

Proteins Are Secreted by both Constitutive and Regulated Secretory Pathways in Lactating Mouse Mammary Epithelial Cells

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Abstract. Lactating mammary epithelial cells secrete high levels of caseins and other milk proteins. The extent to which protein secretion from these cells occurs in a regulated fashion was examined in experiments on secretory acini isolated from the mammary glands of lactating mice at 10 d postpartum. Protein synthesis and secretion were assayed by following the incorporation or release, respectively, of [³⁵S]methionine-labeled TCA-precipitable protein. The isolated cells incorporated [³⁵S]methionine into protein linearly for at least 5 h with no discernible lag period. In contrast, protein secretion was only detectable after a lag of ~1 h, consistent with exocytotic secretion of proteins immediately after passage through the secretory pathway and package into secretory vesicles. The extent of protein secretion was unaffected by the phorbol ester PMA, 8-bromo-cAMP, or 8-bromo-cGMP but was doubled by the Ca²⁺ ionophore ionomycin. In a pulse-label protocol in which proteins were prelabeled for 1 h before

a chase period, constitutive secretion was unaffected by depletion of cytosolic Ca²⁺ but ionomycin was found to give a twofold stimulation of the secretion of presynthesized protein in a Ca²⁺-dependent manner. Ionomycin was still able to stimulate protein secretion after constitutive secretion had terminated. These results suggest that lactating mammary cells possess both a Ca²⁺-independent constitutive pathway and a Ca²⁺-activated regulatory pathway for protein secretion. The same proteins were secreted by both pathways. No ultrastructural evidence for apocrine secretion was seen in response to ionomycin and so it appears that regulated casein release involves exocytosis. Ionomycin was unlikely to be acting by disassembling the cortical actin network since cytochalasin D did not mimic its effects on secretion. The regulated pathway may be controlled by Ca²⁺ acting at a late step such as exocytotic membrane fusion.

PROTEIN secretion by exocytosis occurs in many different cell types and is the mechanism involved in the secretion of a wide range of molecules. Exocytotic secretion occurs either immediately after synthesis and packaging of proteins into secretory vesicles (constitutive exocytosis) or, following variable periods of storage in secretory vesicles or granules, in response to cell activation (regulated exocytosis) (Burgess and Kelly, 1987). Lactating mammary epithelial cells actively secrete large quantities of caseins and other milk proteins by an exocytotic mechanism (Franke et al., 1976; Linzell and Peaker, 1971; Saacke and Heald, 1974) in an apparently constitutive fashion. The possibility that mammary epithelial cells secrete caseins via a regulated secretory pathway has not been rigorously examined and much is still to be learned about the cell biology of secretion from this cell type.

The signaling pathways involved in the differentiation of mammary epithelial cells to a secretory phenotype have been examined in detail, but less is known about the factors that may regulate exocytosis in lactating cells. Relatively little work has been carried out in recent years on the effect of sec-

ond messenger systems on exocytotic secretion of milk proteins. Much effort has gone into the development of mammary cell cultures in which differentiation can be induced in a controlled manner (Howlett and Bissell, 1990) but such cell cultures, particularly those grown on collagen gels (Barcellos-Hoff et al., 1989; Li et al., 1987), secrete relatively low quantities of caseins as a percentage of total protein synthesized. For this reason, we have investigated the effect of second messengers on protein secretion from mammary cells freshly isolated from lactating mouse mammary gland. Recent results have shown that lactating mammary epithelial cells express the Ca²⁺-binding protein annexin II on their apical membrane (Handel et al., 1991). This protein has previously been implicated in Ca²⁺-dependent exocytosis in other cell types (Ali et al., 1989; Burgoyne and Morgan, 1990; Drust and Creutz, 1988, 1991; Turgeon et al., 1990). In addition, it has been found that mammary cells possess Ca²⁺ signaling pathways that can modify cytosolic free Ca²⁺ concentration ([Ca²⁺]_i) (Furuya and Enomoto, 1990; Yoshimoto et al., 1990). We therefore focused on the possible importance of [Ca²⁺]_i. The results suggest that lac-

tating mammary epithelial cells possess both constitutive and regulated pathways for casein secretion. Constitutive exocytosis from lactating mammary cells is unaffected by measures that reduce $[Ca^{2+}]_i$, but regulated exocytosis is stimulated by elevation of $[Ca^{2+}]_i$.

Material and Methods

Materials

Acetoxymethyl (AM)-1,2-bis(2-aminophenoxy)ethane-*N,N,N',N'*-tetraacetic acid (BAPTA), acetoxymethyl-fura2 (fura2-AM), and ionomycin were obtained from Calbiochem Corp., La Jolla, CA. Unless stated otherwise tissue culture media were obtained from Gibco Laboratories, Grand Island, NY, and other reagents from Sigma Chem. Co., St. Louis, MO.

Preparation of Mammary Epithelial Cell Acini from Lactating Mammary Gland

Mammary tissue was removed aseptically from Tuck's No. 1 mice at mid-lactation (10 d postpartum). The tissue was finely chopped with scissors and dissociated by incubation with 0.14% collagenase (Worthington Biochem. Corp., Freehold, NJ) for 90 min at 37°C in HBSS supplemented with 5 μ g/ml insulin and 0.01 μ g/ml cortisol. Digestion was stopped when the majority of acini were reduced to clumps of 10–30 cells and the cell suspension was filtered through a 150- μ m pore nylon mesh (Lockertex, Warrington, UK). The digestion was monitored by light microscopy to ensure the completeness of digestion and cellular integrity. Cells were harvested by centrifugation at 80 g for 5 min and resuspended in HBSS supplemented with 5 μ g/ml insulin, 0.01 μ g/ml cortisol, 0.04 mg/ml DNAase I (Boehringer Mannheim Corp., Indianapolis, IN), and 0.1 mg/ml trypsin inhibitor. Cell pellets were washed four times by centrifugation and filtered through a 53- μ m pore nylon mesh. The filtrate was centrifuged as above and cells were resuspended in culture medium (50% M199, 50% Ham's F12, 5 μ g/ml insulin, 0.01 μ g/ml cortisol, 0.1 μ g/ml prolactin, 0.01 μ g/ml EGF, and 0.001 μ g/ml T₃). The cells were kept in tissue culture flasks for 1 h to remove fibroblasts and macrophages, which attach to the plastic in this time, and then plated out into 24-well tissue culture trays at a cell density of 6×10^6 cells/ml and maintained at 37°C in an atmosphere containing 5% CO₂. Cell viability was assessed by trypan blue exclusion (in the region of 90–95%). Cell number was determined by hemocytometer counts of stained released nuclei after disruption of acini with 0.5 M citric acid.

