media and homogenates were subjected to a chase of amylase activity (19) and for SDS-PAGE (12.5% gels; Coomassie staining, phosphorimaging, and quantitative analysis of 35S-labeled proteins with ImageQuant software (14, 17).

For biosynthetic labeling, analysis of intracellular transport, and secretion of cathepsin B (proenzyme and enzyme), incubation with 35S-amino-acids (0.25 mCi/ml) was increased to a 30-min pulse, and chases were performed for 30-min or hour intervals, as specified. Immunoprecipitations were performed on aliquots of media and detergent extracts (1% Nonidet P-40, 0.25% deoxycholate) of tissue homogenates following addition of protease inhibitors and preclearing of samples with 1 mg of protein A-Sepharose each. Incubations in a Triton X-100/ deoxycholate/SDS-based RIPA buffer were performed overnight at 4 °C with anti-cathepsin B immobilized on protein A-Sepharose. Immunoprecipitates were eluted in Laemmli sample buffer and were processed for SDS-PAGE and analysis as above.

**Morphological Studies**—Immunofluorescent staining of cryosectioned parotid lobules was performed essentially as described before (20). Lobules were fixed by the periodate/llysine/paraformaldehyde procedure (21), frozen in 2.3 M sucrose, 50% polyvinylpyrrolidone (1:1), sectioned (~1 μm) at −50 °C, and mounted on gelatin-coated slides. The sections were blocked in 5% goat serum, 0.05% Triton X-100, and phosphate-buffered saline; suitable dilutions of primary and secondary (Alexa 594-conjugated) antibodies (Molecular Probes, Inc., Eugene, OR) were made in 0.5% goat serum, 0.05% Triton X-100, and phosphate-buffered saline. Stained sections were mounted and examined by fluorescence microscopy.

Labeling of control and stimulated tissue for morphological analysis by bright field microscopy and electron microscopy were fixed in phosphate-buffered 2% formaldehyde, 2% glutaraldehyde, post-fixed in 1% osmium tetroxide, stained en block with 0.5% magnesium uranyl acetate, dehydrated in acetone, and embedded in Epon resin according to standard procedures. Thick (0.5 μm) sections were stained with toluidine blue, and thin sections for EM were mounted on copper grids and stained with uranyl acetate and lead citrate.

**Cannulation of Parotid Ducts and Collection of Stimulated Secretion in Situ**—Rats were anesthetized with urethane, and parotid ducts, one per animal, were surgically exposed and cannulated on the surface of the masseter muscle. Secretagogues freshly dissolved in sterile PBS were administered by local interstitial injections in glandular parenchyma, 100 μl at each of two sites per gland. Stimulations were performed in the order 1 μM carbachol, 5 μM carbachol, 10 μM isoproterenol.

The resulting secretions were collected manually from the cannula into microcentrifuge tubes, and they were assayed for amylase and analyzed by SDS-PAGE, Coomassie staining, and densitometry.

**RESULTS**

**Major Secretory Products in the Parotid Are Expressed in All Acinar Cells**—In analyzing the secretory pathways that are present in parotid acinar cells, we have focused our attention on some of the major secretory products. In particular, we have examined the intracellular transport and discharge of amylase, the most abundant acinar protein in rat, and two other proteins, an ~98-kDa proline-rich protein (PRP) and 25-kDa leucine-rich parotid secretory protein (PSP). As can be seen in the immunofluorescence micrographs shown in Fig. 1, all three proteins are expressed in substantial amounts throughout the entire acinar cell population. Furthermore, none of the proteins accumulates in the acinar extracellular space under the unstimulated or carbamylcholine-stimulated conditions used in this study. Consequently, compositional differences involving these three proteins among different secretory pathways are

1 The abbreviations used are: CCh, carbachol; BFA, brefeldin A; PRP, proline-rich protein; PSP, parotid secretory protein; TGN, trans-Golgi network; SNARE, SNAP receptor where SNAP is soluble NSF attachment protein; PAGE, polyacrylamide gel electrophoresis.

2 A. Y. Huang, A. M. Castle, B. T. Hinton, and J. D. Castle, unpublished data.
almost certainly the consequence of intracellular sorting events occurring generally in acinar cells and also do not reflect different cellular origins for distinct secretory pathways.

