Journal of Cell Science 109, 2591-2599 (1996)
Printed in Great Britain © The Company of Biologists Limited 1996
JCS1250

Two regulated secretory pathways for newly synthesized parotid salivary proteins are distinguished by doses of secretagogues

J. David Castle* and Anna M. Castle

Department of Cell Biology, University of Virginia Health Sciences Center, Charlottesville, VA 22908, USA

*Author for correspondence (e-mail: jdc4r@virginia.edu)

SUMMARY

Low doses of the muscarinic agonist pilocarpine (0.1-1 μM) and the β-adrenergic agonist isoproterenol (0.5-4 nM) stimulate a minor regulated secretory pathway for salivary proteins in rat parotid lobules. Newly synthesized proteins (labeled biosynthetically) are selectively discharged, and they are secreted in the same relative proportions as observed in constitutive-like unstimulated secretion but different from the proportions of older proteins that are discharged by granule exocytosis in response to higher doses of secretagogue. The response to low doses of agonists is transient and involves output of no more than 1-2% of tissue-associated amylase. The same increase in output of pulse-labeled proteins is observed when agonist is added at various chase times (1.5-6 hours), implying that release occurs from a post-Golgi storage pool. Stimulation for 40 minutes significantly depletes the storage pool as a second stimulation elicits smaller output. Stimulation also partially depletes labeled proteins from subsequent constitutive-like secretion after the agonist is removed implying that the constitutive-like and low dose agonist mediated pathways draw on the same pool of secretory proteins. While these results indicate that acinar cells have a second regulated secretory pathway, this new pathway is unlikely to contribute uniquely to the protein composition of parotid secretion. Rather it may serve a different role in secretion at the apical cell surface.

Key words: Regulated secretion, Acinar cell, Salivary protein

INTRODUCTION

Cells that are specialized for regulated secretion store most of their secretory proteins in membrane-bounded granules that undergo exocytosis in response to stimulation. However, recent studies have indicated that these cells also release small amounts of the same proteins by an unstimulated pathway that appears to originate by vesicular budding from maturing granules (Arvan and Castle, 1987; Zastrow and Castle, 1987; Zastrow et al., 1989; Kuliawat and Arvan, 1992; Grimes and Kelly, 1992; Milgram et al., 1994). This pathway has been named the constitutive-like pathway to distinguish it from the Golgi-derived constitutive pathway (Arvan et al., 1991) which is more rapid and contains secretory products that are not found in regulated secretion. A distinctive feature of the constitutive-like pathway is that the relative composition of secretory proteins released by this route differs from that released by stimuli that cause extensive granule exocytosis. Enrichment of certain proteins in the constitutive-like pathway is thought to be the consequence of their less efficient condensation within the granule content core (reviewed by Arvan and Castle, 1992; Kuliawat and Arvan, 1994) or their affinity for budding membranes during granule maturation.

In parotid acinar cells, constitutive-like secretion of newly synthesized proteins has been detected kinetically as a distinct phase of release that is coordinated with granule maturation and precedes the unstimulated exocytosis of mature granules containing secretory proteins of the same post-translational age (Zastrow and Castle, 1987). The secretory products released in this phase are the same as those packaged into storage granules, but the relative proportions of particular proteins are distinct. A minor 22 kDa glycoprotein (recently identified as common salivary protein 1 or CSP1; Girard et al., 1993) is relatively enriched while a major 25 kDa polypeptide (leucine-rich parotid secretory protein or PSP; Mirels and Ball, 1992) is almost absent in constitutive-like secretion (Zastrow and Castle, 1987). α-Amylase, which is the major secretory protein of the tissue, is the major protein released by both constitutive-like and regulated routes, and it has been used to normalize and compare the relative compositions within the two pathways (Zastrow and Castle, 1987; Zastrow et al., 1989). So far the function of the constitutive-like pathway, which has slower export kinetics as compared to constitutive secretion (Arvan and Castle, 1987; Zastrow and Castle, 1987; Grimes and Kelly, 1992; Milgram et al., 1994), is not clear.

In the present study, we show that very low doses of the secretory agonists pilocarpine and isoproterenol each enhance the export of biosynthetically labeled proteins in the identical proportions that are observed in constitutive-like secretion. At the doses used, stimulation clearly enhances export of newly synthesized protein preferentially with little or no evidence of increased exocytosis of mature storage granules. The cholinergic and β-adrenergic stimuli both draw on the same pool of secretory proteins that contributes to constitutive-like output in the absence of stimulation. These findings provide evidence for a second stimulus-regulated pathway for parotid acinar

proteins that is linked to the constitutive-like pathway. Because the pathway is minor as compared to secretion granule exocytosis and does not transport unique proteins, we suggest that it has roles in the secretory process other than protein export.

MATERIALS AND METHODS

Materials

Male Sprague-Dawley rats 125-200 g obtained from Hilltop (Scottsdale, PA) were used in all experiments, and were fasted overnight prior to sacrifice. Tran[^{35}S]label used for biosynthetic labeling was obtained from ICN (Irvine, CA); secretagogues D,L-isoproterenol and pilocarpine were obtained from Sigma (St Louis, MO).

Experimental incubations

In each experiment, the parotid tissue of one animal was used. Animals were sacrificed by cardiac incision under ether anesthesia, and excised parotid glands were dissected into lobules in Dulbecco's MEM equilibrated with 95% O_2, 5% CO_2. All lobules were pooled in a single 25 ml flask, preincubated for three 10 minute intervals at 37°C in RPMI 1640 medium lacking methionine and cystine and then pulse-labeled 5 minutes in the same medium containing 0.6-0.8 mCi/ml Tran[^{35}S]label. Following the pulse, the lobules were washed by swirling twice with 37°C chase medium (Dulbecco's MEM supplemented with 300 μg/ml methionine and 60 μg/ml cystine), divided into groups of 20-25 lobules each and transferred to 10 ml Erlenmeyer flasks for chase incubations. All chase incubations were performed in 1 ml medium, and the samples were gassed every 5 minutes with 95% O_2, 5% CO_2. At specified time intervals during the chase, the medium was removed in entirety from each flask and replaced with 1 ml fresh medium. Each sample of medium was centrifuged 2 minutes at 10,000 ***g*** in a microcentrifuge, and the supernatant was transferred to a fresh tube and frozen for storage at −20°C. Secretagogues were added at specified experimental timepoints (10 μl of 100× stock solutions prepared in water within 2 hours of use and kept at 0°C in the dark). In experiments where low doses of secretagogues were used transiently at various chase times, massive granule exocytosis was induced later during the final 40 minutes of incubation by adding ≥10 μM isoproterenol. The latter samples were used to confirm tissue responsiveness for the duration of incubation and to compare the specific radioactivity to that of proteins released during the earlier intervals. Following the last time interval, the lobules were washed once in chase medium and thoroughly homogenized in 1 ml phosphate buffered saline.