Incorporation of [³⁵S]Methionine into Cellular Proteins and Assay of Protein Synthesis and Secretion

For continuous labeling cells were incubated with 5 μ Ci/ml L-[³⁵S]methionine (cell labeling grade, sp act >1,300 Ci/mmol; Amersham plc, Amersham, UK) for various times with or without drug additions. Drugs were in aqueous solution or dissolved in DMSO. Controls were included to assess the effect of DMSO which was at a final concentration of 1%. For pulse-chase experiments cells were incubated for 1 h with 25 μ Ci/ml L-[³⁵S]methionine. An equal volume of ice-cold culture medium was added to the cells and the cell suspension was centrifuged at 80 g for 5 min. The supernatant was discarded and the cell pellets were resuspended in cold culture medium. The cells were aliquoted and incubated with or without drug additions for various periods at 37°C. Incubations were terminated by centrifugation at 6,500 rpm in a microfuge for 1 min. The supernatant was removed and duplicate 400- μ l aliquots were mixed with equal volumes of 20% TCA. The pellets were resuspended in 800 μ l of 10% TCA, and 100 μ l of 1% BSA was added. The samples were kept on ice for 20 min and centrifuged at 6,500 rpm for 10–15 s. The pellets were washed twice by resuspension and centrifugation with 800 μ l 10% TCA, and TCA-precipitable protein was finally pelleted by centrifugation at 13,000 rpm for 5 min. The supernatants were discarded and the bottoms of the tubes containing precipitates were removed using a hot scalpel and placed in scintillation vials. Cocktail T

1. *Abbreviations used in this paper:* AM, acetoxymethyl; BAPTA, 1,2-bis(2-aminophenoxy)ethane-*N,N,N',N'*-tetraacetic acid; $[Ca^{2+}]_i$, cytosolic free Ca²⁺ concentration.

(BDH; 8 ml/vial) was added, the vial vortexed, and incorporated ³⁵S determined by scintillation counting.

SDS-PAGE and Fluorography

Cells were labeled by incubation with 25 μ Ci/ml of L-[³⁵S]methionine for varying times. In some cases cells were pulse-labeled for 1 h and chased for various periods as above, and the incubation was terminated by centrifugation at 6,500 rpm for 1 min. Pellets were solubilized in 100 μ l of SDS dissociation buffer. Equal volumes of cold methanol were added to each of the supernatants and after 20 min at -70°C precipitated protein was pelleted by centrifugation at 13,000 rpm in a microfuge for 2 min. The pellets were washed by centrifugation with 1 ml of cold methanol and the final pellets solubilized in 100 μ l of SDS dissociation buffer. Proteins were separated by PAGE on 12.5% slab gels. Molecular weight standards and mouse casein standards were also run and gels stained with Coomassie brilliant blue. For fluorography, gels were soaked in Amplify (Amersham Corp.) for 30 min and dried onto filter paper using a slab gel dryer (Bio-Rad Laboratories, Richmond, CA). Hyperfilm MP (Amersham Corp.) was exposed to the dried gels at -70°C before developing. Exposed fluorograms were quantitated using a video densitometer (Bio-Rad Laboratories).

Staining of Cells with Rhodamine-Phalloidin

Control cells or cells treated with 10 μ M cytochalasin D for 1, 2, or 3 h were fixed by addition to the cell suspension of an equal volume of 8% formaldehyde in PBS and the fixed cell suspensions stored overnight at 4°C. The cells were then centrifuged at 6,500 rpm for 30 s and washed three times with PBS by centrifugation. The cell pellets were then resuspended in 200 μ l of 0.1% Triton X-100, 0.3% BSA in PBS (PBT) and incubated for 30 min. The cells were pelleted as before and resuspended in 1 ml PBT containing 2.5×10^{-8} M rhodamine-phalloidin and incubated in the dark for 30 min. Cells were washed three times in PBS, and an aliquot of the final cell suspension was mounted on a slide and examined using a Zeiss fluorescence microscope with the appropriate filters for detection of rhodamine fluorescence.

Electron Microscopy

Cells at a density of 10^8 /ml were incubated for 1 h with or without 10 μ M ionomycin as for the pulse-chase protocol above. For fixation, 0.5 ml of cell suspension was added to an equal volume of 6% glutaraldehyde in 75 mM phosphate buffer, pH 7.3, gently mixed, and incubated for 15 min at room temperature. The cells were pelleted by centrifugation at 13,000 rpm for 1 min in a microfuge and stored at 4°C overnight. The cell pellets were postfixed in 1% osmium tetroxide in 56 mM sodium acetate, pH 7.3, containing 131 mM sucrose for 60 min, dehydrated in graded concentrations of acetone, and embedded in an epon/araldite resin mixture. Ultrathin sections were cut, stained with uranyl acetate and lead citrate, and viewed and photographed with a Philips EM 300 at 60 kV.

Measurement of $[Ca^{2+}]_i$ Using fura2

For determination of $[Ca^{2+}]_i$, mammary cells were suspended at a density of 6×10^6 /ml in buffer A (145 mM NaCl, 5 mM KCl, 1.3 mM MgCl₂, 0.78 mM CaCl₂, 1.2 mM NaH₂PO₄, 10 mM glucose, 20 mM HEPES, pH 7.4, equilibrated with 95% O₂/5% CO₂) containing 0.5% BSA and loaded by incubation at room temperature with 10 μ M fura2-AM for 45 min. The cells were washed with buffer A, incubated for an additional 45 min, washed twice, and resuspended in buffer A and fluorescence monitored in an LS5 luminescence spectrometer (Perkin-Elmer Corp., Norwalk, CT) (λ_{ex} , 340 nm; λ_{em} , 510 nm). Fluorescence was calibrated by cell lysis with 200 μ M digitonin followed by Ca²⁺ chelation with 40 mM EGTA and $[Ca^{2+}]_i$ determined using a K_d of 135 nM (Gryniewicz et al., 1985).

Incorporation of [¹⁴C]Acetate into Cellular Triglycerides and Assay of Triglyceride Secretion

Cells were incubated with 5 μ Ci/ml [¹⁴C]acetic acid, sodium salt (sp act 55 mCi/mmol, Amersham Corp.) for 2 h at 37°C. Cells were then centrifuged at 80 g for 5 min and the supernatant was discarded. Cell pellets were then resuspended in fresh culture medium and incubated at 37°C for 1 h. After this period, cells were again centrifuged at 80 g for 5 min and the supernatant was discarded. Cells were then resuspended in fresh culture media with or without 10 μ M ionomycin and incubated for 1 h. Incubations

were terminated by centrifugation at 6,500 rpm in a microfuge for 1 min. The supernatant was removed and duplicate 400- μ l aliquots were mixed with equal volumes of distilled water, and lipid extractions were performed by the method of Bligh and Dyer (1959). It has previously been shown that under the conditions used the majority of the label in the chloroform extract is in the form of triglyceride (Hansen and Knudsen, 1991) and so the extent of triglyceride release was determined by scintillation counting of aliquots of this extract.