“Resting Secretion” by Parotid Lobules in Vitro and by Tissue in Situ Occurs Almost Entirely by the Constitutive-like and Minor Regulated Pathways—More than 20 years ago, it was established in parotid acinar cells that secretory granules appear to be discharged in order of their synthesis with older granules being released first (22). Previously, we used biosynthetic labeling and analysis of radioactive secretory products to identify the constitutive-like and minor regulated pathways in parotid and to distinguish them from granule exocytosis as routes that preferentially export newly synthesized proteins (12, 14). Furthermore, we found that the two non-granule pathways exported amylase but almost no PSP, whereas both proteins were abundant in secretion released by granule exocytosis. (PRP was not analyzed because it contains no methionine or cysteine and thus was not labeled by the $^{35}$S-amino-acid mixture.) Examples of these differences in radiochemical composition are illustrated in Fig. 2A. As noted previously (14), the compositional differences are independent of the chase interval selected for observation. Whereas the biosynthetic labeling strategy enabled us to detect and characterize the minor pathways over any secretion resulting from exocytosis of unlabelled granules, we did not previously address their relative contributions as putative sources of resting secretion. Consequently, we have now examined the composition of total secretory proteins released from parotid lobules in the absence of stimulation and in the presence of low dose carbachol stimulation and compared it with the composition released by high dose isoproterenol stimulation and with the content of purified secretory granules. As shown in Fig. 2B, the Coomassie staining profiles for unstimulated secretion and carbachol-stimulated secretion are highly similar to each other but differ from those of isoproterenol-induced secretion and granule content, which are identical to each other. Just as observed for the radiolabeled secretory profiles (Fig. 2A), PSP is nearly absent from the protein staining profiles of unstimulated and carbachol-stimulated secretions, although it is prominent in granule content and isoproterenol-stimulated secretion. In addition, PRP (which is not labeled by $^{35}$S in Fig. 2A) is poorly represented in unstimulated and carbachol-stimulated secretions even though it is prominent in stored and released granule contents (Fig. 2B).

To document that the secretion observed in vitro reflects what occurs in situ, we injected low concentrations of carbachol and subsequently a high concentration of isoproterenol into the glandular interstitium of an anesthetized rat and collected secretion from the cannulated parotid duct. Local application of a cholinergic agonist avoids significant activation of adrenal catecholamine secretion and consequent adrenergic stimulation of parotid tissue as can occur following systemic administration (3). As can be seen in Fig. 2C, the composition of secreted proteins following low doses of carbachol closely resembles that obtained in vitro with unstimulated and carbachol-stimulated lobules, implying that resting secretion is derived from the constitutive-like and minor regulated pathways and that the pathways are apically directed.

Unstimulated Exocytosis of Secretory Granules Contributes to Resting Secretion by Parotid Acinar Cells of Fasted Animals but Is Not a Dominant Pathway—Rats are nocturnal feeders, and the total amylase content in parotid typically decreases by ~40% overnight as a result of secretion (23). In initiating morning experiments using parotid tissue from ad libitum fed animals, we are examining the secretory pathways in acinar cells that have a complement of storage granules that is below capacity. Although our experimental conditions satisfactorily approximate the physiological state, we were interested to examine whether overnight fasting, which should maximize the cellular storage pool of granules, affects the composition of resting secretion and the pathways that contribute to it. Thus, with tissue from fasted animals, we carried out biosynthetic labeling using a pulse-chase protocol, and we analyzed the radiochemical and total protein compositions of unstimulated secretion and secretion stimulated by 40 nM carbachol and by 10 μM isoproterenol. As shown in Fig. 2D, the radiochemical composition released by the constitutive-like and minor regulated pathways is similar to that observed with tissue from ad libitum fed animals. Secretion of labeled PSP is underestimated as compared with the level observed upon stimulating granule exocytosis with isoproterenol. However, the spectrum of total proteins secreted, as revealed by Coomassie staining, now includes increased amounts of PRP and PSP, suggesting participation of granule exocytosis (Fig. 2E). To distinguish whether the increased amount of PSP represents newly synthesized or older protein, we compared the specific radioactivities of amylase and PSP (normalized to Coomassie staining) in secretions following 40 nM carbachol and 10 μM isoproterenol.
addition. Whereas the specific radioactivity of amylase was 6-fold higher after carbachol than after isoproterenol, the specific radioactivity of PSP was lower (by 1.2-fold) after carbachol than after isoproterenol. Thus basal exocytosis of older (unlabeled) granules becomes a contributing pathway to resting secretion along with the other two pathways in acinar cells that are replete with granules.

Fig. 2 summarizes the output of newly synthesized and total amylase in the absence and presence of stimulation in a typical experiment. The data illustrate that the pathways contributing to resting secretion account for only small fractions of newly synthesized and stored amylase, whereas stimulated granule exocytosis releases much larger fractions. It is also apparent that the fractional output of amylase in resting secretion is increased during fasting.

Stimulation of the Minor Regulated Pathway Correlates with Expansion of Apical Luminal Profiles Occurring Rapidly in a Large Fraction of Acinar Cells—In an attempt to visualize morphological changes in response to low doses of carbachol and to assess whether the minor regulated pathway is activated broadly among acinar cells, we incubated lobules for brief times in the presence and absence of 40 nM carbachol and then chilled, fixed, and processed the samples for light and electron microscopy. Fig. 3, A and B, shows light micrographs of toluidine blue-stained sections that have been printed in reverse contrast to enhance visualization and comparison of extracellular spaces bordering acinar cells, and particularly the apical lumina. In controls, the lumina appear mostly as small dark spots bordered by the light adjoined acinar cells. Following 1 min and especially 3 min of stimulation, a substantial fraction...