Assays

Aliquots of each sample of incubation medium and homogenate were assayed for amylase activity (Bernfeld, 1955); only values within the linear (zero-order kinetics) range of the assay were used so that activity accurately reflected the amount of enzyme present. Samples normalized to equal activity were subjected to SDS-PAGE on 11% gels and fluorography (Castle et al., 1992). (Amylase represents about 40% of the total secretory protein produced in rat parotid tissue so this normalization is proportional to that which would be achieved by normalizing to total protein). Within individual experiments, all samples were processed and fluorographed together. Incorporated radioactivity in individual gel bands exposed in the linear range of the X-ray film was quantitated by laser densitometry. Since all gel samples contained equal amylase activity, the fluorographic density of the 58 K amylase band is a direct measure of specific radioactivity.

RESULTS

Visualization of enhanced secretion by low doses of agonists

Constitutive-like secretion has been detected in pulse-chase labeling experiments as a transient phase of increased output of radiolabeled proteins in the absence of stimulation. It substantially precedes the larger and sustained output of radiolabeled proteins by unstimulated exocytosis of secretion granules (Arvan and Castle, 1987). Because unstimulated exocytosis of secretion granules is a continuous low-level activity in exocrine cells, constitutive-like secretion can only be detected in the presence of this background output using the radiolabeling and kinetic approach (Arvan and Castle, 1987; Zastrow and Castle, 1987). For testing the effects of low doses of secretory agonists on secretion, we used the same experimental strategy. Thus we introduced the agonists at various intervals during chase incubation following brief pulse labeling with Tran[^{35}S]label and examined the effect of stimulation on the output of both labeled proteins and amylase activity. The agonists pilocarpine, isoproterenol or a combination of the two all had the same effect and elevated the secretion of radiolabeled proteins. An example of the results that were obtained with the combined agonists is shown in Fig. 1. The early phase of constitutive-like secretion peaks at 80 minutes and is enriched in four products: amylase, 38 kDa and 32 kDa polypeptides, and 22 kDa CSP1. The constitutive-like secretion is preceded by the more rapid release of higher M_r proteins (≥90,000; at 40 minutes in Fig. 1) presumably by constitutive, rather than constitutive-like, pathways (Arvan and Castle, 1987; Zastrow and Castle, 1987). Low dose stimulation was applied between 240-280 minutes chase to the sample shown in Fig. 1b, and it resulted in an enhanced output of radiolabeled proteins in very similar proportions to those observed in the early phase of constitutive-like secretion. The specific radioactivity of proteins released following low dose stimulation was comparable to that at the peak of constitutive-like output and was clearly higher than that observed in response to 10 μM isoproterenol (added between 400-440 minutes chase). As illustrated in Fig. 1c, the low dose agonist effect is accompanied by an increased release of ~1.5% of total amylase activity during the interval of stimulation. This is 20-fold smaller than the 30% release achieved by granule exocytosis during the 400-440 minute interval (not shown).

Dose dependence and duration of agonist effects

We were intrigued by the observation that both pilocarpine and isoproterenol cause the low dose response shown in Fig. 1 because at higher doses, these agonists have very different effects on secretion. Pilocarpine, a muscarinic agonist, mainly elicits fluid and electroyte secretion and only moderately increases protein secretion from the parotid while isoproterenol, a β-adrenergic agonist stimulates discharge of a protein-rich, highly concentrated saliva. Therefore, we followed the output of biosynthetically labeled proteins (particularly the major secretory protein amylase) in response to different doses of each agonist and used a higher resolution timecourse in order to compare the responses. Fig. 2 shows one example from four experiments in which varying levels of pilocarpine or isoproterenol were added at 90 minutes of chase incubation (just after the peak of constitutive-like secretion of newly synthesized proteins) and maintained until 130 minutes; changes to fresh (secretagogue-containing) medium were made at 100 and 110 minutes to follow the kinetics of the response. The specific radioactivity of amylase and the fraction of total amylase enzyme activity discharged during each interval were

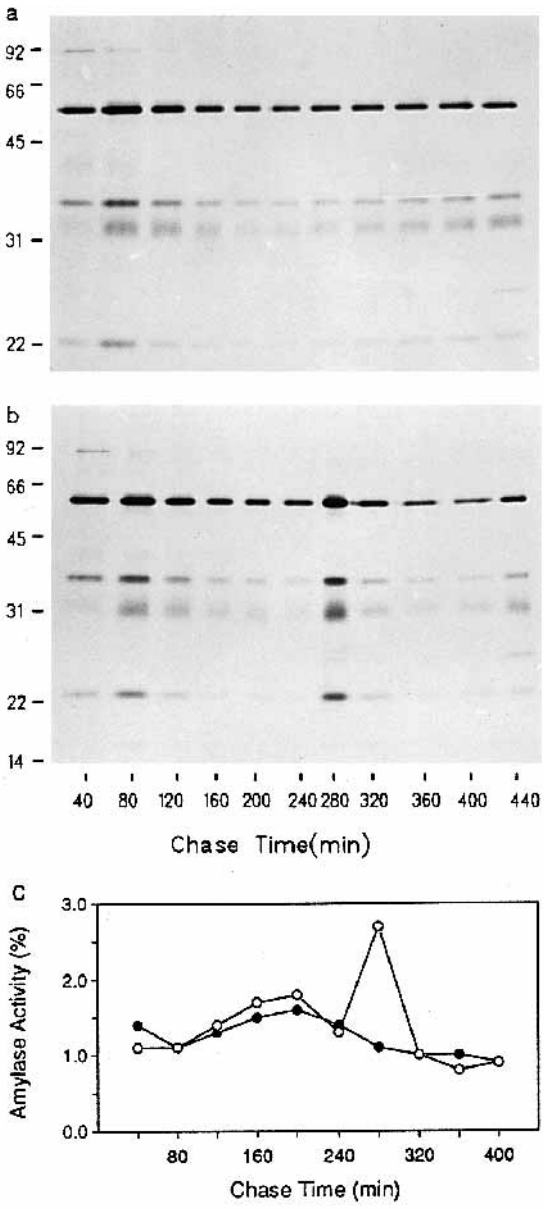


Fig. 1. (a,b) Fluorographs illustrating the timecourse of output of secretory proteins during successive 40 minute intervals of chase incubation following pulse biosynthetic labeling. The times indicated are the end of each interval. Results for control (unstimulated) tissue and tissue stimulated with 0.1 μM pilocarpine at 240-280 minutes are shown in a and b, respectively. During the final time interval (400-440 minutes), 10 μM isoproterenol was added to induce massive granule exocytosis. The samples in each lane contain 0.8 U amylase activity. Note that the autoradiographic densities of proteins released during peak constitutive-like secretion (80 minutes) are very similar in the two panels (indicating that the samples are well normalized to each other) and that similar densities are observed as a consequence of low dose stimulation (280 minutes in b). (c) Plots showing the rate of secretion of amylase enzyme activity for each of the samples in a,b, and illustrating the small stimulatory effect of low doses of agonists between 240 and 280 minutes. During the final time interval (400-440 minutes), 30 and 29% of total amylase was released in response to 10 μM isoproterenol for a and b, respectively.