Results

Mammary epithelial cells in secretory acini were isolated from lactating mouse mammary gland at 10 d postpartum by enzymatic digestion with conditions chosen to produce intact groups of 10–30 cells. Light microscopical examination of the isolated cells indicated that \sim 85% of the cell population was in cell groups or acini. The majority of the cells excluded trypan blue and remained functional when maintained in suspension in culture medium for up to 18 h. To assess the synthetic and secretory ability of these cell suspensions, cells were labeled with [35 S]methionine for varying periods and the incorporation of radioactivity into TCA-precipitable protein in cells and in medium was determined. Determination of the total counts incorporated into TCA-precipitable protein in both cells and medium (a measure of total protein synthesis) indicated that the cells synthesized protein without a detectable lag and continued to do so for between 5 and 18 h (data not shown). It can be seen (Fig. 1) that the cells synthesized protein over the 3-h period but that protein secretion into the medium only occurred after a lag period of \sim 45–60 min, which is consistent with constitutive secretion of proteins immediately after movement through the secretory pathway, and comparable with data for secretion *in vivo* (Saacke and Heald, 1974). After 3 h the secreted protein accounted for 32.6% of total synthesized protein. From a series of cell preparations, the amount of 35 S-labeled protein secreted after 3 h corresponded to $38.9 \pm 6.4\%$ ($n = 10$) of total synthesized protein, a value again comparable with that found for secretion *in vivo* (Saacke and Heald, 1974). Release of 35 S-labeled protein was not a result of cell lysis since the percentage of cells excluding trypan blue was unchanged over the incubation periods used and release was entirely microtubule dependent being abolished by treatment with nocodazole (Rennison et al., 1992).

The nature of the proteins synthesized and secreted from isolated cells was determined by SDS-PAGE and fluorography of 35 S-labeled proteins (Fig. 2). The majority of label was incorporated into a limited number of proteins. Little secreted protein was detected in the medium after 1 h but considerable levels of proteins comigrating with mouse α - and β -caseins were detected after 2 h. From densitometric quantitation of the data in Fig. 2 after correction for gel loading, it was found that the secreted polypeptides corresponding to the α - and β -caseins represented 27.8 and 25.8%, respectively, of the total level of each of these proteins synthesized. This result is consistent with the values found from assay of total TCA-precipitable 35 S-labeled protein. A low molecular weight polypeptide was selectively retained by the cells (open arrow in Fig. 2) under these conditions.

To investigate the effect of various second messengers on secretion, cells were labeled with [35 S]methionine over a 3-h period in the presence of 8-bromo-cAMP (0.1 mM), 8-bromo-cGMP (0.1 mM), the Ca^{2+} ionophore ionomycin

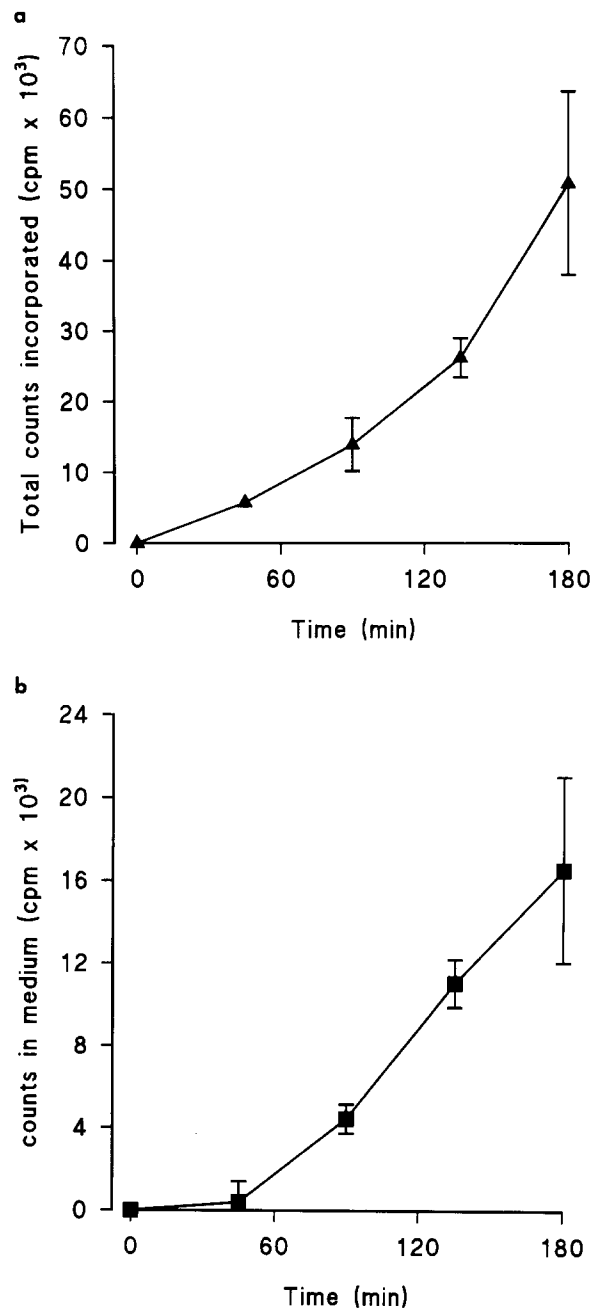


Figure 1. Time course of protein synthesis and secretion by dissociated lactating mammary acini. Mammary cells in suspension culture were incubated for various times with 5 μ Ci/ml [35 S]methionine. The extent of protein synthesis and secretion was determined by assaying the counts incorporated into cell-associated or medium TCA-precipitable protein. The data show total counts incorporated (a) and counts released into medium (b), and are shown as mean \pm SEM ($n = 3$).

(10 μ M), or the intracellular Ca^{2+} chelator BAPTA-AM (25 μ M) (Table I). Both 8-bromo-cAMP and 8-bromo-cGMP reduced the extent of total protein synthesis but the proportion of protein secreted was no different from that of control cells. The Ca^{2+} chelator BAPTA used as the acetoxymethyl ester form would be expected to be taken up, cleaved, and the free BAPTA chelate cytosolic Ca^{2+} . BAPTA-AM produced a

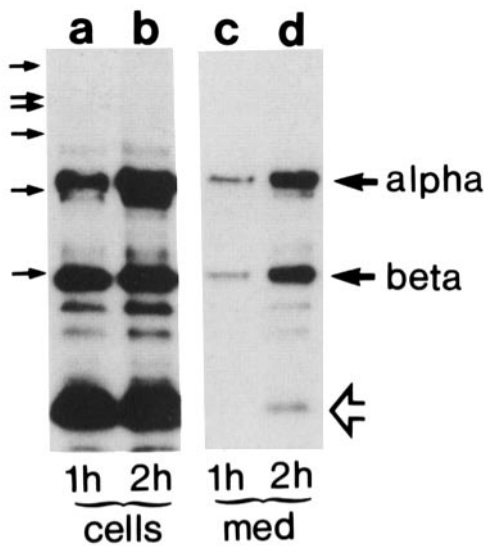


Figure 2. Characterization of proteins synthesized and secreted by lactating mammary acini. Mammary cells in suspension culture were incubated for 1 h (a and c) or 2 h (b and d) with 25 μ Ci/ml [35 S]methionine, and labeled proteins were detected by SDS-PAGE and fluorography. The labeled proteins present in the cells and in medium (med) are shown. The positions of migration of mouse α - and β -caseins and molecular mass standards (arrows) run on the same gel are shown (top to bottom, 200, 116, 94, 67, 42, and 29 kD). The open arrow on the right indicates a low molecular weight protein selectively retained within the cells.

marked inhibition of total synthesis consistent with the findings that depletion of cytosolic Ca^{2+} inhibits protein synthesis (Brostrom et al., 1990) and the early stages of the secretory pathway (Balch, 1989), and increases protein degradation in the ER (Wileman et al., 1991). The variability of the results with BAPTA-AM due to the low levels of counts incorporated made it impossible to reliably determine the effect of this chelator on the extent of protein secretion. The Ca^{2+} ionophore ionomycin also produced some inhibition of total synthesis but in this case the proportion of protein secreted was increased to twice that of control cells.