Fig. 2. Composition of parotid secretory proteins collected under different conditions. A and B, proteins released in vitro by parotid lobules of ad libitum fed rats that have been incubated without stimulation (Ctl), with 40 nM carbachol (CCh), or with 10 μM isoproterenol (Iso). Amylase (Amy), proline-rich protein (PRP), and parotid secretory protein (PSP) are identified in A and B. A, radiochemical profile following pulse labeling with Expre35S and chase incubation. The samples have been normalized to load equal radioactivity (band density) of amylase in order to emphasize the relative compositional differences of other proteins, particularly PSP, in the different secretions. B, composition of total secreted protein as viewed by Coomassie staining. The composition of the content of isolated secretory granules (Gran) is also shown. C, Coomassie staining of total salivary proteins collected in situ by cannulation of the parotid duct. Stimulation was by sequential interstitial injection of 1 and 5 μM carbachol and 10 μM isoproterenol. The results shown are representative of three separate cannulation experiments. D and E, radiochemical and Coomassie staining profiles for unstimulated, carbachol-stimulated, and isoproterenol-stimulated secretions released in vitro by parotid lobules from rats fasted overnight. The Coomassie profile for granules purified from the glands of fasted animals is shown in E for comparison. The 22-kDa protein observed at variable levels in A–E is common salivary protein, a product mainly of intercalated duct cells (58). It has not been considered in the present analysis of acinar products. F, summary of secretion of newly synthesized amylase (35S) and amylase enzyme (Enz. Act.) during a 30-min interval expressed as fractions of total (tissue + all media). Ctl values for 35S-amylase are taken from the peak (90–120 min) of unstimulated secretion (14); CCh values reflect stimulated release between 150 and 180 min; isoproterenol values reflect stimulated release between 240 and 270 min.
of the luminal spaces within individual lobules has expanded, implicating structural reorganization at the apical surface and possible secretion. Stereological measurements of the surface area of >1500 luminal profiles from multiple acini of unstimulated specimens and >2400 profiles from stimulated specimens made using the Openlab program (Improvision Inc.) indicated that the mean luminal diameter increased 1.5–2-fold by 3 min of stimulation with at least a 25% shift in the distribution of total profiles to larger dimensions. Not only is the response observed broadly in the acinar tissue, but also it occurs quite rapidly and correlates well with the rapid export of secretory products observed in our earlier study (Fig. 2 in Ref. 14). Examination by EM (Fig. 3, C and D) indicated that luminal expansion decreases the incidence of microvilli protruding into the lumen and brings the apical surface into closer proximity to the stored granule population. These observations suggest that the actin-rich terminal web may be reorganized in a way that is conducive to granule exocytosis, although there is no evidence suggesting that low dose carbachol treatment causes granule exocytosis.

**The Minor Regulated and Constitutive-like Pathways Form in Parallel, but Their Outputs Have Different Specific Radioactivities**—We showed previously that the minor regulated pathway could be stimulated to export pulse-labeled salivary proteins of high specific radioactivity at prolonged chase times, well after most of the constitutive-like secretion of the labeled proteins had occurred (14). This distinguished the minor regulated pathway as a separate secretagogue-responsive storage compartment and discounted the possibility that stimulation was accelerating the constitutive-like pathway. Although the two pathways are distinct, they draw on the same pool of secretory proteins including several involved in post-Golgi transport isozymes of ADP-ribosylation factor family GTPases (26). We were interested to compare the sensitivities of the constitutive-like and minor regulated pathways to BFA. Accordingly, we used our standard pulse labeling protocol and compared the sensitivities of both pathways to BFA. GDP/GTP exchange on certain ADP-ribosylation factor family GTPases (26).

Formation of many kinds of vesicular carriers including several involved in post-Golgi transport is inhibited by BFA (e.g. Refs. 18, 24, and 25), which blocks GDP/GTP exchange on certain ADP-ribosylation factor family GTPases (26). We were interested to compare the sensitivities of the constitutive-like and minor regulated pathways to BFA. Accordingly, we used our standard pulse labeling protocol and added BFA during the chase for time intervals beginning at 80 min or later. Earlier times of addition were not used in initial experiments in order to minimize the well known effects of BFA’s inhibition of more proximal (endoplasmic reticulum-Golgi) intracellular transport steps. When 10 μg/ml BFA was added at 80 min, it inhibited the subsequent output of radio-labeled proteins in response to 40 nM carbachol by ~80%. In
The minor regulated and constitutive-like secretory pathways form in parallel, but secretions released by the two pathways have different specific radioactivities. Parotid lobules from ad libitum fed or fasted animals were pulse-labeled with 35S-amino-acids and divided into separate samples for chase incubation with medium changes at 30-min intervals. At specified time points, individual samples were stimulated for 30 min with 40 nM carbachol to elicit secretion by the minor regulated pathway. Secretions from all samples and all time points were assayed for amylase enzyme activity and subjected to SDS-PAGE, and radioactive amylase was quantitated by phosphorimaging. A, examples of specific radioactivity profiles of amylase for unstimulated lobules (open circles) and lobules stimulated for a single 30-min interval beginning at 90 (open triangles) and 210 min (open inverted triangles). B, plots summarizing the results of experiments conducted as in A from ad libitum fed (squares) and fasted (circles) animals. The open symbols show the specific radioactivity of amylase in unstimulated secretion. The filled symbols correspond to the net specific radioactivity of amylase in CCh-stimulated secretion. Each data point derives from a separate sample where carbachol was applied at a single time interval as shown. Net specific radioactivity of amylase was calculated after correcting the radioactivity and enzyme activity of amylase for the contributions from unstimulated secretion. The data are representative of at least six experiments using tissue from fed animals and three experiments using tissue from fasted animals.