quantitated throughout the timecourse. Pilocarpine elicits a sharp rise in specific radioactivity at doses of 0.1 μM and 10 μM (Fig. 2a). In the presence of 0.1 μM pilocarpine, the specific radioactivity increased 2.6±0.8-fold (mean ± s.e.m.; four separate experiments) above the level observed during the preceding peak of constitutive-like secretion whereas the increase was 2.2±0.9-fold (mean ± s.e.m.; two separate experiments) for 10 μM pilocarpine. The rise in specific radioactivity is transient, and its initial decline toward control levels is more rapid at higher doses. The profile obtained with 1 μM pilocarpine is very similar to that for 10 μM, but this intermediate dose has not been used routinely. Secretion of amylase enzyme activity was stimulated only a small amount above control values at both high and low doses of pilocarpine (<1% of total for 0.1 μM and <3% for 10 μM at each timepoint; Fig. 2c). In combination, the data in Fig. 2a,c indicate that the preferential output of newly synthesized amylase is highest at the lowest dose of pilocarpine.

Isoproterenol at doses ≤0.1 μM has similar effects on the specific radioactivity of amylase discharged (Fig. 2b), although the effects appear somewhat smaller than with pilocarpine. The specific radioactivity increased 1.6±0.2-fold (mean ± s.e.m.; four separate experiments) in the presence of 5 nM isoproterenol and 1.4±0.0-fold (mean ± s.e.m.; two separate experiments) in the presence of 0.1 μM isoproterenol. At the lower dose, the rate of rise of specific radioactivity is slower and the rise is sustained longer than at higher doses. As with pilocarpine, these responses reflect only a very small increased output of amylase enzyme activity (Fig. 2c) and thus represent preferential discharge of newly synthesized amylase. In contrast, 10 μM isoproterenol causes a more delayed rise in amylase specific radioactivity (Fig. 2b). However, substantial discharge of amylase enzyme activity is already evident at the 100 minute timepoint (Fig. 2c) indicating massive exocytosis of mature unlabeled secretion granules, consistent with the previously reported preferential export of older secretory products under comparable stimulatory conditions (Sharoni et al., 1976).

In separate experiments (not shown), we examined the sensitivity of the low dose response to lower concentrations of secretagogues, and we examined the duration of the response in the continued presence of stimulation. Judging by detection of increased specific radioactivity of secreted amylase, we found that the lower limits of sensitivity of the response were 0.5 nM for isoproterenol and 10 nM for pilocarpine. By extending incubation times in the presence of stimuli, we found that for either 5 nM isoproterenol or 0.1 μM pilocarpine applied continuously, 90 minutes was required for the specific radioactivity of amylase to decrease almost to control levels. Summation of the autoradiographic densities of the amylase released throughout this timecourse and that remaining in the tissue indicated that 10% of the newly synthesized amylase was discharged in response to low-dose secretagogue stimulation. By analogous measurement, about 20% of newly synthesized CSP1 was released concomitantly.

Composition of agonist-evoked secretion

The radiochemical composition of secretion at each time interval under the various conditions of stimulation just discussed is shown in Fig. 2d-i. In all cases except the sample stimulated with 10 μM isoproterenol, the enhanced secretion is enriched in the same polypeptides (amylase, 38 kDa, and 22

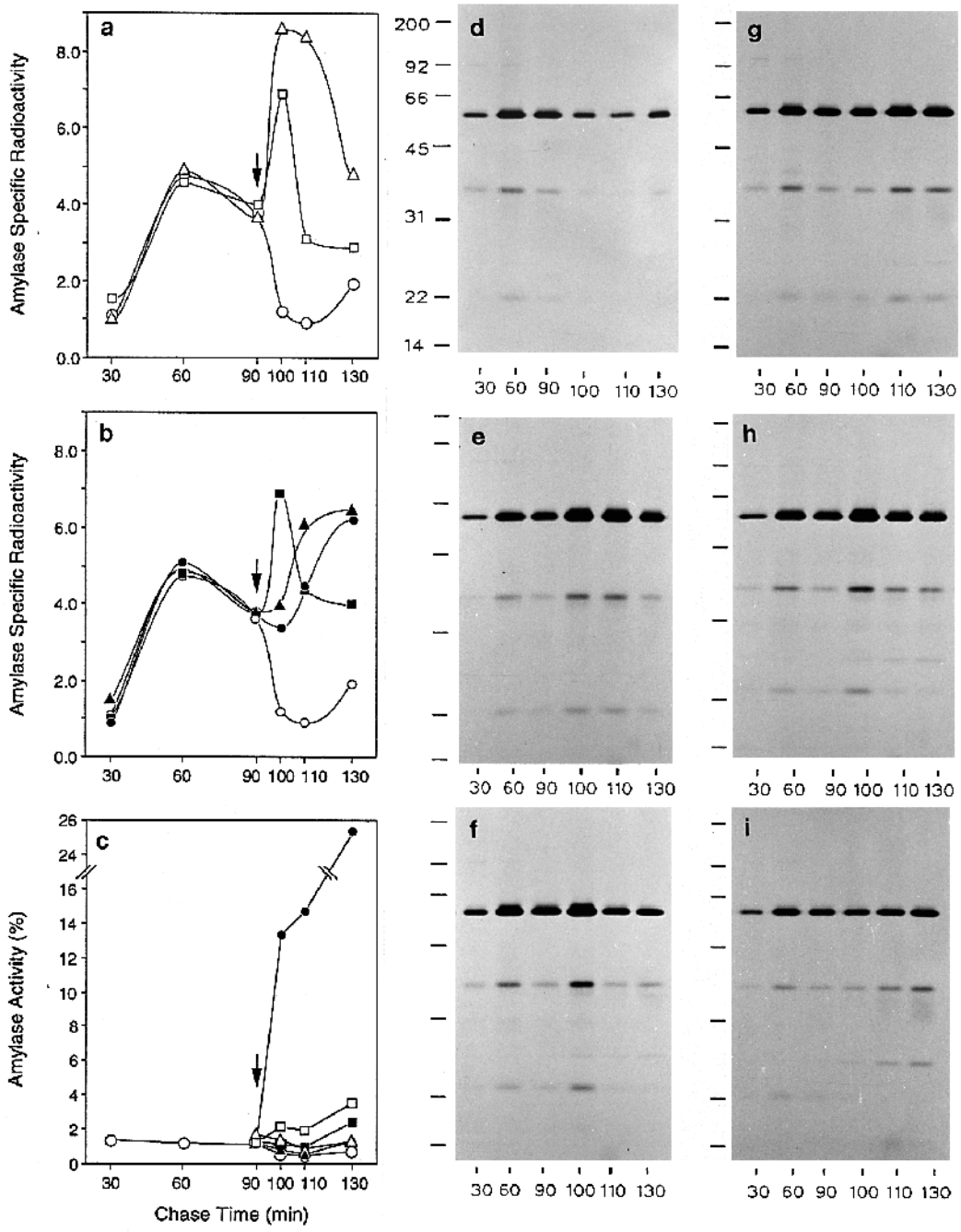
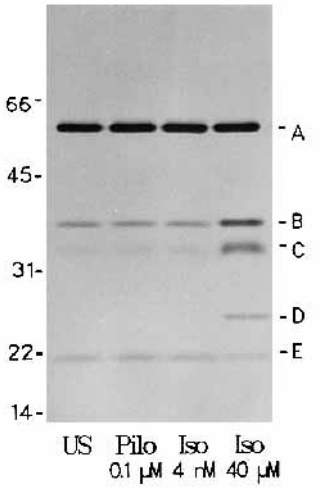


