Characterization of the effects of Ca^{2+} depletion on the synthesis, phosphorylation and secretion of caseins in lactating mammary epithelial cells

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We have examined the effects of depleting luminal Ca^{2+} on the synthesis, phosphorylation and secretion of caseins in lactating mouse mammary cells by using inhibitors of the endoplasmic reticulum Ca^{2+} -ATPase or the ionophore ionomycin in the absence of external Ca^{2+} . Treatment with these drugs resulted in a transient increase in the cytosolic Ca^{2+} concentration due to Ca^{2+} mobilization. Protein synthesis over a 1 h period was substantially inhibited by Ca^{2+} depletion, but in a pulse-chase protocol secretion of pre-synthesized proteins was unaffected by Ca^{2+} depletion. Analysis of polysome profiles showed that Ca^{2+} depletion resulted in a loss in polysomes, consistent with an

inhibition of initiation of protein synthesis. Neither treatment with Ca^{2+} -ATPase inhibitors to deplete endoplasmic reticulum Ca^{2+} nor treatment with ionomycin/EGTA had any effect on an early phase of phosphorylation of α - or β/γ -caseins, but Ca^{2+} depletion resulted in a decrease in a late phase of casein phosphorylation. These results indicate that luminal Ca^{2+} is required to maintain protein synthesis in lactating mammary cells but is not required for protein secretion, and that Ca^{2+} accumulation in the Golgi cisternae is required for a late but not for an early phase of casein phosphorylation.

INTRODUCTION

Cytosolic free Ca^{2+} is required for vesicular transport between the endoplasmic reticulum (ER) and the Golgi complex and between compartments of the Golgi complex [1], as well as stimulating regulated exocytosis in many cell types [2]. Ca^{2+} within the lumen of the ER and possibly the Golgi cisternae has additional roles in the secretory pathway [3]. The ER accumulates Ca^{2+} to high levels due to the action of a Ca^{2+} -ATPase [4], and high levels of Ca^{2+} within the Golgi cisternae have also been observed [5]. Depletion of Ca^{2+} from these intracellular organelles has several effects on the secretory pathway, including inhibition of protein synthesis at the level of translation [6,7] following phosphorylation of initiation factor 2 [8,9], acceleration of protein degradation within the ER [10], increased secretion of resident ER proteins [11], decreased processing of N-linked oligosaccharides on α_1 -antitrypsin and decreased secretion of the latter (but not of albumin) from HepG2 cells [7], and inhibition of brefeldin A-induced retrograde transport from the Golgi complex to the ER [12]. These effects have been demonstrated by removal of external Ca^{2+} and treatment with Ca^{2+} ionophores, or more specifically with inhibitors of the organellar, particularly ER, Ca^{2+} -ATPases. It has been established that these disruptions of the secretory pathway are not a consequence of Ca^{2+} release into the cytoplasm and the subsequent increase in the cytosolic Ca^{2+} concentration, but are due to depletion of luminal Ca^{2+} . It is believed, therefore, that Ca^{2+} within the ER and/or the Golgi plays an important role in the control of the folding and stability of nascent proteins, and in protein interactions within these organelles, and thereby controls the processing and traffic of secretory proteins.

Lactating mammary epithelial cells possess $\text{Ins}(1,4,5)\text{P}_3$ -sensitive Ca^{2+} stores [13]. Ca^{2+} uptake and the presence of Ca^{2+} -ATPase activity has been demonstrated in both ER and Golgi vesicles derived from these cells [14–17]. Ca^{2+} accumulation into

these organelles is required to allow substantial secretion of Ca^{2+} along with lactose and caseins during the exocytosis of casein vesicles to provide the high level of Ca^{2+} (up to 100 mM) found in milk. In addition, calcium phosphate has been shown to be required for the formation and stability of the typical casein micelle structure, which is essentially due to Ca^{2+} -dependent aggregation of the caseins following their phosphorylation [18,19]. It is not clear whether intraluminal Ca^{2+} is required for casein stability prior to secretion or for casein phosphorylation *in vivo*, which occurs in the Golgi complex [20–23]. A number of other secretory proteins have been found to be phosphorylated during their passage through the secretory pathway, including progastrin [24] and the chromogranins [25], but the requirement for luminal Ca^{2+} for phosphorylation of these proteins has not been examined. Casein kinase activity *in vitro* is cation dependent and is stimulated by Ca^{2+} , but Mg^{2+} is as effective as Ca^{2+} and Mn^{2+} is yet more effective [23,26,27], and so the requirement for luminal Ca^{2+} for casein phosphorylation *in vivo* is unclear. We show here that depletion of intracellular Ca^{2+} stores by treatment with Ca^{2+} -ATPase inhibitors or the Ca^{2+} ionophore ionomycin inhibits protein synthesis in lactating mouse mammary cells but has no effect on the secretion of the major milk caseins. The phosphorylation of the caseins occurs in two phases, and Ca^{2+} depletion inhibited the late but not the early phase of casein phosphorylation.