contrast, when BFA was added at 160 min (after most carriers containing labeled proteins had formed) and followed by carbachol stimulation, secretion was decreased by only ~40% (Fig. 5). Although BFA blocked the output of labeled protein, it caused at most a small inhibition of carbachol-stimulated output of amylase enzyme activity (data not shown). Thus the results indicate that BFA inhibits formation of carriers of the minor regulated pathway but not their discharge. Although BFA inhibited formation of the minor regulated pathway, it enhanced unstimulated secretion as reflected in the discharge of amylase detected enzymatically (Fig. 6A) and radiochemically. Notably, the specific radioactivity of amylase appearing in unstimulated secretion increased ~3-fold following BFA addition at 80 min of chase (Fig. 6B). The radiochemical compo-

sition of the enhanced secretion immediately following BFA was not noticeably changed from before drug addition (Fig. 6C). Two alternative explanations for this result come to mind. First, inhibition of formation of minor regulated carriers leads to a compensatory amplified export of the newly synthesized proteins in the constitutive-like pathway. As these proteins have a high specific radioactivity, they would increase the specific radioactivity of constitutive-like secretion, which is much lower (Fig. 4). Second, BFA may itself cause exocytosis of minor regulated secretory carriers thereby adding a separate high specific radioactivity component to “unstimulated” output. To distinguish between these possibilities, we examined the effect of BFA addition at increasing chase times beyond 80 min. According to the first alternative, the increment of specific radioactivity of amylase in the medium following BFA addition at increasing times should decrease in parallel with the normal decrease in specific radioactivity observed in constitutive-like secretion. In contrast, the second alternative predicts that the BFA-induced increase in specific radioactivity of amylase in the medium at increasing times of drug addition should decrease only slightly (~30%; Fig. 4) reflecting secretion from the high specific radioactivity compartment. As can be seen in Fig. 6D, the increase in specific radioactivity of secreted amylase following BFA addition progressively declines and is more than 10 fold lower by 320 min. Thus the first alternative applies, and BFA blockade appears to divert newly synthesized protein to the constitutive-like pathway.

The observation that BFA enhanced output in the constitutive-like pathway was quite interesting on two accounts. First, it seemed to contrast with previous observations suggesting that formation of constitutive-like secretory carriers from immature granules is blocked by BFA (e.g. Ref. 24). Second, it appeared potentially analogous to the recently reported enhanced constitutive-like secretion of procathepsin B in pancreatic β-cells in response to BFA action at a site distal to exit from the immature granule (18). Consequently, we wondered whether there might be two sites of BFA blockade, one at exit and one beyond exit from immature granules, and that drug addition at 80 min post-pulse might have missed the first one. Thus in an effort to evaluate how BFA action on early post-Golgi events might affect the pathways of resting secretion, we incubated parotid lobules post-pulse for 2 h at 19 °C to accumulate labeled proteins in the TGN (18). In parallel samples, we either made no addition or added BFA either at the same time or 25 min after raising incubation temperature to 37 °C.

FIG. 4. The minor regulated and constitutive-like secretory pathways form in parallel, but secretions released by the two pathways have different specific radioactivities. Parotid lobules from ad libitum fed or fasted animals were pulse-labeled with 35S-amino-acids and divided into separate samples for chase incubation with medium changes at 30-min intervals. At specified time points, individual samples were stimulated for 30 min with 40 nM carbachol to elicit secretion by the minor regulated pathway. Secretions from all samples and all time points were assayed for amylase enzyme activity and subjected to SDS-PAGE, and radioactive amylase was quantitated by phosphorimaging. A, examples of specific radioactivity profiles of amylase for unstimulated lobules (open circles) and lobules stimulated for a single 30-min interval beginning at 90 (open triangles) and 210 min (open inverted triangles). B, plots summarizing the results of experiments conducted as in A from ad libitum fed (squares) and fasted (circles) animals. The open symbols show the specific radioactivity of amylase in unstimulated secretion. The filled symbols correspond to the net specific radioactivity of amylase in CCh-stimulated secretion. Each data point derives from a separate sample where carbachol was applied at a single time interval as shown. Net specific radioactivity of amylase was calculated after correcting the radioactivity and enzyme activity of amylase for the contributions from unstimulated secretion. The data are representative of at least six experiments using tissue from fed animals and three experiments using tissue from fasted animals.