Fig. 2. Specific radioactivity and radiochemical composition of secretion released in response to various doses of secretagogues. (a,b) Amylase specific radioactivity at each interval of chase incubation. Agonists, pilocarpine (0.1 μM, △; 10 μM, □) in a and isoproterenol (5 nM, ▲; 0.1 μM, ■; 10 μM, ●) in b were added at 90 minutes chase (arrow) and at each time interval thereafter (when the medium was changed). A plot for the unstimulated control is included in each panel (○). (c) Secretion of amylase enzyme activity (% total: tissue + all media) for the samples shown in a and b. Again, the arrow refers to the time of agonist addition. Control (○); pilocarpine (0.1 μM, △; 10 μM, □); isoproterenol (5 nM, ▲; 0.1 μM, ■; 10 μM, ●). (d-i) Fluorographs showing the relative compositions of radiolabeled secretory proteins in constitutive-like secretion and in secretion released in the absence of stimulation (d) or in response to 0.1 μM pilocarpine (e), 10 μM pilocarpine (f), 5 nM isoproterenol (g), 0.1 μM isoproterenol (h), and 10 μM isoproterenol (i).

kDa CSP1) that are observed in the preceding phase of constitutive-like secretion (60 minutes chase) and in the control (Fig. 2d). In contrast, the secretion induced by 10 μM isoproterenol (Fig. 2i) contains very little radiolabeled CSP1 but is enriched in 25 kDa PSP. Labeled PSP is also observed at lower levels in samples stimulated with 0.1 μM isoproterenol (Fig. 2h) and can be detected in longer exposures of the fluorograph of samples stimulated with the highest dose of pilocarpine (Fig. 2f). We interpret our findings to indicate that at least two distinct regulated secretory pathways can be distinguished in parotid tissue based on agonist dosage and relative secretory composition. At low doses of either agonist, there is selective stimulation of a pathway that discharges newly synthesized secretory products with a composition that resembles that observed in the constitutive-like pathway. We have named this pathway the minor regulated pathway because the total amount of secretory protein released is very small. At high doses of isoproterenol, the major regulated pathway comprising granule exocytosis predominates. At intermediate doses of isoproterenol and high doses of pilocarpine, the secretory composition suggests superimposition of the minor pathway with limited output by the major pathway.

Because intermediate doses of isoproterenol seem to elicit limited granule exocytosis and high doses of pilocarpine and other muscarinic agonists cause changes in acinar cell morphology that may be non-physiologic (Leslie and Putney, 1983; Foskett and Melvin, 1989; our own unpublished observations), we have carried out further studies of the minor

a



b

Band Density Ratios

Sample	B/A	C/A	E/A
Unstim	0.13	0.02	0.08
0.1 μM Pilo	0.10	0.04	0.08
4 nM Iso	0.11	0.05	0.08
40 μM Iso	0.29	0.27	0.03

Bands A,B,C,E are labeled in panel a

Fig. 3. Radiochemical compositions of secretion from constitutive-like and regulated pathways. (a) Fluorograph of samples in which the amylase specific radioactivities were matched during gel loading. Constitutive-like unstimulated secretion (US; released between 40 and 80 minutes post-pulse), secretion released by the minor regulated pathway in response to 0.1 μM pilocarpine (Pilo; released between 200 and 240 minutes post-pulse) and 4 nM isoproterenol (Iso; released between 200 and 240 minutes post-pulse), and secretion released by the major regulated pathway (granule exocytosis) in response to 40 μM isoproterenol (added between 400 and 440 minutes post-pulse) are shown. The accompanying table (b) compares the densities of corresponding bands normalized to the density of the amylase band in each sample. The latter determinations were made on fluorographs exposed in the linear range of the X-ray film.

regulated pathway using only low doses of agonists. In Fig. 3, we present a quantitative comparison of the radiochemical profiles of constitutive-like secretion and secretion stimulated by 5 nM isoproterenol, 0.1 μM pilocarpine, and 40 μM isoproterenol. The intensities of the most prominent bands relative to amylase were measured on samples from a single experiment in which equal amylase specific radioactivity (measured in a separate fluorograph) was loaded in each lane. As can be seen in Fig. 3b the intensity ratios for major bands are essentially identical for constitutive-like secretion and secretion released by low doses of each agonist, and they clearly differ for secretion discharged by 40 μM isoproterenol. In three separate experiments, the ratios B/A and E/A indicated in Fig. 3 were measured. Although the absolute values varied between experiments, the ratios within each experiment reiterated the results shown in Fig. 3b. B/A ratios for unstimulated, 0.1 μM pilocarpine, and 4 nM isoproterenol samples all differed from each other by a factor of 1.08±0.05 (s.e.m.) while the ratio for the 40 μM isoproterenol-stimulated samples was always more than 1.7-fold higher. E/A ratios for unstimulated, 0.1 μM pilocarpine and 4 nM isoproterenol samples differed from each other by a factor of 1.33±0.05 (s.e.m.) while the ratios for the 40 μM isoproterenol-stimulated samples were more than threefold higher.