Table I. Effect of 8-bromo-cAMP, 8-bromo-cGMP, BAPTA-AM, and Ionomycin on Protein Synthesis and Protein Secretion from Lactating Mammary Acini

Treatment	cpm incorporated into total protein	cpm in protein secreted	Synthesized protein secreted %
Control	25,483 \pm 1,575	8,953 \pm 526	35.1 \pm 2.1
8-br-cAMP	17,126 \pm 1,405	5,922 \pm 1,173	34.6 \pm 6.8
8-br-cGMP	17,606 \pm 2,974	4,761 \pm 1,082	27.0 \pm 6.1
DMSO control	24,268 \pm 2,148	8,762 \pm 1,002	36.1 \pm 4.1
BAPTA-AM	2,022 \pm 1,001	520 \pm 770	25.7 \pm 38.1
Ionomycin	8,438 \pm 152	6,852 \pm 18	81.2 \pm 2.1*

Dissociated acini from lactating mouse mammary gland were incubated for 3 h with [35 S]methionine under various conditions as indicated and the incorporation of counts into TCA-precipitable protein in cells and medium was determined to allow calculation of total counts incorporated into protein and into secreted protein. The data are means \pm SEM ($n = 3$).

* $p < 0.001$ compared to DMSO control.

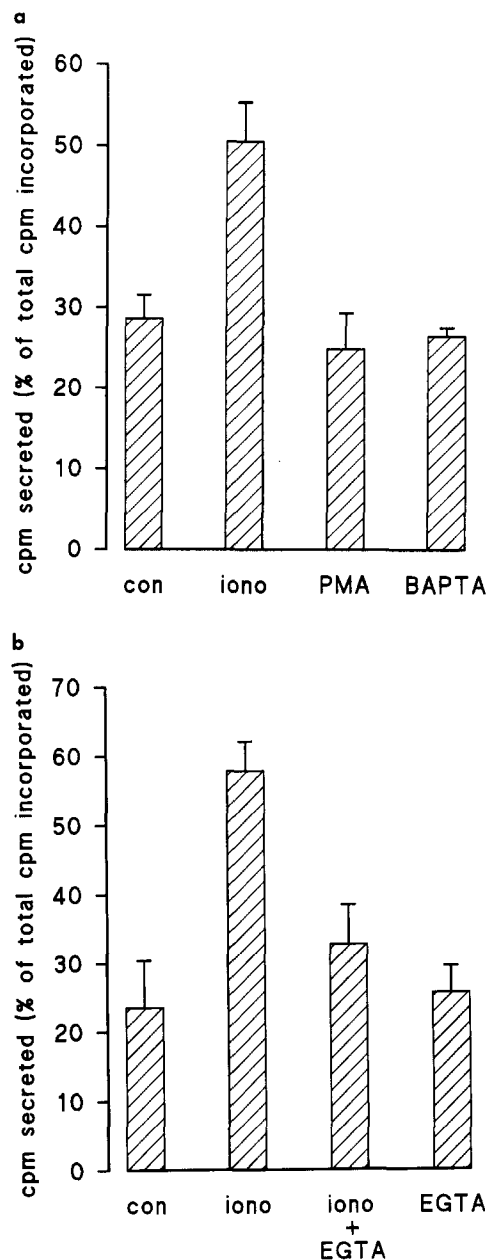


Figure 3. Effect of ionomycin, BAPTA-AM, EGTA, and PMA on protein secretion from dissociated lactating mammary acini in a pulse-chase protocol. Mammary cells in suspension culture were incubated for 1 h with 25 μ Ci/ml [35 S]methionine. The cells were washed and then incubated for an additional hour with or without 10 μ M ionomycin, 100 nM PMA, 25 μ M BAPTA-AM, or 2 mM EGTA in the medium. The extent of protein synthesis and secretion was determined by assaying the counts incorporated into cell-associated or medium TCA-precipitable protein. The data show the percentage of total counts that were secreted and are shown as mean \pm SEM ($n = 3$ for a; $n = 4$ for b). Secretion was increased in the presence of ionomycin (iono) compared to controls (con). PMA and BAPTA-AM were without effect (a). The stimulatory effect of ionomycin was prevented by the additional inclusion of EGTA (b).

To avoid the problem of possible interference with protein synthesis or early stages of the secretory pathway we adopted a pulse-chase approach to examine the importance of $[\text{Ca}^{2+}]_i$ in the regulation of exocytosis. Cells were labeled

with [³⁵S]methionine for 1 h, over which time they would secrete little protein (Figs. 1 and 2), washed with ice-cold buffer, and then incubated with or without additions. From a series of cell preparations the amount of ³⁵S-labeled protein secreted over 1 h after this pulse-labeling and washing was $28.3 \pm 2.4\%$ ($n = 9$) of total synthesized protein for control cells. Secretion of pulse-labeled protein occurred mainly within the first hour of the chase. Release did not appear to be due to cell damage since it was abolished by incubation at 4°C. As shown in Fig. 3 *a* secretion of pre-labeled protein was unaffected by 25 μM BAPTA-AM or the phorbol ester PMA (100 nM), but was again markedly stimulated by 10 μM ionomycin. These results suggest that an increase in [Ca²⁺]_i by ionophore treatment can stimulate secretion and that this stimulation can not be mimicked by activation of protein kinase C. The result with BAPTA-AM suggests that depletion of cytosolic Ca²⁺ does not impair exocytosis. It is possible that BAPTA-AM did not have sufficient time to accumulate within the cells to exert a marked effect on [Ca²⁺]_i during the chase period. Therefore, an additional experiment was carried out in which the cells were incubated with or without ionomycin with excess EGTA added to the medium in the pulse-chase protocol (2 mM EGTA added, medium Ca²⁺ concentration was 0.78 mM). Fig. 3 *b* shows that removal of external Ca²⁺ did not affect basal secretion. The stimulation of secretion by ionomycin was abolished when EGTA was also present. Treatment of cells with ionomycin in the presence of EGTA would be expected not only to prevent the ionomycin-induced increase in cytosolic Ca²⁺ concentration but also to result in a depletion of cellular calcium (see below). Under these conditions the extent of protein secretion was reduced down to but not below control levels. These results show that increased [Ca²⁺]_i can increase protein secretion but that depletion of [Ca²⁺]_i does not inhibit constitutive exocytosis.