FIG. 5. Inhibition of formation of the minor regulated pathway by BFA. BFA was added to pulse-labeled tissue at either 80 or 160 min chase at 37 °C and maintained until 240 min at which time the minor regulated pathway was stimulated with 40 nM carbachol. The results are normalized to a control in which BFA was not added but stimulation was performed at 240 min. The results shown are from three experiments (mean ± S.E.) for BFA added at 80 min and two experiments (mean; vertical line is range) for BFA added at 160 min.
The results are shown in Fig. 6, E–G. Warming the tissue promptly increased the release of amylase enzyme activity to ~1% of total per 30 min in the control (the level observed in our other experiments), and BFA at both times of addition elevated unstimulated release above control (Fig. 6E). The effects of BFA on the specific radioactivity of amylase and on the radiochemical composition of secreted proteins, however, depended on when the drug was added (Fig. 6, F and G). When added at the time of warm-up, BFA inhibited the rise in amylase-specific radioactivity. Although the output of radioactive proteins was reduced, the composition of the secretion also changed such that it was enriched in labeled PSP along with other acinar proteins as if imposing early blockade had resulted in low level discharge of unsorted proteins. When BFA was added after warm-up, the specific radioactivity of secreted amylase immediately increased (as in the experiments conducted entirely at 37 °C), and the radiochemical composition of the amplified secretion was comparable to that typically observed in the constitutive-like and minor regulated pathways of untreated tissue. Quantitation of the PSP/amylase band density ratio of the 180-min samples from two separate experiments yielded a ratio of 0.01–0.03 (range) for control and for BFA added 25 min after warm-up and 0.10–0.15 (range) for BFA added at warm-up. These outcomes document decreased sorting as an indirect consequence of inhibiting vesicular budding by early BFA addition. Taken together, the observations indicate that BFA imposes two blocks, an early one that impairs the constitutive-like pathway and a later one that inhibits formation of minor regulated carriers distal to exit from immature granules where sorting occurs.

Wortmannin Has No Effect on the Minor Regulated Pathway but Extends the Duration of Constitutive-like Secretion of Newly Synthesized Proteins Over the Same Period—The phosphatidylinositol 3-kinase inhibitor wortmannin at concentrations ≤100 nM perturbs a variety of post-Golgi trafficking pathways with little or no effects on constitutive and regulated secretion (15, 18, 27–30). Because wortmannin has been reported to interfere with lysosomal prohydrolase trafficking from immature secretory granules to endosomes in endocrine cells (18), we were interested to test whether it affected the exocrine constitutive-like and minor regulated secretory pathways. We applied it immediately after pulse labeling parotid lobules and monitored its effects on unstimulated and carbachol-stimulated secretion during subsequent chase incubation. In three separate experiments, we observed that wortmannin did not affect delivery to either the constitutive-like pathway or minor regulated pathway, the latter tested by stimulation at 80 and 160 min. Newly synthesized amylase appeared in the medium from both pathways of drug-treated tissue at the same rates as in control tissue, and the radiochemical composition of the secretions was not altered (data not shown). At prolonged times (>160 min

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Fig. 6. The effects of BFA on unstimulated secretion. A–C show results of incubation entirely at 37 °C. Parotid lobules were pulse-labeled and chased, and media were replaced and analyzed every 40 min. BFA (open circles) or Me2SO (closed circles) was added at 80 min post-pulse and maintained throughout. A, amylase activity was assayed, and unstimulated secretion of amylase was plotted as a function of time. B, specific radioactivity of secreted amylase with time evaluated as described under “Experimental Procedures.” C, profiles of radiolabeled proteins secreted before (40 and 80 min) and after BFA addition (120 min). The results in A–C are representative of three separate experiments. D, effect of BFA addition at increasing chase times on the specific radioactivity of amylase in the ensuing 40-min interval of unstimulated secretion. Results are normalized to the increment in specific radioactivity observed in the interval beginning at 80 min. E–G show results where lobules were incubated for 2 h at 19 °C following pulse labeling and subsequently warmed to 37 °C for the rest of incubation. Me2SO (filled circles) or BFA was added at the time of warming (120 min, filled triangles) or at 25 min after warming (145 min, open circles) and maintained throughout. Media were replaced every 30 min. E, amylase enzyme activity; F, amylase-specific radioactivity. Data from two identical experiments were normalized to the maximal values obtained with BFA added 25 min post warm-up, and means are plotted. Vertical bars identify ranges that differ by more than the width of the symbol. G, profiles of radiolabeled secretory proteins detected in secretions at 180 min for untreated lobules (Ctl), lobules where BFA was added at 120 min (warm-up), and lobules where BFA was added at 145 min (after warm-up). For untreated cells, the profile is quite similar to the control in C except that the level of labeled PSP is slightly increased as a consequence of imposing the 19 °C block. Positions of amylase (Amy) and PSP are indicated.