MATERIALS AND METHODS

Materials

Ionomycin was purchased from Calbiochem-Novabiochem, Nottingham, U.K., and 2,5-di-(*t*-butyl)-1,4-benzohydroquinone (tBHQ) was a gift from Dr. C. W. Taylor (University of Cambridge, U.K.). Unless otherwise stated, tissue culture media were obtained from Gibco BRL Life Technologies Ltd., Paisley,

Abbreviations used: ER, endoplasmic reticulum; tBHQ, 2,5-di-(*t*-butyl)-1,4-benzohydroquinone.

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Scotland, U.K., and other reagents were from Sigma Chemical Co., Poole, Dorset, U.K.

Cell preparation

Mammary glands were dissected from mid-lactation (day 10–11) primigravida Tuck's no.1 mice. The tissue was digested into acini of 10–30 cells by incubation with 0.14% collagenase (Lorne Laboratories, Twyford, Berks., U.K.) in Hanks balanced salt solution containing 5 µg/ml insulin and 0.01 µg/ml cortisol. The digestion was terminated when the cell suspension was filtered through a 150 µm pore nylon mesh, and the cells were washed by centrifugation and resuspension in a medium containing 0.1 mg/ml trypsin inhibitor, 0.04 mg/ml DNase I (Boehringer Mannheim), 5 µg/ml insulin and 0.01 µg/ml cortisol. The cell suspension was then filtered through a 53 µm pore nylon mesh, washed again and finally resuspended in culture medium [0.1 µg/ml prolactin, 0.01 µg/ml epidermal growth factor, 0.001 µg/ml tri-iodothyronine, 5 µg/ml insulin, 0.01 µg/ml cortisol, 50% (v/v) M199 medium, 50% (v/v) Ham's F12, 2.5% (w/v) BSA]. The cells were then counted using a haemocytometer and diluted to a final concentration of 1×10^6 cells per ml of culture medium. Cell viability was assessed by Trypan Blue exclusion and was around 90–95%.

Measurement of protein synthesis and secretion

To study protein synthesis or secretion [28,29], the cells at 1×10^6 were incubated (1 ml per incubation) for 1 h at 37 °C in an atmosphere containing 5% CO₂ and 95% O₂ with 10 µCi/ml L-[³⁵S]methionine (cell labelling grade; specific radioactivity > 13000 Ci/mmol; Amersham International).

For assay of protein synthesis over this time, the cells were incubated in the presence or absence of drugs and the incubations were terminated by centrifugation of the suspended cells for 1 min in a Microfuge at 2500 g. The cell pellets were mixed with 100 µl of 1% (w/v) BSA and 800 µl of 10% (w/v) trichloroacetic acid and left for 1 h at 4 °C. The precipitated protein was extracted by centrifugation in a Microfuge at 2500 g for 1 min and then washed twice in 10% trichloroacetic acid. The final pellets were mixed with 10 ml of Cocktail T (BDH) and the incorporated ³⁵S was determined by scintillation counting.

For assay of protein secretion, cells labelled for 1 h in the absence of drug additions were chased for a further 1 h in medium lacking radiolabel in the absence or presence of added drugs. After centrifugation, duplicate aliquots of the supernatants were mixed with equal volumes of 20% trichloroacetic acid, the trichloroacetic acid precipitates were washed and ³⁵S in secreted proteins was determined by scintillation counting. The extent of secretion of radiolabelled proteins was calculated and expressed as a percentage of total label incorporated.