No data is available on [Ca²⁺]_i in isolated lactating mammary cells and, therefore, we determined basal [Ca²⁺]_i and the effect of ionomycin by monitoring the fluorescence of the Ca²⁺ indicator fura2 in loaded cells in suspension. The lactating mammary cells had a basal [Ca²⁺]_i of 87 ± 17 nM ($n = 8$). Addition of 10 μM ionomycin in the presence of external Ca²⁺ resulted in a sustained rise in [Ca²⁺]_i up to ~ 1 μM (Fig. 4 *a*). Addition of excess EGTA, as used in secretion experiments, resulted in an immediate drop in fluorescence and a continued fall in [Ca²⁺]_i over time (Fig. 4 *b*). In the presence of excess EGTA, ionomycin did not elicit a rise in [Ca²⁺]_i. These results show that ionomycin in the presence of external Ca²⁺ does indeed raise [Ca²⁺]_i and that [Ca²⁺]_i is reduced to levels well below basal concentration in the presence of external EGTA.

The effect of ionomycin appeared to be due to Ca²⁺-dependent stimulation of secretion and not to nonspecific cell damage. As noted above, the effect of ionomycin was dependent on external Ca²⁺. In addition, cells treated with ionomycin for 3 h did not show any increase in permeability to trypan blue compared to control cells (90–95% of cells excluded trypan blue in both conditions). Cells treated in the pulse-chase protocol with or without ionomycin were also examined by EM and no indication of cell damage due to ionomycin was detected. The treated cells showed normal morphology of intracellular organelles (such as mitochondria and ER) which can be rapidly affected after cell damage

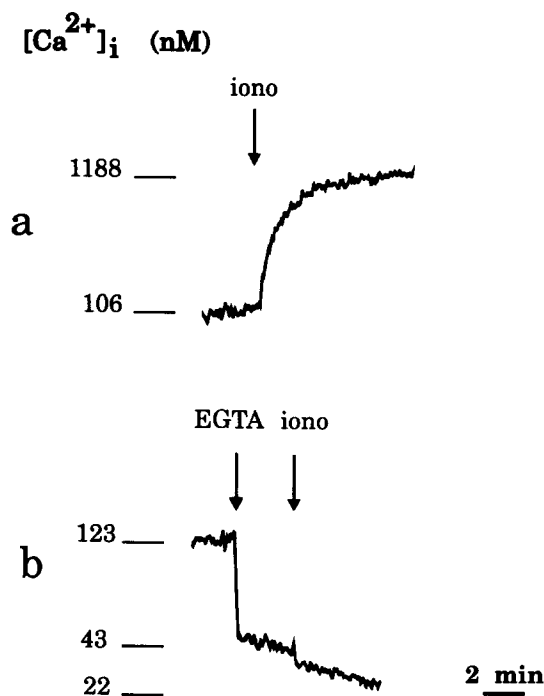


Figure 4. Effect of ionomycin and EGTA on [Ca²⁺]_i in mammary cells. Isolated acini were loaded with fura2 and fluorescence was monitored and calibrated as in Materials and Methods. (*a*) In the presence of external Ca²⁺, ionomycin (10 μM) produced a sustained elevation of [Ca²⁺]_i. (*b*) Addition of excess EGTA (2 mM) prevented the ionomycin-induced rise in [Ca²⁺]_i and resulted in progressive fall of basal [Ca²⁺]_i.

(Fig. 5). Considerable variation between cells in the number of secretory vesicles was found and no attempt was made to assess the effect of ionomycin on vesicle number.

It is believed that by far the majority of casein secretion from mammary cells occurs by exocytosis and release of casein micelles (Franke et al., 1976; Nickerson and Akers, 1984). After disruption and fractionation of mouse mammary cells, we found that no more than 1% of β-casein was present in a soluble form, the remainder being associated with micelles. This supports the notion that the casein released by constitutive and regulated secretion was derived from micelle-containing vesicles. It has been suggested that caseins could be secreted by a process of apocrine secretion whereby casein vesicles and other cytoplasmic organelles are carried from the cells as budding of lipid droplets from the apical surface of the cell occurs (Wooding, 1971). This mechanism, however, is now believed to make little significant contribution to protein secretion (Kanno, 1990) and the lipid droplets possessing cytoplasmic crescents have been shown to be only minor components of secreted milk (Huston and Patton, 1990). Nevertheless, we examined the effect of ionomycin on the extent of triglyceride release as a measure of lipid secretion. Over a 1-h period, triglyceride release due to ionomycin ($27,317 \pm 1,058$ cpm, $n = 4$) was found to be increased compared with control cells ($13,481 \pm 1,089$ cpm, $n = 4$). To assess whether lipid and protein secretion in response to ionomycin occurred by apocrine secretion, we carried out an extensive ultrastructural survey to search for evi-

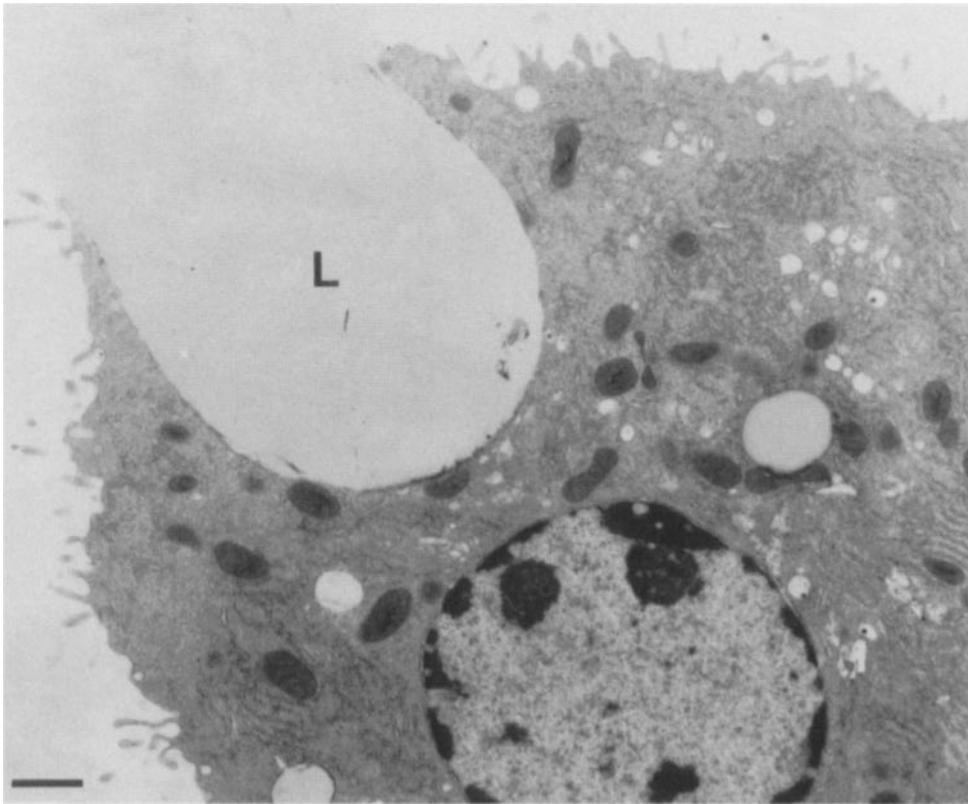


Figure 5. Electron microscopy of ionomycin-treated dissociated mammary cells. Cells were incubated with ionomycin for 1 h as in the pulse-label secretion experiments. Ionomycin-treated cells showed normal morphology of intracellular organelles (such as the mitochondria and ER) and no indication of cell damage due to the ionophore. Images were frequently seen of lipid being released from cells as a consequence of apparent leakage from large lipid droplets (*L*) still within the cell, rather than due to budding out with associated apical plasma membrane. Bar, 1 μm .