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We also tested the addition of BFA 15 min before warming, but we have not shown the results because they resemble the findings for BFA added at warm-up and do not provide further insight.
chase), however, wortmannin had two minor effects. It maintained the unstimulated release of labeled protein above control levels without increasing the overall level of unstimulated (amylase enzyme) secretion, and it subtly reduced carbachol-stimulated discharge of amylase activity without altering secretory composition. Isoproterenol-induced discharge of labeled proteins by granule exocytosis was not altered by wortmannin (data not shown).

The Constitutive-like and Minor Regulated Pathways Are Not Significant Export Routes for Lysosomal Hydrolase Precursors—Lysosomal hydrolase precursors are useful markers for monitoring traffic from immature granules to endosomes (18, 31–33). By having established that the constitutive-like and minor regulated pathways are distinct yet collaborate to form resting secretion, we were interested to evaluate whether the trafficking of a prohydrolase under normal conditions or upon addition of wortmannin aided in further understanding the interrelationship of the two pathways. Also, regulated exocytosis of mature lysosomal hydrolases has been reported in various cell types (34, 35), raising the possibility that there might be a relationship of this process to the operation of the minor regulated pathway. In order to address these issues, we have used extended (30 min) pulse labeling and immunoprecipitation of procathepsin B from the media and detergent extracts of homogenized lobules. Initially, we confirmed that procathepsin B enters and exits immature granules (32) by showing that 10 μM isoproterenol added between 0–1 and 1–2 h of chase, respectively, stimulated secretion of about 70% of total amylase at 1–2 h of chase, respectively.

FIG. 7. The constitutive-like and minor regulated pathways are not significant export routes for lysosomal hydrolase precursors (or enzymes). Lobules pulse-labeled 30 min were chased and stimulated (or not) with 10 μM isoproterenol (Iso) between 0–1 and 1–2 h or with 40 nM carbachol (CCh) between 1.5 and 2 h of chase. Samples of solubilized cell lysates and medium were immunoprecipitated with anticathepsin B antibody under conditions that quantitatively immunoprecipitate procathepsin B/cathepsin B. The fluorographs of cell samples identify the mobilities of procathepsin B (ProB) and mature cathepsin B (B) taken from samples at 0 and 2.5 h of chase, respectively. For secretions, isoproterenol stimulates export of 48% of total labeled procathepsin B/cathepsin B during the first time interval and 22% during the second time interval. The * identifies a secretory contaminant migrating slightly slower than procathepsin B in unstimulated secretion. CCh stimulated export of only a small amount (<0.3% of total) of procathepsin B. Lanes showing samples with and without CCh stimulation were immunoprecipitated from samples three times the amount of medium used for the isoproterenol sample.

In the present studies, we have made a compelling case through examination of the total composition of parotid secretory proteins that secretion under resting (basal) conditions and under conditions of acute strong stimulation is supported by different intracellular pathways. Surprisingly, we have been able to deduce that granule exocytosis, which customarily has been equated with most forms of regulated exocrine secretion of proteins, is largely reserved for the latter response where massive quantities of protein are needed to efficiently process ingested food. In contrast, the minor regulated secretory pathway, which is a derivative of the maturing secretory granule, normally handles the need for regulated secretion under resting conditions. Secretory granule exocytosis makes a contribution to resting secretion only when acinar cells approach full-capacity storage (during fasting), and even then the contribution is modest (Fig. 2). The selective mobilization of a smaller capacity and more sensitive-responding pathway to support resting secretion represents a clever and economic specialization.

Previously, we deduced that the minor regulated pathway was capable of releasing as much as 10% of newly synthesized amylase and thus concluded that the pathway is broadly derived from the acinar cell population (14). In the present study, we have shown that low dose carbachol stimulation visibly affects the organization and dimensions of the apical surfaces of a sizable fraction of the acinar cell population (Fig. 3) and that these changes correlate with production of an apically directed secretion in situ (Fig. 2C). Although a morphological response to stimulation is not evident in all cells, we suspect that the breadth of the effect may be limited by the dose of stimulant. Indeed, our previous results show that the magnitude of the minor regulated secretory response increases with secretagogue dose (Fig. 2 in Ref. 14), suggesting that acinar

DISCUSSION

Minimal Contribution of Granule Exocytosis to Resting Secretion—In the present studies, we have made a compelling case through examination of the total composition of parotid secretory proteins that secretion under resting (basal) conditions and under conditions of acute strong stimulation is supported by different intracellular pathways. Surprisingly, we have been able to deduce that granule exocytosis, which customarily has been equated with most forms of regulated exocrine secretion of proteins, is largely reserved for the latter response where massive quantities of protein are needed to efficiently process ingested food. In contrast, the minor regulated secretory pathway, which is a derivative of the maturing secretory granule, normally handles the need for regulated secretion under resting conditions. Secretory granule exocytosis makes a contribution to resting secretion only when acinar cells approach full-capacity storage (during fasting), and even then the contribution is modest (Fig. 2). The selective mobilization of a smaller capacity and more sensitive-responding pathway to support resting secretion represents a clever and economic specialization.