Analysis of casein phosphorylation

Cells at 1×10^6 per ml were incubated (1 ml per incubation) with 75 µCi/ml [³²P]P_i (Amersham) and 100 µg/ml cycloheximide, with or without thapsigargin (1 µM) or ionomycin (10 µM) + 2 mM EGTA for 30 min. The cells were then sedimented by centrifugation for 15 s at 2500 g, and the cell pellets from the duplicate incubations were resuspended, pooled and washed once in PBS before resuspension in 100 µl of SDS dissociation buffer and boiling for 5 min. The samples were separated in duplicate by SDS/PAGE (12.5% gels) along with molecular mass markers and mouse casein standards. The gels were stained with Coomassie Brilliant Blue before drying on a Bio-Rad Slab Gel Drier and exposing them to a Molecular

Dynamics PhosphorScreen and processing using a PhosphorImager (Molecular Dynamics). The images were quantified using Image Quant software (Molecular Dynamics). For each determination, three aliquots of labelled cells were pooled and analysed on duplicate gels to produce a mean value. In each experiment six independent values were determined in this way for control and experimental conditions.

Polysome profile

Cells (1×10^6 per ml) were incubated for 1 h in the presence or absence of drug additions under the conditions described above. A 15 ml sample of the cell suspension was then centrifuged at 80 g for 3 min and the cell pellet resuspended in 0.9 ml of lysis buffer (50 mM Tris, pH 7.4, 26 mM KCl, 5 mM MgCl₂, 1% Triton X-100, 1% sodium deoxycholate, 1 mg/ml heparin, 10 µg/ml cycloheximide), before centrifugation at 10000 g for 10 min at 4 °C. Aliquots of the supernatants (300 µl) were applied to 13 ml sucrose gradients. The gradients were formed of 17–51% (w/v) sucrose in 50 mM Tris, pH 7.4, 25 mM KCl, 5 mM MgCl₂ and were prepared using an Autodensiflow IIC gradient fractionator (Buchler Instruments). The gradients were centrifuged for 2 h at 270000 g at 4 °C, and then analysed by extraction by the Autodensiflow IIC gradient fractionator and pumped through a Monitor UV-M (Pharmacia LKB Biotechnology, Uppsala, Sweden); absorbance was monitored at 254 nm.

Measurement of cytosolic free Ca²⁺ concentration

Mammary acini at a density of 1×10^6 cells per ml were suspended in a buffer containing 145 mM NaCl, 5 mM KCl, 1.2 mM NaH₂PO₄, 10 mM glucose, 20 mM Hepes, 0.78 mM CaCl₂, 1.3 mM MgCl₂ and 0.5% BSA, pH 7.4. The cell suspension was incubated at room temperature for 45 min with 10 µM fura-2/AM, washed twice in fresh buffer, incubated for a further 45 min at 37 °C, and washed three times before resuspension in fresh buffer at 37 °C. Fluorescence was measured using an LS5 Luminescence Spectrophotometer (Perkin-Elmer Corp., Norwalk, CT, U.S.A.). Excitation wavelengths were 340 nm and 380 nm, the emission wavelength was 509 nm and values for the ratio of emission at 380/340 nm (*R*) were determined. The fluorescence was calibrated by cell lysis with 200 µM digitonin followed by chelation using 40 mM EGTA. The Ca²⁺ concentration ([Ca²⁺]_i) was calculated by determining emission ratios (*R*) at basal (EGTA) and peak (digitonin) Ca²⁺ levels and substituting the values for *R* at each time point into the following formula:

$$[\text{Ca}^{2+}]_i = 873 \times (R - 105) / (19.5 - R)$$

RESULTS

This study was carried out with freshly isolated acini from lactating mouse mammary glands. These mammary cells have been extensively characterized and shown to remain polarized, viable and active in protein synthesis and secretion over several hours [28–30]. Protein secretion into the medium is temperature- and microtubule-dependent [29]. The predominant proteins synthesized and secreted by these cells are the secretory milk proteins, which are mainly α-, β- and other caseins [28–30]. Assay of [³⁵S]methionine incorporation into total cellular protein or into proteins released into the medium, therefore, provides a reliable estimate of casein synthesis and secretion.