dence for this mode of secretion. No evidence in favor of apocrine secretion was obtained. No released cytoplasmic crescents were seen in any preparation and, in fact, budding lipid droplets were relatively rare in isolated control and ionomycin-treated cells. When lipid budding was observed few or no casein vesicles were associated with the lipid droplets. As shown for ionomycin-treated cells (Fig. 5) lipid droplets were frequently seen to be releasing their content by a nonbudding mechanism. Similar images have previously been reported for lactating mouse mammary cells (Burwen and Pitelka, 1980). This route is likely to account for the release of triglyceride measured in control and ionomycin-treated cells, and is not simply a feature of the isolated cells since we also observed it in the fixed intact gland. We conclude that casein released in response to ionomycin is due to exocytosis of micelle-containing vesicles.

The additional increment of protein secretion due to ionomycin treatment after pulse-labeling could be due either to an increase in the rate of ongoing constitutive secretion or the presence of an additional regulated pathway. The data shown in Fig. 6 is from an experiment designed to distinguish these two possibilities and suggests that the mammary cells possess distinct constitutive and regulated pathways. After pulse-labeling, ^{35}S -labeled proteins were secreted within 1 h and no further release was detected at later time points (Fig. 6). When ionomycin was added at 1 or 2 h after the chase began, additional protein secretion was detected. Thus, ionomycin can elicit a phase of regulated exocytosis after constitutive release has terminated, suggesting the existence of a separate population of secretory vesicles in which proteins are stored until $[\text{Ca}^{2+}]_i$ is elevated. Ionomycin is

likely to be acting on a late stage of the secretory pathway since release elicited by ionomycin was complete within 15 min and was unaffected by the antimicrotubule drug nocodazole (our unpublished observations). To determine whether the same proteins are secreted by the constitutive and regulated routes, the polypeptide patterns of released ^{35}S -labeled proteins were examined by gel electrophoresis and fluorography (Fig. 7). The same pattern of secreted proteins was seen after constitutive or ionomycin-induced release with major polypeptides in both cases corresponding to α - and β -caseins.

In a number of cell types showing Ca^{2+} -dependent regulated exocytosis, Ca^{2+} acts at the level of exocytosis and also by stimulating reorganization of the cortical actin network which appears to act as a barrier to exocytosis in these cells (Burgoyne and Cheek, 1987; Vitale et al., 1991; Cheek and Burgoyne, 1991). One possible mechanism of action of an increase in $[\text{Ca}^{2+}]_i$ due to ionomycin could be that Ca^{2+} activates breakdown of the actin network in mammary cells. To test the possibility that the cortical actin network could be a regulator of exocytosis in mammary cells we examined the effect of disruption of the actin network on secretion. If the cortical network was a barrier to exocytosis in mammary cells, then disruption of this actin should result in an increase in the extent of protein secretion. Previous studies have used high concentrations of cytochalasin B (Smith et al., 1982a, b; Nickerson and Akers, 1984) which inhibited protein secretion. The effect of the drug on actin was not determined and cytochalasin B is known to inhibit glucose uptake by blocking the glucose transporter in many cell types including mammary cells (Madon et al., 1990) and could, as a conse-

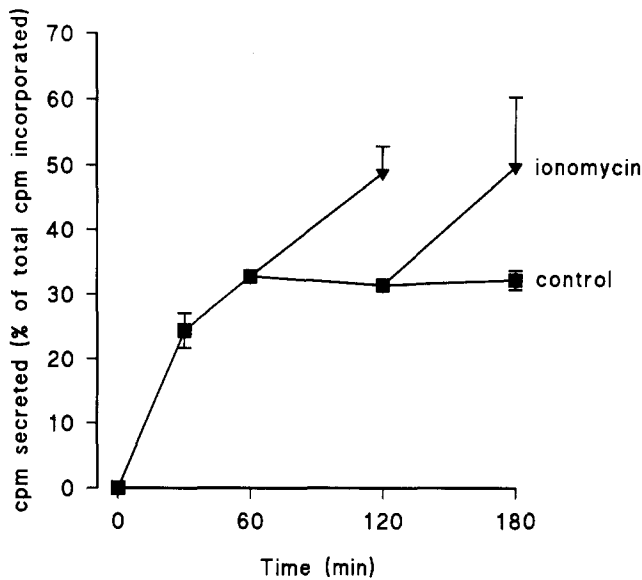


Figure 6. Effect of ionomycin on protein secretion from lactating mammary cells when added at late times during a chase period. Mammary cells in suspension culture were incubated for 1 h with 25 $\mu\text{Ci/ml}$ [^{35}S]methionine. The cells were washed and then incubated for various times before assay of protein secretion. At indicated times, 10 μM ionomycin was added to cells and the extent of release estimated after 1 h. The data show the percentage of total counts that were secreted and are shown as mean \pm SEM ($n = 3$). Addition of ionomycin at times after constitutive secretion had terminated still resulted in an increase in protein secretion.

quence, inhibit energy-dependent processes such as secretion independently of actions on actin. Therefore, we examined the effect of the more specific analogue cytochalasin D. The organization of polymerized actin in mammary cells was examined using rhodamine-phalloidin staining and treatment of the cells with 10 μM cytochalasin D was found to markedly disrupt the actin network (Fig. 8). In control cells, intense staining around the cell periphery was seen due to the cortical actin network. No indication of any polarity of actin organization was seen either in isolated lactating mammary cells (Fig. 8) or in cells in situ in tissue sections (data not shown). After treatment with cytochalasin D, the periph-

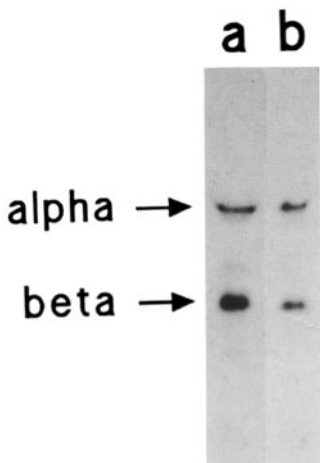


Figure 7. Effect of ionomycin on the nature of ^{35}S -labeled proteins secreted during the chase period. Mammary cells in suspension culture were incubated for 1 h with 25 $\mu\text{Ci/ml}$ [^{35}S]methionine. The cells were washed and then incubated for 1 h. Medium was analyzed for constitutively released proteins (a) or cells were washed and released proteins analyzed after an additional 1 h in the presence of 10 μM ionomycin (b).