Characteristics of the minor regulated pathway and its relationship to constitutive-like secretion

Given the close similarity in secretory composition between the constitutive-like and minor regulated pathways, additional experiments were conducted to evaluate the interrelationship of the pathways further and to assess whether the same pool of proteins is drawn upon by both cholinergic and β-adrenergic stimulation. From our original characterization of constitutive-like secretion, we knew that the presence of secretory antagonists (atropine, phentolamine and propranolol) had no effect on the magnitude or timing of unstimulated output (Zastrow and Castle, 1987). Therefore, we felt that it was unlikely that low doses of agonists were acting simply as accelerators of export by the constitutive-like pathway. Rather, the minor regulated pathway might serve as a supplementary export route. Results shown in Fig. 4a using agonist stimulation at increasing times during extended chase incubation support this interpretation. Pilocarpine (0.1 μM) added for 40 minutes beginning at 80 minutes of chase incubation increases the specific radioactivity of secreted amylase above the level observed at the peak of constitutive-like secretion, indicating that the minor regulated pathway is additive with the constitutive-like pathway. Further, 0.1 μM pilocarpine causes essentially the same increase in amylase specific radioactivity when applied at 80, 160, and 320 minutes chase. Thus, release of radiolabeled proteins can be stimulated long after most of the constitutive-like secretion has taken place, and the minor regulated pathway exhibits the characteristics of a storage pool with a slow turnover.

Fig. 4a also shows that stimulation by 0.1 μM pilocarpine at 80-120 minutes chase partially depletes a subsequent 40 minute stimulation of the same sample at 320-360 minutes chase. In addition to confirming the small size of the low dose releaseable pool, the latter finding suggests that any replenishment following the first stimulation involves protein of lower specific radioactivity.

While the study employing stimulation at increasing chase times suggests that the minor regulated and constitutive-like pathways are distinct, at least in part, two observations argue that they have a common origin. First, we noticed that after terminating low dose agonist stimulation, the subsequent unstimulated secretion of radiolabeled proteins always decreases to a level that is about half that measured in the control (Fig. 4a). This effect was observed with pilocarpine, isoproterenol or both agonists together and is visualized best in the actual autoradiographs for amylase (Fig. 4b, chase times 200-400 minutes; see also Fig. 1b, chase times 360 and 400 minutes). Apparently the pools of secretory protein supplying the constitutive-like and minor regulated pathways are interrelated.

The second observation that seems to relate the origins of

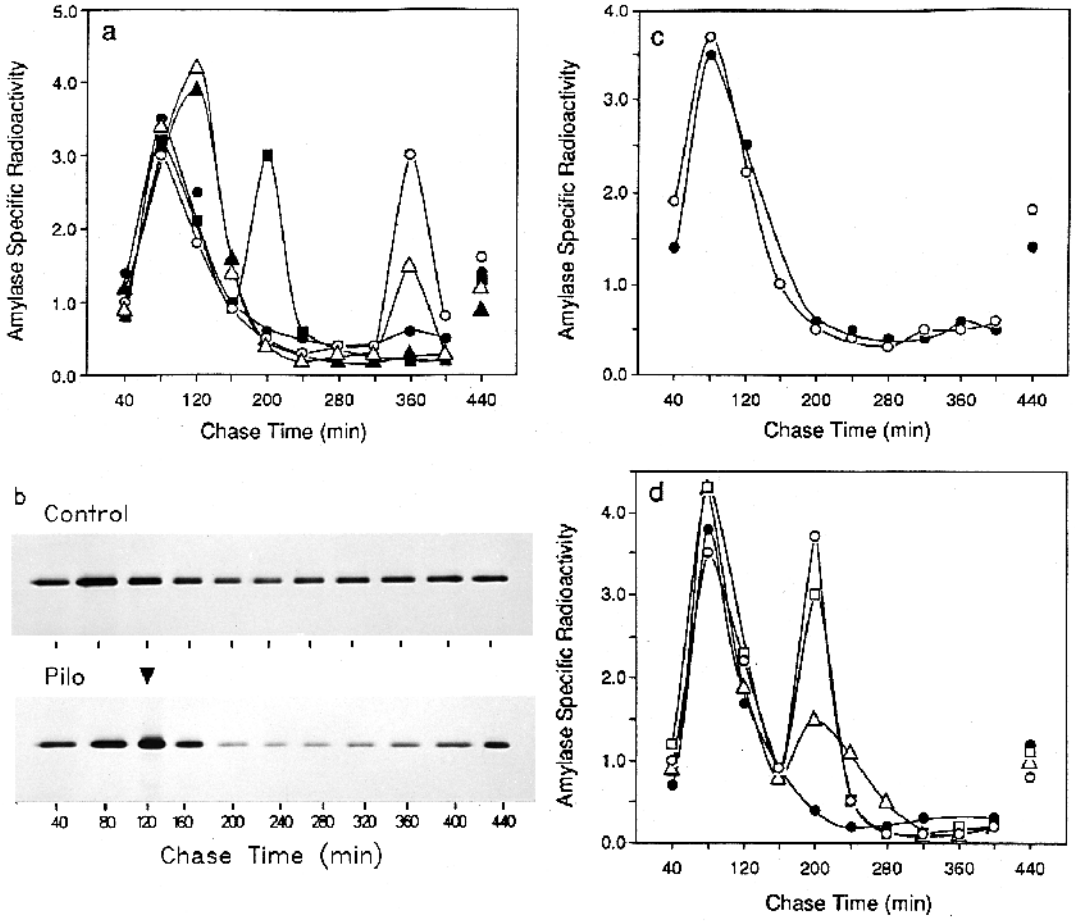


Fig. 4. Further characterization of the minor regulated secretory pathway. (a) Comparison of amylase specific radioactivity following application of 0.1 μM pilocarpine at 80-120 minutes (▲), 160-200 minutes (■), 320-360 minutes (○), and during two intervals at 80-120 minutes and 320-360 minutes (△). Control (●). The data points at 440 minutes which are not attached to the curves report the specific radioactivity of amylase released by the major regulated pathway following addition of 40 μM isoproterenol during the final interval. (b) Fluorographs of the amylase band in samples that were unstimulated throughout 400 minutes incubation (top) or stimulated with 0.1 μM pilocarpine at 80-120 minutes (bottom, arrowhead). (c) Timecourse showing the specific radioactivity of amylase in the medium when 0.1 μM pilocarpine was applied at 0-40 minutes chase (○) as compared to the unstimulated control (●). (d) The effects of the β-adrenergic antagonist propranolol on secretion stimulated by pilocarpine and of stimulation of the minor regulated pathway by pilocarpine and isoproterenol in consecutive time intervals. Timecourses of amylase specific radioactivity in the medium are shown for an unstimulated control (●) and for samples stimulated with 0.1 μM pilocarpine (160-200 minutes) in the presence of 10 μM propranolol (120-200 minutes, ○); 0.1 μM pilocarpine (160-200 minutes) immediately followed by 4 nM isoproterenol (200-240 minutes, □); and 4 nM isoproterenol (160-240 minutes, △). In both c and d the detached data points at 440 minutes refer to amylase released by the major regulated pathway as in a.