The approaches used in this study to deplete luminal Ca²⁺ used the Ca²⁺-ATPase inhibitors thapsigargin [31] and tBHQ [32] or treatment with the Ca²⁺ ionophore ionomycin in the absence

Figure 1 Changes in cytosolic Ca^{2+} concentration in lactating mouse mammary cells in response to thapsigargin, tBHQ or ionomycin

Isolated acini from lactating mouse mammary glands were loaded with the Ca^{2+} indicator fura-2 and challenged with $1 \mu\text{M}$ thapsigargin (TG) (A) or $100 \mu\text{M}$ tBHQ (B) in the presence of external Ca^{2+} , or with $10 \mu\text{M}$ ionomycin (iono) plus 2 mM EGTA to chelate external Ca^{2+} (C).

of external Ca^{2+} . All three treatments led to a burst of Ca^{2+} mobilization, seen as a transient rise in the cytosolic Ca^{2+} concentration detectable with the Ca^{2+} indicator fura-2 (Figure 1). Ionomycin in the absence of external Ca^{2+} would be expected to deplete all internal luminal Ca^{2+} stores, whereas the Ca^{2+} -ATPase inhibitors are expected to be specific for the ER (although no data are available on the potential sensitivity of any Golgi ATPase to these drugs). The mean peak rises in cytosolic Ca^{2+} concentration were $98.2 \pm 17.0 \text{ nM}$ ($n = 7$), $118.8 \pm 17.0 \text{ nM}$ ($n = 8$) and $43.5 \pm 8.4 \text{ nM}$ ($n = 4$) above basal levels for $1 \mu\text{M}$ thapsigargin, $100 \mu\text{M}$ tBHQ and $10 \mu\text{M}$ ionomycin respectively. Lower concentrations of thapsigargin (100 nM or less) did not produce detectable rises in the cytosolic Ca^{2+} concentration.

The effect of Ca^{2+} depletion on protein synthesis was assayed by examining the effect of the drugs during a 1 h labelling period with [^{35}S]methionine. The effect on secretion was assayed by pulse-labelling the cells for 1 h and then following secretion over a subsequent 1 h chase period in the presence or absence of drugs. These conditions were chosen based on previous work on isolated lactating mouse mammary cells which showed that little secretion occurs over the 1 h pulse-labelling period and that constitutive secretion of caseins is essentially complete over a 1 h chase period [28–30]. Thapsigargin at concentrations up to $1 \mu\text{M}$ produced a dose-dependent inhibition of synthesis in mammary cells, but had no effect on protein secretion (Figure 2A). Similar results were seen with five different batches of cells, with a maximal inhibition of protein synthesis of $60 \pm 5.5\%$ ($n = 5$) at $1 \mu\text{M}$ thapsigargin. On SDS/PAGE and fluorography this was seen as a general reduction of labelling of all synthesized polypeptides, which are mainly caseins (Figure 2C). To confirm that this effect was due to the inhibition of the ER Ca^{2+} -ATPase activity, the effect of the structurally unrelated Ca^{2+} -ATPase inhibitor tBHQ was examined. This drug also produced a marked

Figure 2 Effects of thapsigargin and tBHQ on protein synthesis and secretion in lactating mouse mammary cells

Isolated acini from lactating mouse mammary glands were incubated with [^{35}S]methionine for 1 h in the absence or presence of the indicated concentrations of thapsigargin (A) or tBHQ (B), and the extent of protein synthesis over this period was determined from the incorporation into total cellular trichloroacetic acid-precipitable protein. Separate batches of cells were radiolabelled for 1 h, washed and then incubated for 1 h without or with added thapsigargin or tBHQ. The extent of secretion over the chase period was determined from the level of radiolabelled trichloroacetic acid-precipitable protein present in the medium and expressed as a percentage of the total incorporation of radiolabel ($n = 4$). (C) SDS/PAGE and PhosphorImager analysis of [^{35}S]methionine incorporation into control cells (lane a) and cells treated with $1 \mu\text{M}$ thapsigargin (lane b).

inhibition of protein synthesis (Figure 2B), with relatively little effect on protein secretion except at high concentrations ($100 \mu\text{M}$) that were already maximal for inhibition of protein synthesis. Similar results were found with tBHQ in two separate batches of cells, with maximal inhibition of 84 and 52 % at $100 \mu\text{M}$ tBHQ in the two experiments. The data on the effect of thapsigargin on protein secretion shown in Figure 2(A) were based on exam-

Figure 3 Effect of thapsigargin on the time course of protein secretion in lactating mouse mammary cells

Isolated acini from lactating mouse mammary glands were pulse-labelled with [^{35}S]methionine for 1 h, washed and then protein secretion was monitored during the chase periods from analysis of incorporation into trichloroacetic acid-precipitable protein released into the medium. The cells were incubated during the chase period without (control) or with 1 μM thapsigargin. Data shown are means $\pm$ S.E.M. ($n = 4$).

ination of a single time point (1 h). In order to examine possible effects of thapsigargin on the rate of secretion, the time course of protein secretion in the absence or presence of 1 μM thapsigargin was examined. No inhibitory effects of thapsigargin on protein secretion were seen at any time point (Figure 3).