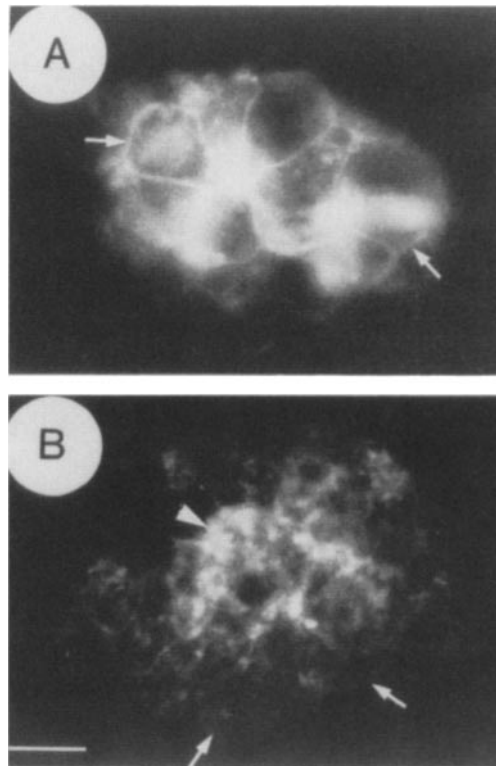


Figure 8. Effect of cytochalasin D treatment on the organization of actin filaments in dissociated mammary acini. Mammary cells in suspension culture were incubated for 3 h without (A) or with (B) 10 μM cytochalasin D and stained with rhodamine-phalloidin. In controls it was seen that rhodamine-phalloidin staining was clearly concentrated at the cell periphery consistent with concentration of actin filaments in the cell cortex (arrows). After treatment with cytochalasin D (B) cortical staining was lost and many cells at the edge of the acini were now virtually unstained (arrows). Punctate staining with rhodamine-phalloidin was also apparent in these treated cells (arrowhead). Bar, 50 μm .

eral staining disappeared indicating that the cortical actin had disassembled and instead intense punctate staining appeared particularly after treatment for 2–3 h (Fig. 8). Ionomycin was able to elicit additional protein secretion after pulse-labeling at late times in the chase period. In contrast, treatment of cells with the cytochalasin D had essentially no effect or a small inhibitory effect on the extent of protein secretion during the chase period after pulse-labeling (Fig. 9). Similar results were found when cytochalasin D was present during a 3-h labeling period. These results show that the cortical actin network does not normally function as a barrier to exocytosis in these cells and that its disruption does not enhance the extent of exocytosis.

Discussion

Exocytotic secretion of protein from lactating mammary epithelial cells has usually been presumed to be a purely constitutive process that is not under the acute control of second messenger pathways. The intracellular requirements and regulation of exocytosis from regulated secretory cells has been studied in detail in recent years but much less information is available on constitutive exocytosis, despite the fact

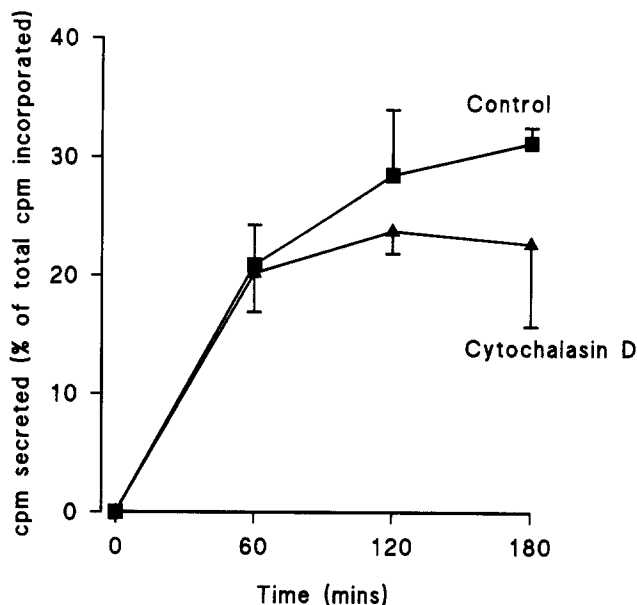


Figure 9. Effect of cytochalasin D on protein secretion from dissociated lactating mammary acini. Mammary cells in suspension culture were incubated for 1 h with 25 $\mu\text{Ci/ml}$ [^{35}S]methionine, washed, and then incubated for further indicated times with or without 10 μM cytochalasin D. The extent of protein synthesis and secretion was determined by assaying the counts incorporated into cell-associated or medium TCA-precipitable protein. The data show the percentage of total counts that was secreted and are shown as mean \pm SEM ($n = 3$).

that it can be reconstituted in an *in vitro* system (Woodman and Edwardson, 1986). It has not been clear to what extent cells with constitutive secretory pathways also possess a regulated pathway or whether constitutive exocytosis is dependent upon or regulated by $[\text{Ca}^{2+}]_i$. Studies on constitutive secretion from intact cells show varying Ca^{2+} dependencies of the constitutive pathway. Exocytotic secretion of immunoglobulin and constitutive protein secretion from fibroblasts was inhibited by treatment with EGTA and the ionophore A23187 to reduce basal $[\text{Ca}^{2+}]_i$, but secretion from macrophages was unaffected by this treatment (Tartakoff and Vassalli, 1978). More recently it was found that transport of Semliki Forest virus glycoproteins from the *trans*-Golgi network to the plasma membrane in permeabilized BHK cells was inhibited by EGTA (de Curtis and Simons, 1988). In contrast, constitutive secretion of either membrane or fluid-phase markers from permeabilized CHO cells in culture was unimpaired even at low (10^{-9} M) $[\text{Ca}^{2+}]_i$ and occurred at the same rate over a wide range of $[\text{Ca}^{2+}]_i$, indicating that in this cell type—constitutive exocytosis was not dependent upon or activated by $[\text{Ca}^{2+}]_i$ (Helms et al., 1990; Miller and Moore, 1991). These findings suggest that, depending on the cell type, constitutive exocytosis is either dependent on or independent of resting $[\text{Ca}^{2+}]_i$. Our present results on protein secretion from intact mammary cells suggest that constitutive protein secretion in these cells is independent of resting $[\text{Ca}^{2+}]_i$. Constitutive protein exocytosis in lactating mammary cells was unimpaired by treatments, including incubation with BAPTA-AM and ionomycin with excess EGTA, expected to reduce basal $[\text{Ca}^{2+}]_i$. We have also found that mammary cells possess an additional regu-

lated pathway for protein secretion that is activated by an elevation of $[\text{Ca}^{2+}]_i$. The presence of both constitutive and regulated pathways for protein secretion has been reported for thyroglobulin secretion from thyroid epithelial cells in culture (Arvan and Lee, 1991). In lactating mammary cells, the regulated pathway appeared to be distinct from the constitutive pathway since, in a pulse-chase protocol, regulated secretion could be activated after constitutive secretion had terminated. These findings suggest the possible existence of two populations of secretory vesicles in lactating mammary cells, and PAGE analysis of the secreted proteins showed that these vesicles would be expected to contain the same secretory proteins. The exact site of action of Ca^{2+} in the secretory pathway in regulated secretion in mammary cells is not clear, but the ability of ionomycin to rapidly stimulate protein secretion at late times after pulse labeling would be consistent with a stimulation of exocytosis of preformed secretory vesicles as seen in other cell types showing Ca^{2+} -dependent regulated secretion. Despite the fact that ionomycin also stimulated triglyceride release, we can rule out the possibility that caseins were secreted by an apocrine mechanism (Wooding, 1971), since no evidence for this mechanism was observed by electron microscopy and since lipid droplets with associated cytoplasm are extremely rare in secreted milk of a variety of species (Huston and Patton, 1990).