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Chase
Time (h)
0 2.5
0 - 1
1 - 2
1.5 - 2

Cells
Iso
- -
- -
- +
- +

Secretion
Iso
- -
- -
- +
- +

ProB
B-
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cells may be differentially sensitive to stimulation. Physiological resting secretion may normally involve only a fraction of cells responding to low dose parasympathetic stimulation at any given time. Differential sensitivity to secretory stimulation within populations of regulated secretory cells appears to be a general property of exocrine and endocrine glands (36–39). Notably, whereas only a portion of total acinar cells may supply resting secretion at any point in time, the formation of new carriers probably proceeds continuously in all cells since filling and discharge of the minor regulated pathway seem to be indifferent to the fasting/feeding state of the animal (Figs. 2 and 4).

Intracellular Organization of Minor Regulated and Constitutive-like Pathways—Our findings regarding the intracellular relationships of the minor regulated and constitutive-like pathways have led to the model shown in Fig. 8. Both pathways originate by a common step of vesicular budding that is linked to maturation of secretory granules and is shown to derive from both condensing vacuoles and immature granules. The two pathways overlap at this level with the route followed by lysosomal prohydrolases. They diverge subsequently by formation of minor regulated carrier vesicles, which are maintained as a storage pool until they are induced to undergo exocytosis at the apical surface. After the branch point, constitutive-like carriers proceed to a junction with endosomes. Here the lysosomal prohydrolase pathway diverges, and the proteins destined for constitutive-like secretion are delivered to the cell surface via recycling endosomes. Notably, the separation from the prohydrolase pathway appears especially efficient in the acinar cell as compared with the pancreatic β-cell (Fig. 7, (18)), possibly reflecting enhanced membrane (receptor) association of procathepsin B at the elevated pH characterizing apical endosomes (40).

Formation of the two pathways of resting secretion together is supported by the observations that the relative radiochemical compositions of the major salivary proteins in both pathways are the same (Fig. 2) and that initial detection of radio-labeled proteins in unstimulated and carbachol-stimulated secretions occurs concurrently (Fig. 4). Divergence of the two pathways distal to the common origin is supported by at least three findings. First, we showed previously that the minor regulated pathway alone behaves as a storage compartment from which biosynthetically labeled proteins could be discharged long after the bulk of unstimulated (constitutive-like) secretion of labeled proteins had occurred (14). Second, we have observed a striking difference in the specific radioactivity of amylase released by the two pathways, with that of the minor regulated pathway being substantially higher than that of the constitutive-like pathway (Fig. 4). Third, the two pathways are distinguished by their differential sensitivity to BFA, particularly when it is applied at chase times that implicate an effect distal to immature granule exit (Figs. 5 and 6).

 Whereas passage of the constitutive-like pathway through endosomes has been analyzed previously by following trafficking of lysosomal prohydrolases in pancreatic β-cells (18, 32, 33), we have been motivated to propose this route in acinar cells to explain the difference in specific radioactivities of secretory proteins released by the constitutive-like and minor regulated pathways. Accordingly, we view endosomes as the probable site distal to branching of the minor regulated pathway where proteins traveling in the constitutive-like pathway are diluted into a pool of unlabeled (or less radioactive) proteins. It seems quite likely that the steady state level of secretory protein present within acinar endosomes is significant as a consequence of continual internalization by endocytosis from the apical lumen. Apical luminal spaces of exocrine tissues, which have a small caliber and are tortuous, may be uniquely capable of accumulating discharged secretory proteins as prospective cargo for endocytosis if passive drainage of the ductal system is slow, as it clearly is when there is little or no stimulation. In contrast to the constitutive-like carriers, minor regulated carriers, once formed, are stored until discharged directly by exocytosis. The peak and subsequent decline in specific radioactivity of amylase in the minor regulated pathway (Fig. 4) suggests that there is continual turnover within the storage pool.