We have previously demonstrated that treatment of mammary cells with the Ca^{2+} ionophore ionomycin also results in inhibition of protein synthesis [28]. These earlier experiments were carried out with external Ca^{2+} present and could have been due to sustained elevation of cytosolic Ca^{2+} due to Ca^{2+} entry rather than luminal Ca^{2+} depletion. To avoid problems of interpretation, the effects of ionomycin in the presence of 2 mM EGTA, to deplete external Ca^{2+} and thus prevent external Ca^{2+} entry, were examined. Under these conditions, where the sustained effect of ionomycin should be solely due to depletion of luminal Ca^{2+} , 10 μM ionomycin produced a $61 \pm 1.5\%$ ($n = 4$) inhibition of protein synthesis in the lactating mammary cells.

Depletion of luminal Ca^{2+} has been shown in other cell types to inhibit translation, and this can be seen as a reduction in polysome content and an increase in 80 S monosomes in analyses of polysomal profiles using density gradients. Such a loss of polysomes and an increase in the size of the 80 S monosome peak were seen following treatment of lactating mammary cells with 1 μM thapsigargin or 100 μM tBHQ for 1 h (Figure 4). The traces shown are representative of five gradients each for control and thapsigargin treatment and three each for experiments with tBHQ.

Having established that treatment with Ca^{2+} -ATPase inhibitors or with ionomycin in the absence of external Ca^{2+} leads to release of luminal Ca^{2+} and inhibition of protein synthesis, we examined the effects of these drugs on casein phosphorylation by following ^{32}P incorporation into α -, β - and γ -caseins. The caseins are the major phosphoproteins synthesized in lactating mammary cells [22]. Since the treatments inhibit protein synthesis, this would reduce the amount of caseins available for phosphorylation. In order to remove differences between treatments, all cells were treated with 100 $\mu\text{g}/\text{ml}$ cycloheximide at the time of drug addition to ensure that only phosphorylation of pre-synthesized caseins

Figure 4 Effects of thapsigargin and tBHQ on polysome profiles in lactating mouse mammary cells

Isolated acini from lactating mouse mammary glands were incubated for 1 h under control conditions (A, C) or with the addition of 1 μM thapsigargin (B) or 100 μM tBHQ (D) in two separate experiments. Cell extracts were separated on sucrose gradients to examine the polysome/monosome profile. The position of the 80 S monosome peak is indicated, and the polysome peak in control cells is shown by the thick arrow.

was monitored. Cycloheximide at this concentration inhibited protein synthesis by 93%. β - and γ -caseins migrated close to each other (Figure 5) and were quantified together. In previous work, a phase of casein phosphorylation was observed that was complete within 20–30 min [23]. In initial experiments, therefore, we examined the effect of thapsigargin on the extent of casein phosphorylation following a 30 min incubation (Figure 5). Under these conditions no effect of thapsigargin on casein phosphorylation was detected in six experiments. It is not known whether thapsigargin would act on Ca^{2+} transport within the Golgi in addition to its effects on the ER, and so we also examined the effect of Ca^{2+} depletion using ionomycin in the presence of EGTA, which would be expected to deplete Ca^{2+} from all cellular organelles. In addition, the time course of casein phosphorylation in the absence and presence of ionomycin/EGTA was followed for up to 3 h. Over a short time course (Figures 6A and 6B), it was apparent that there was an early phase of casein phosphorylation over the first 30 min that was essentially unaffected by Ca^{2+} depletion. The rate of casein phosphorylation then increased, and in experiments over a longer time course the later phase of casein phosphorylation was found to continue for up to 150 min (Figures 6C and 6D). The second phase of casein phosphorylation was partially inhibited by Ca^{2+} depletion using ionomycin/EGTA and reached a new lower plateau of phosphorylation after incubation for 150 min (Figures 6C and 6D). Similar results were seen for α -casein (Figures 6A and 6C) and for β/γ -caseins (Figures 6B and 6D).