Many cell types possess both constitutive and regulated secretory pathways but it is generally believed that particular proteins are secreted by only one route (Burgess and Kelly, 1987). Dense-core vesicles are found on regulated secretory pathways and it has been suggested that condensation of the vesicle-core proteins is a key event in sorting into the regulated pathway (Burgess and Kelly, 1987; Huttner et al., 1991). Proteins that do not precipitate with the vesicle core would pass into the constitutive pathway by default (Burgess and Kelly, 1987). Mammary epithelial cells possess secretory vesicles containing dense cores (casein micelles) and are unusual in the extent to which protein synthesis is directed towards production of large amounts of a limited number of secretory proteins (see Fig. 2). One possibility, therefore, is that the appearance of caseins in the constitutive pathway may be due to incomplete condensation of caseins at the level of the *trans*-Golgi network thereby allowing a proportion to pass by default into constitutive secretory vesicles. A similar situation appears to exist in exocrine pancreas where 15% of regulated secretory proteins exit through the constitutive pathway (Arvan and Castle, 1987). It is not clear, however, to what extent casein vesicles can be regarded as being similar to regulated secretory vesicles. The appearance of dense core is not necessarily diagnostic of a regulated pathway.

Previous work on protein secretion from lactating mammary epithelial cells had not detected a regulatory role for $[\text{Ca}^{2+}]_i$. The effect of ionomycin on protein secretion from these cells has not previously been examined. It was found that the ionophore A23187 had no effect on secretion from mammary cells in culture (Ortiz, G. L., and V. Rocha, 1986. *J. Cell Biol.* 103:406a). This ionophore has been reported to be much less effective than ionomycin in stimulating regulated secretion in other cell types (Poggioli et al., 1982). In addition, the experiments of Ortiz and Rocha were carried out on mammary cells differentiated in culture. Such cells do develop the ability to secrete caseins but their secretory com-

petence is poor compared with freshly isolated lactating mammary cells (our unpublished results), and it is conceivable that aspects of the machinery that regulates exocytosis may be absent from these cultured cells. Smith et al. (1982c) did find an increase in protein output from lactating rat mammary cells in response to A23187, but this effect could be attributed to increased synthesis.

In this study we found, using a continuous labeling protocol, that a prolonged exposure to ionomycin produced a substantial inhibition of protein synthesis. Such a phenomenon has previously been reported for other cell types (Brostrom et al., 1990; Wileman et al., 1991). Under these conditions ionomycin still increases the extent of secretion but problems of interpretation were avoided by the use of a pulse-chase protocol in later experiments.

The Ca^{2+} -dependent secretion of caseins is not a peculiarity of ionomycin. We have also directly observed Ca^{2+} - and ATP-dependent casein secretion from digitonin-permeabilized mammary cells (our unpublished observations) that occurred over a similar range of micromolar Ca^{2+} concentration to those that activate exocytosis in regulated secretory cells (e.g., Ali et al., 1989). Little is known of the factors involved in acute physiological regulation of casein secretion but it has recently been reported that prolactin will stimulate casein secretion measured in a pulse-chase protocol consistent with an effect on regulated exocytosis (Seddiki and Olliver-Bousquet, 1991). It has been shown that lactating mammary epithelial cells possess Ca^{2+} stores sensitive to the intracellular messenger inositol 1,4,5-trisphosphate (Yoshimoto et al., 1990) indicating that $[\text{Ca}^{2+}]_i$ in these cells could be under rapid hormonal control. Perhaps more significant is the recent finding that mammary cells in culture show spontaneous oscillations of $[\text{Ca}^{2+}]_i$ and that mechanical stimulation stimulated increases in $[\text{Ca}^{2+}]_i$ (Furuya and Enomoto, 1990). This raises the possibility that mechanical stimulation of mammary cells in the lactating gland during milk ejection could lead to increased protein exocytosis through a Ca^{2+} -activated pathway. It has recently been found that exocytosis of surfactant from lung epithelial cells is activated by a rise in $[\text{Ca}^{2+}]_i$ elicited by mechanical stretching of the cells (Wirtz and Dobbs, 1990).

In regulated exocytosis the cortical actin network appears to act as a barrier to prevent exocytotic fusion until second messenger-mediated breakdown of the cortical network occurs (Burgoyne and Cheek, 1987; Cheek and Burgoyne, 1991; Koffer et al., 1990; Hays and Lindberg, 1991; Vitale et al., 1991). It has been suggested based on the coexistence of both constitutive and regulated exocytosis in the same cells that the movement of constitutive secretory vesicles may not be impaired by the actin cytoskeleton (Linstedt and Kelly, 1987). It appears from the present results, based on the use of cytochalasin D, that disruption of the actin cytoskeleton does not modify the extent of protein secretion implying that the actin network does not normally inhibit exocytosis in mammary cells. Previously reported inhibitory effects of cytochalasin B (Smith et al., 1982b) could have resulted from inhibition of glucose uptake. From rhodamine-phalloidin staining the mammary cells possess a cortical actin network around the whole cell but it is not clear from such images how dense the actin network is. From electron microscopical observations on mammary cells it appears that the cortical network is not extensive in these cells

(our unpublished observations). It is also of interest that in many epithelial cells that show polarized regulated exocytosis (such as pancreatic and parotid acinar cells) the cortical actin network is particularly prominent in resting cells at the secretory apical membrane (Drenckhahn and Mannherz, 1983) where it could act as a barrier to regulate exocytosis at this membrane. No indication for such polarity of the cortical actin network was seen in isolated mammary cells or even in cells in the intact tissue. We conclude, therefore, that the cortical actin network in mammary cells is unlikely to regulate exocytosis and the stimulatory effect of raising $[\text{Ca}^{2+}]_i$ is unlikely to be mediated through disassembly of the cortical actin network as occurs in the case of regulated exocytosis in, for example, adrenal chromaffin (Burgoyne and Cheek, 1987) and mast cells (Koffer et al., 1990).

One possible target for the action of Ca^{2+} in stimulating exocytosis is the Ca^{2+} - and phospholipid-binding protein annexin II (calpactin). This protein is able to bind to and aggregate secretory granules from adrenal chromaffin cells in the presence of micromolar Ca^{2+} (Drust and Creutz, 1988) and is a component of secretory granule membranes from anterior pituitary (Turgeon et al., 1990). Work on permeabilized chromaffin cells has suggested that annexin II may be involved in Ca^{2+} -activated exocytosis (Ali et al., 1989; Ali and Burgoyne, 1990; Burgoyne and Morgan, 1990) possibly by mediating the linkage of secretory granules to the plasma membrane (Nakata et al., 1990). We have recently found that annexin II is expressed in lactating mammary epithelial cells where it is localized specifically to the apical membrane (Handel et al., 1991). Whether annexin II plays any role in Ca^{2+} -activated exocytosis in mammary epithelial cells remains to be determined.

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