the minor regulated and constitutive-like pathways is that low dose agonist stimulation has no apparent effect on the output of biosynthetically labeled proteins until constitutive-like secretion is in progress. This is clearly seen in Fig. 4c where agonist applied for 40 minutes immediately post-pulse (prior to arrival of labeled proteins in post-Golgi compartments) had no effect on the timing or specific radioactivity of ensuing constitutive-like secretion. Evidently this contrasts with the results in Figs 2 and 4a where the minor regulated pathway contributes incrementally to the specific radioactivity of secreted amylase during and following the peak of constitutive-like output.

We also examined whether pilocarpine and isoproterenol were stimulating release from a common pool of radiolabeled proteins in the minor regulated pathway by using the muscarinic and β-adrenergic agonists sequentially. We compared output when pilocarpine was followed by isoproterenol with output when isoproterenol was used repetitively. When 4 nM isoproterenol is used at 160 minutes and 200 minutes, the specific radioactivity of amylase rises moderately and remains elevated through both time intervals. However, when 4 nM isoproterenol is added after pilocarpine (0.1 μM), the rise and fall of amylase specific radioactivity are identical to that observed with pilocarpine used alone in the first interval (Fig. 4d). Apparently, initial pilocarpine stimulation efficiently depletes secretory protein accessed by low dose isoproterenol stimulation, arguing that the two secretagogues access the same pool and that a single minor regulated pathway is regulated by muscarinic and β-adrenergic stimuli.

In order to rule out the possibility that the stimulation by 0.1 μM pilocarpine is preganglionic and is mediated through endogenous catecholamines present in the heavily innervated parotid, we examined the effect of pretreatment with 10 μM propranolol, a β-adrenergic antagonist, on stimulation. Results presented in Fig. 4d show that propranolol has no inhibitory

effect on pilocarpine stimulation of the minor regulated pathway. Rather, if anything, it slightly augments the specific radioactivity of amylase released. The efficacy of propranolol in this type of experiment was independently confirmed by its partial inhibition of secretion elicited by 40 μM isoproterenol (not shown).

DISCUSSION

We have been able to detect the release of parotid salivary proteins by a novel minor regulated pathway against a background of unstimulated secretion by using a combination of pulse-chase radiolabeling and low dose agonist stimulation. This pathway is distinguished from the major regulated secretory pathway, exocytosis of acinar cell granules, by several features including: its high sensitivity to agonists; its comparable (if not greater) response to a muscarinic as compared to a β-adrenergic agonist; its very low capacity, more rapid depletion (as compared to stored granules) and selectivity for newly synthesized secretory proteins; and its relative composition of salivary proteins.

The composition of proteins discharged in the minor regulated pathway is identical to that of constitutive-like secretion provided the dose of secretagogue is below the level that stimulates the major regulated pathway (Figs 2, 3). Notably, the minor regulated and constitutive-like pathways each increase the specific radioactivity of total secreted amylase, signifying that stimulation elicits secretion and not merely washout of already secreted proteins. Increases due to the minor regulated and constitutive-like pathways are additive during the phase of constitutive-like secretion, while the increase caused by the minor regulated pathway can still occur at late chase times (e.g. Fig. 4a). Both features signify that the pathways ultimately are distinct; however, several characteristics other than identical secretory composition argue that they are closely interrelated. First, the increment in specific radioactivity of amylase released by low dose stimulation is about the same as the increment observed during the peak of constitutive-like secretion when examined over uniform time intervals (Figs 1b, 4a), suggesting origination from a common pool of protein. Second, the ensuing partial depletion of radiolabeled proteins in constitutive-like secretion following transient activation of the minor regulated pathway (Figs 1b, 4b), also suggests that the two pathways stem from a common source but that the reserve of exportable labeled cargo may be limited. Third, release of radiolabeled secretory proteins by the minor regulated pathway does not precede constitutive-like secretion, and stimulation immediately following biosynthetic labeling does not alter the specific radioactivity of susequently secreted amylase (Fig. 4c). We believe that these features, taken together, focus the origin of the minor regulated pathway on the maturing secretion granule.

The mechanism for the minor regulated pathway is not likely to involve stimulated exocytosis of immature granules. The minor regulated pathway in parotid exports only a small fraction of newly synthesized secretory protein, and certain acinar secretory products are very underepresented in its content (Fig. 3). In addition, the minor regulated pathway is unlikely to reflect exocytosis of secretion granules from a minor cell population that produces less than 1-2% of the total amylase of the tissue. As 10% of newly synthesized amylase can be released by the minor regulated pathway, we believe that the pathway must originate in a large fraction of, if not all, acinar cells. While the enrichment of CSP1 in the secretory output of the minor regulated pathway might be taken as cause to consider an origin in intercalated duct cells where production of this protein is most concentrated (Girard et al., 1993), we regard this possibility as quite unlikely because intercalated ducts cells in rat parotid do not produce and store amylase (Vugman and Hand, 1995). In combination with our recent detection of low levels of CSP1 in acinar cells by EM immunolabeling (J. D. Castle and H. Stukenbrok, unpublished observations), these observations argue in favor of an acinar cell origin for the minor regulated pathway by a mechanism that is linked to granule maturation. Thus, as for constitutive-like secretion (Arvan and Castle, 1992; Milgram et al., 1994; Carnell and Moore, 1994; DeLisle and Bansal, 1996), we focus on processes that involve vesicular budding from maturing granules where the secretory content mainly includes polypeptides that are least efficiently retained for storage in granules.