The time course of casein phosphorylation seen in these experiments was slow compared with the transit times (45–60 min) previously found for newly synthesized casein to pass through the secretory pathway and be constitutively secreted [28,29]. It should be noted, however, that a portion of newly synthesized casein is not secreted, but instead accumulates in a regulated exocytotic compartment [28]. Since these phosphorylation experiments were carried out on cycloheximide-treated

Figure 5 SDS/polyacrylamide gel analysis of proteins phosphorylated in lactating mouse mammary cells in the absence or presence of thapsigargin

Isolated acini from lactating mouse mammary glands were incubated with [^{32}P]P $_i$ for 30 min, in the presence of cycloheximide to prevent further protein synthesis, under control conditions (lane a) or with 1 μM thapsigargin (lane b). Incorporated radiolabel was detected using a PhosphorScreen and a Molecular Dynamics PhosphorImager. The positions of α -casein and the β/γ -casein doublet are indicated. In some experiments the incorporation into α -casein was greater than that into β/γ -casein, as seen previously [22].

cells, we examined the time course of secretion of [^{35}S]methionine pulse-labelled proteins from the cells. Under these conditions casein secretion was slowed and continued for up to 150 min (Figure 7). The data in Figure 7 additionally show that casein

secretion was not inhibited at any time point by ionomycin/EGTA treatment.

DISCUSSION

We have examined the effects of luminal Ca^{2+} depletion by Ca^{2+} -ATPase inhibitors and treatment with ionomycin/EGTA on casein synthesis, phosphorylation and secretion. Both thapsigargin and tBHQ produced only a transient rise in the cytosolic Ca^{2+} concentration when tested in the presence of external Ca^{2+} , indicating that their major effect on lactating mammary cells was mobilization of stored Ca^{2+} without any sustained activation of Ca^{2+} entry as occurs in certain other cell types [33,34]. The finding that the rises in cytosolic Ca^{2+} were transient would argue against long-term effects of the inhibitors being due to elevation of cytosolic Ca^{2+} rather than depletion of luminal Ca^{2+} . In addition, treatment with ionomycin in the presence of EGTA also produced a transient elevation of cytosolic Ca^{2+} , and under these conditions we would expect all luminal Ca^{2+} pools to be depleted.

Treatment with thapsigargin, tBHQ or ionomycin/EGTA resulted in each case in a substantial inhibition of protein synthesis based on a decrease in [^{35}S]methionine incorporation. This was seen as a general depression in radiolabel incorporation

Figure 6 Effects of Ca^{2+} depletion by treatment with ionomycin/EGTA on the early and late phases of casein phosphorylation in lactating mouse mammary cells

Isolated acini were incubated with [^{32}P]P $_i$ for various times in the presence of 100 $\mu\text{g}/\text{ml}$ cycloheximide to block protein synthesis and with or without 10 $\mu\text{g}/\text{ml}$ ionomycin and 2 mM EGTA. Samples were analysed on duplicate SDS/polyacrylamide gels and ^{32}P incorporation was detected using a PhosphorScreen and quantified using a Molecular Dynamics PhosphorImager and Image Quant software. In order to allow pooling of data from separate experiments, the data are expressed as percentages of the maximum value obtained. The data shown (means $\pm$ S.E.M.) are from short time courses (A, B; $n = 6$ from three cell batches) or longer time courses (C, D; $n = 4$ from two cell batches) and give quantification for α -casein (A, C) and for β/γ -casein (B, D).