The use of BFA has identified two levels where the pathways comprising resting secretion are perturbed (Fig. 8) and has been key in placing the branch point of the constitutive-like and minor regulated pathways in the overall transport process (Figs. 5 and 6). We were able to block production of the characteristic constitutive-like secretion, presumably by inhibiting vesicular budding from condensing vacuoles and immature granules, if we added BFA while biosynthetically labeled proteins were accumulated in the TGN. Under these conditions, the radiochemical composition of the ensuing secretion (Fig. 5G) suggested that sorting had been preempted, whereas the reduced specific radioactivity of amylase (Fig. 5F) probably reflected extracellular dilution by older unlabeled enzyme, whose export is facilitated by the later effect of BFA (Fig. 6, A and E). Blockade of constitutive-like secretion at its origin agrees with previous observations (24, 25), and the secretion of newly synthesized proteins that occurs under these conditions is likely to derive proximally, possibly from components of the TGN vesiculated by BFA (41, 42). If we added BFA later during chase, either with or without prior accumulation of labeled secretory proteins in the TGN, we found that the drug had the opposite effects of increasing the specific radioactivity of exported amylase and preserving the composition characteristic of constitutive-like secretion. Moreover, these effects were...
linked in a compensatory relationship to inhibition of formation of the minor regulated pathway. Simultaneously, the results identified the second level of action of BFA on resting secretion and signified the presence of the branch point of its two pathways. Interestingly, this compensatory relationship where constitutive-like secretion is increased when the minor pathway is inhibited is the reverse of one noted previously where discharge of the minor regulated pathway transiently reduced constitutive-like secretion of labeled amylase during replenishment of the minor regulated carriers (14).

In contrast to BFA, wortmannin did not have major effects on the constitutive-like and minor regulated pathways over the same time course. The minor effects observed late during chase appear to be secondary to the formation of both pathways and may be related to well known perturbations within the endosomal system (15). The lack of perturbation of protein exit from immature granules by wortmannin in salivary acinar cells appears distinct from the effect observed in insulin-secreting cells (18). However, we presently cannot fully rule out the possibility that decreased drug access to the transport machinery in acinar cells of parotid lobules might contribute to the difference.

Potentially Related Pathways and Functions in Other Cell Types—The preferential export of newly synthesized secretory proteins and the enhanced secretagogue sensitivity characterizing the minor regulated pathway of parotid acinar cells are reminiscent of various earlier studies in endocrine cells, for example pancreatic β-cells where preferential secretion of newly synthesized insulin in response to moderate glucose elevation was reported (43). Furthermore, a potentially related pathway involving rapid "piecemeal" secretion of stored secretory products has been described in hematopoietic cells (44). Consequently, there is good reason to believe that an analog of the exocrine minor regulated pathway might exist in most if not all types of regulated secretory cells and possibly even other cell types (45). In the case of endocrine cells, it may be particularly intriguing to consider in future studies whether a minor regulated pathway analog functions in pulsatile secretion that widely characterizes resting secretion of hormones (46).

Whether the minor regulated pathway and its prospective analogs carry any unique secretory cargo also remains a significant issue. Our data show that the content of the parotid minor regulated pathway is essentially identical to that of the constitutive-like pathway and mainly contains proteins that are less efficiently retained in maturing granules (Fig. 2). Nevertheless, we do not discount the possibility that the pathway might be used selectively to export minor components that are required to maintain oral physiology during periods between ingestion of food. At present, however, it seems clear that the minor regulated pathway does not function in stimulus-evoked export of active lysosomal enzymes (Fig. 7), a process characterized elsewhere (34, 35).

Although we have no information about the function of the minor regulated pathway in the trafficking of membrane components, it is notable that there are several potentially interesting avenues to be explored here. Exocrine granule maturation entails progressive reduction in the surface density of intramembranous particles as viewed by freeze fracture (47), suggesting that vesicles emanating from maturing granules may engage in selective membrane protein removal. SNARE and associated proteins have been implicated in nucleating membrane protein sorting during vesicle formation (48–50), and one or more SNAREs that eventually support exocytosis of vesicles derived from immature granules may also function in forming the vesicles. A recent study identified a BFA-sensitive remodeling process involving vesicle-associated membrane protein-

protein-4 and synaptotagmin-4 removal from maturing neuroendocrine granules that appears to enhance stimulus responsiveness of the granules (51). Although removal of a negative regulator of granule exocytosis, synaptotagmin-4, was emphasized, there is likely to be an accompanying role of the exiting vesicles as secretory carriers. We are intrigued by the possibility that the vesicles are related to constitutive-like and/or minor regulated carriers in acinar cells.

We anticipate that other membrane proteins may be concentrated in minor regulated carriers as well. In particular, the membrane machinery regulating fluid and electrolyte secretion may be included in the vesicles, especially as the minor regulated pathway is stimulated by the same secretagogues that cause fluid and electrolyte secretion and is mobilized by levels of stimulation that are just sufficient to initiate salivary flow. As acinar cells are epithelial, it is possible that the minor regulated pathway may be related to apical carriers/endosomes, which undergo increased export to the apical surface in response to cell signaling (52–57). The identification of markers and the isolation of minor regulated carriers should greatly enhance our ability to explore these relationships and to address possible general secretory and transport functions.

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Resting (Basal) Secretion of Proteins Is Provided by the Minor Regulated and Constitutive-like Pathways and Not Granule Exocytosis in Parotid Acinar Cells

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