Fig. 5 illustrates two scenarios for the operation of the minor regulated pathway involving immature granules in acinar cells as an origin. In the first, the formation of constitutive-like carrier vesicles from maturing granules is regulated by low doses of secretory agonists. This process may be an extension of signal-mediated vesicle formation and transport from the TGN (Luini and De Matteis, 1993), especially to the apical surface in other epithelial cells (Pimplikar and Simons, 1994; Brewer and Roth, 1995; Brignoni et al., 1995). In acinar cells, low dose stimulation may increase the rate of formation of carrier vesicles destined for the cell surface and also activate this process in granules that are more advanced in the maturation process in order to account for both summation of constitutive-like and minor regulated output at early chase times and continued responsiveness at later chase times (Fig. 4a). As vesicle formation is necessarily coupled to and limited by

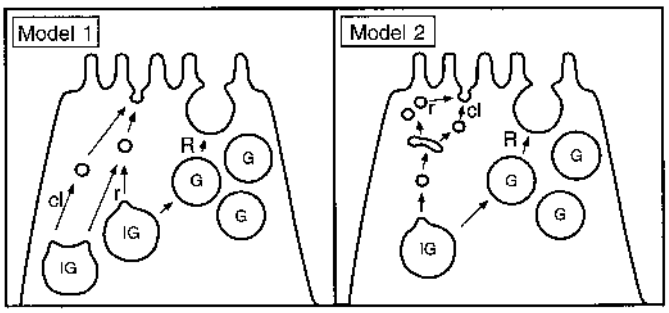


Fig. 5. Two models for the minor regulated pathway and its relationship to the constitutive-like secretory pathway in parotid acinar cells. In Model 1, the minor regulated (r) and constitutive-like (cl) secretory pathways derive directly from immature granules (IG) and are coupled to the process of formation of mature granules (G). Regulation by low doses of muscarinic and β-adrenergic agonists is exerted at the level of carrier vesicle formation. Granule exocytosis, the major regulated pathway (R), is stimulated mainly by higher doses of β-adrenergic agonists. In Model 2, vesicular carriers budding from maturing granules intersect an apical recycling endosome-like compartment. The constitutive-like pathway (cl) continuously carries part of the secretory cargo from this compartment to the cell surface while the minor regulated pathway (r) performs this function in response to low doses of agonists.

granule maturation, it follows that elevated specific radioactivity in the secretion is transient (Fig. 2) and that the specific radioactivity of constitutive-like secretion would be decreased relative to control after cessation of stimulation (Figs 1b, 4b).

In the second scenario, a common carrier for the constitutive-like and minor regulated pathways buds from the immature granule but then intersects another compartment where the constitutive-like and minor regulated components of secretion are divided. Part of the secretory cargo advances from this site in a carrier that is bound for release at the cell surface as the component of constitutive-like secretion. The rest is efficiently retained in intracellular vesicles that undergo exocytosis in response to low doses of agonists. Upon stimulation, release would be additive while following cessation of stimulation, the reduced specific radioactivity of secretion relative to control (Figs 1b, 4b) would reflect transiently increased diversion of content to replenish the minor regulated pool. A significant complication of this scenario is that continuous formation and efficient intracellular accumulation of carriers of the minor regulated pathway suggests build-up as a significant intracellular pool into which the pulse-labeled secretory proteins would be diluted. This is not readily reconciled with the results shown in Fig. 4a where essentially the same increments in amylase specific radioactivity are observed in constitutive-like secretion and low dose stimulated output at early and late chase times and in Fig. 4c where the specific radioactivity of constitutive-like secretion is unaffected by a preceding stimulation.

Evidently, we do not presently have data that directly address the validity of these (or possibly other) scenarios. To date, our efforts to use electron microscopy to search for a morphological correlate of the minor regulated pathway in tissue that has been stimulated with low doses of agonists have not yielded any real insight, and further progress is likely to require the identification of a specific marker for the pathway. Even without visible evidence, we are quite intrigued by the presence and potential functional significance of two regulated secretory pathways with different sensitivities to stimulation in parotid acinar cells. This arrangement has an interesting parallel in neurons where low frequency stimulation elicits synaptic vesicle exocytosis and higher frequency stimulation is required for large dense core vesicle exocytosis (reviewed by Lundberg and Hokfelt, 1983; DeCamilli and Navone, 1987). Just as the neuronal pathways are used for two different purposes, we believe the same is likely to be true for the minor and major regulated pathways in acinar cells. In considering the possible purpose of the minor regulated pathway, we suggest that its secretory content is unlikely to be significant, especially as the same proteins are being released continuously by basal-level granule exocytosis in the absence of stimulation. Rather we believe that the minor regulated pathway acts to relocate membrane components (with secretory content merely marking intravesicular volume) and thus serves as a sensitive means to upregulate machinery that is utilized at the cell surface for secretion.

It is well known that parotid acinar cells are the source of much of the protein and fluid/electrolyte components of saliva (reviewed by Baum, 1987; Nauntofte, 1992) and that output of these components is differentially regulated by parasympathetic and sympathetic neural input to the gland. Parasympathetic stimulation mainly elicits fluid and electrolyte secretion and produces parotid saliva that has a large volume and low protein concentration. In contrast, sympathetic stimulation, particularly by β-adrenergic pathways, produces a concentrated protein-rich saliva (reviewed by Emmelin, 1967; Young et al., 1987). Interestingly, an earlier report has shown that parotid secretion discharged in situ by parasympathetic stimulation contains relatively increased amounts of a polypeptide corresponding to CSP1 among a spectrum of polypeptides that is otherwise quite similar to that discharged by sympathetic stimulation (Anderson et al., 1984). As muscarinic agonists mimic parasympathetic stimulation, we are intrigued by the possibility that the elevated levels of CSP1 that we have observed in the secretion of the constitutive-like and minor regulated pathways may signify a role of these pathways in cellular events that are related to fluid/electrolyte secretion. Our interest in this possibility is increased by analogy to other cell types, especially adipocytes and renal epithelial cells where it is well established that insulin and vasopressin, respectively, regulate glucose and fluid transport by accelerating the relocation of transporter containing vesicles to the cell surface (reviewed by James et al., 1994; Brown and Sabolic, 1993). If this view proves to be correct, then the presence of both major and minor regulated pathways in acinar cells may largely reflect the dual secretory functions in protein and fluid/electrolyte secretion. Finally, because the minor regulated pathway also is activated by low doses of isoproterenol, we raise the unusual possibility that this pathway could provide the cell surface with components that are needed for granule exocytosis.

We are grateful to Charles Hubbard for his assistance in performing laser densitometry on the fluorographs used in this study. These studies were supported by NIH grant DE08941.

REFERENCES

Anderson, L. C., Garrett, J. R., Johnson, D. A., Kauffman, D. L., Keller, P. J. and Thulin, A. (1984). Influence of circulating catecholamines on protein secretion into rat parotid saliva during parasympathetic stimulation. *J. Physiol.* **352**, 163-171.

Arvan, P. and Castle, J. D. (1987). Phasic secretion of newly synthesized secretory proteins in the unstimulated rat exocrine pancreas. *J. Cell Biol.* **104**, 243-252.

Arvan, P., Kuliawat, R., Prabakaran, D., Zavacki, A., Elahi, D., Wang, S. and Pilkey, D. (1991). Protein discharge from immature secretory granules displays both regulated and constitutive characteristics. *J. Biol. Chem.* **266**, 14171-14174.

Arvan, P. and Castle, J. D. (1992). Protein sorting and secretion granule formation in regulated secretory cells. *Trends Cell Biol.* **2**, 327-331.

Ball, W. D., Hand, A. R. and Johnson, A. O. (1988). Secretory proteins as markers of cellular phenotypes in rat salivary glands. *Dev. Biol.* **125**, 265-279.

Baum, B. J. (1987). Regulation of salivary secretion. In *The Salivary System* (ed. L. M. Sreebny), pp. 123-134. CRC Press.

Bernfeld, P. (1955). Amylases α and β. *Meth. Enzymol.* **1**, 149-158.

Brewer, C. B. and Roth, M. G. (1995). Polarized exocytosis in MDCJK cells is regulated by phosphorylation. *J. Cell Sci.* **108**, 789-796.

Brignoni, M., Pignataro, O. P., Rodriguez, M. L., Alvarez, A., Vega-Salas, D. E., Rodriguez-Boulan, E. and Salas, P. J. I. (1995). Cyclic AMP modulates the rate of constitutive exocytosis of apical membrane proteins in Madin-Darby canine kidney cells. *J. Cell Sci.* **108**, 1931-1943.

Brown, D. and Sabolic, I. (1993). Endosomal pathways for water channel and proton pump recycling in kidney epithelial cells. *J. Cell Sci.* **Suppl. 17**, 49-59.

Cameron, R. S., Cameron, P. L. and Castle, J. D. (1986). A common

spectrum of polypeptides occurs in secretion granule membranes of different exocrine glands. *J. Cell Biol.* **103**, 1299-1313.

Carnell, L. and Moore, H.-P. H. (1994). Transport via the regulated secretory pathway in semi-intact PC12 cells: Role of intra-cisternal calcium and pH in the transport and sorting of secretogranin II. *J. Cell Biol.* **127**, 693-705.

Castle, A. M., Stahl, L. E. and Castle, J. D. (1992). A 13-amino acid N-terminal domain of a basic proline-rich protein is necessary for storage in secretory granules and facilitates exit from the endoplasmic reticulum. *J. Biol. Chem.* **267**, 13093-13100.

DeCamilli, P. and Navone, F. (1987). Regulated secretory pathways of neurons and their relation to the regulated secretory pathway of endocrine cells. *Ann. NY Acad. Sci.* **493**, 461-479.

DeLisle, R. and Bansal, R. (1996). Brefeldin A inhibits constitutive-like secretion of sulfated protein in pancreatic acinar cells. *Eur. J. Cell Biol.* (in press).

Emmelin, N. (1967). Nervous control of salivary glands. In *Handbook of Physiology*, section 6, The Alimentary Canal vol II. (ed. C. F. Code), pp. 595-632. Williams and Wilkins, Baltimore, MD.

Foskett, J. K. and Melvin, J. E. (1989). Activation of salivary secretion: Coupling of cell volume and $[Ca^{2+}]_i$ in single cells. *Science* **244**, 1582-1585.

Girard, L., Castle, A., Hand, A., Castle, D. and Mirels, L. (1993). Characterization of common salivary protein 1, a product of rat submandibular, sublingual, and parotid glands. *J. Biol. Chem.* **268**, 26592-26601.

Grimes, M. and Kelly, R. B. (1992). Intermediates in the constitutive and regulated secretory pathways release in vitro from semi-intact cells. *J. Cell Biol.* **117**, 539-549.

James, D. E., Piper, R. C. and Slot, J. W. (1994). Insulin stimulation of GLUT-4 translocation: a model for regulated recycling. *Trends Cell Biol.* **4**, 120-125.

Kuliawat, R. and Arvan, P. (1992). Protein targeting via the constitutive-like secretory pathway in isolated pancreatic islets: passive sorting in the immature granule compartment. *J. Cell Biol.* **118**, 521-529.

Kuliawat, R. and Arvan, P. (1994). Distinct molecular mechanisms for protein sorting within immature secretory granules of pancreatic β-cells. *J. Cell Biol.* **126**, 77-86.

Leslie, B. A. and Putney, J. W. (1983). Ionic mechanisms in secretagogue-induced morphological changes in rat parotid gland. *J. Cell Biol.* **97**, 1119-1130.

Luini, A. and De Matteis, M. A. (1993). Receptor-mediated regulation of constitutive secretion. *Trends Cell Biol.* **3**, 290-292.

Lundberg, J. M. and Hokfelt, T. (1983). Coexistence of peptides and classical neurotransmitters. *Trends Neurosci.* **6**, 325-333.

Mirels, L. and Ball, W. D. (1992). Neonatal rat submandibular gland protein SMG-A and parotid secretory protein are alternatively regulated members of a salivary protein multigene family. *J. Biol. Chem.* **267**, 2679-2687.

Milgram, S. L., Eipper, B. A. and Mains, R. E. (1994). Differential trafficking of soluble and integral membrane secretory granule-associated proteins. *J. Cell Biol.* **124**, 33-41.

Nauntofte, B. (1992). Regulation of electrolyte and fluid secretion in salivary acinar cells. *Am. J. Physiol.* **263**, G823-G837.

Pimplikar, S. and Simons, K. (1994). Activators of protein kinase A stimulate apical but not basolateral transport in epithelial Madin-Darby canine kidney cells. *J. Biol. Chem.* **269**, 19054-19059.

Sharoni, Y., Eimerl, S. and Schramm, M. (1976). Secretion of old versus new exportable protein in rat parotid slices. *J. Cell Biol.* **71**, 107-122.

Vugman, I. and Hand, A. R. (1995). Quantitative immunocytochemical study of secretory protein expression in parotid glands of rats chronically treated with isoproterenol. *Microsc. Res. Tech.* **31**, 106-117.

Young, J. A., Cook, D. I., van Lennep, E. W. and Roberts, M. (1987). Secretion by the major salivary glands. In *Physiology of the Gastrointestinal Tract* (ed. L. R. Johnson), pp. 773-815. Raven Press, NY.

Zastrow, M. v. and Castle, J. D. (1987). Protein sorting among two distinct export pathways occurs from the content of maturing exocrine storage granules. *J. Cell Biol.* **105**, 2675-2684.

Zastrow, M. v., Castle, A. M. and Castle, J. D. (1989). Ammonium chloride alters secretory protein sorting within the maturing exocrine storage compartment. *J. Biol. Chem.* **264**, 6566-6571.

(Received 15 May 1996 - Accepted 22 July 1996)