Figure 7 Time course of protein secretion in the presence of cycloheximide, and effect of ionomycin/EGTA

Isolated acini from lactating mammary glands were pulse-labelled with [^{35}S]methionine for 1 h, washed and then protein secretion was monitored during the chase periods from analysis of incorporation into trichloroacetic acid-precipitable protein released into the medium. The cells were incubated during the chase period in the presence of 100 $\mu\text{g}/\text{ml}$ cycloheximide with or without 10 μM ionomycin/2 mM EGTA. Data shown are means $\pm$ S.E.M. ($n = 4$).

into all synthesized proteins, which are predominantly the secreted caseins [28,29]. These data are consistent with earlier work using explants of rabbit mammary gland which showed that inclusion of EGTA in the culture medium resulted in inhibition of protein synthesis [35]. This earlier study did not, however, allow discrimination between the depletion of cytosolic or luminal Ca^{2+} . In cells treated with thapsigargin or tBHQ, changes in the polysome profile with a loss of polysomes and an increase in the size of the 80 S monosome peak suggested that the inhibition of protein synthesis was likely to be due to an effect on initiation of translation. This is in agreement with the effects of luminal Ca^{2+} depletion in other cell types [6–9]. Thapsigargin, tBHQ and treatment with ionomycin/EGTA had no effect on the constitutive secretion of pre-synthesized caseins. In studies on other secretory proteins, variable effects of Ca^{2+} depletion on secretion have been observed. Notably, in HepG2 cells the secretion of α_1 -antitrypsin is inhibited following thapsigargin treatment but secretion of albumin is unaffected [7]. Since caseins bind Ca^{2+} , and Ca^{2+} is required for the stability of the casein micelle, it might be expected that Ca^{2+} depletion would impair the transit of these proteins through the secretory pathway, but the data presented here show that this did not appear to be the case.

Active Ca^{2+} uptake occurs into both ER and Golgi vesicles from lactating mammary cells [14–17]. Uptake of Ca^{2+} into the Golgi cisternae could be necessary to allow accumulation of Ca^{2+} in the forming secretory vesicles for secretion of Ca^{2+} , or it might have additional functions in the Golgi cisternae. Intraluminal Ca^{2+} is required for the normal processing of glycosylated secretory proteins [6,7], and Ca^{2+} or other bivalent cations are required for maximal casein kinase activity *in vitro* [23,25,26]. Using this assay we have previously shown that treatment of lactating mammary cells with brefeldin A, which causes collapse of *cis*-Golgi cisternae into the ER, results in inhibition of the phosphorylation of β - and γ -caseins but not of α -casein [22]. These results indicate that phosphorylation of α -casein was likely to occur normally in the ER or *cis*-Golgi, whereas that of β - and

γ -caseins would occur predominantly in *trans*-Golgi cisternae or later post-Golgi compartments. Other secretory proteins have been reported to be phosphorylated in one or other of these locations depending on the particular protein [21,36]. Treatment with thapsigargin had no effect on the extent of ^{32}P incorporation into α -, β - or γ -caseins during a 30 min incubation. It is not known whether the mammary Golgi ATPase is sensitive to thapsigargin, and one possibility was that thapsigargin did not lead to depletion of Ca^{2+} from Golgi cisternae. A requirement for luminal Ca^{2+} for an early phase of casein phosphorylation was ruled out, however, by the observation that treatment with ionomycin/EGTA also failed to significantly inhibit phosphorylation over a 30 min incubation, and ionomycin would be expected to act on all intracellular membranes and deplete all luminal Ca^{2+} stores. In longer incubations, however, it was apparent that a second and more extensive phase of casein phosphorylation occurred which continued for up to 150 min of incubation and that this later phase of phosphorylation was partially inhibited by treatment with ionomycin/EGTA. This inhibitory effect was not on the rate of phosphorylation but on the extent of phosphorylation, as a new lower plateau of phosphorylation was observed with ionomycin/EGTA. The reduced phosphorylation of caseins at later times is unlikely to be a consequence of reduced cellular ATP levels, since this would be expected to result in a reduction in the rate rather than the extent of phosphorylation. In addition, under the conditions used (cycloheximide present), secretion, which is ATP-dependent [37], continued over the same time course and was unaffected by ionomycin/EGTA. One explanation for the two phases of casein phosphorylation and the differential effects of Ca^{2+} depletion would be the presence of two casein kinases differing in Ca^{2+} sensitivity, with the Ca^{2+} -independent kinase acting at an earlier stage of the secretory pathway than the Ca^{2+} -dependent kinase. Independent evidence in favour of this explanation comes from earlier work which demonstrated two biochemically distinct casein kinase activities in bovine mammary gland Golgi vesicles, one of which was Ca^{2+} -independent and the other Ca^{2+} -dependent [38]. Functional dissection of the Golgi complex by treatment of mouse mammary cells with brefeldin A has also suggested the possible presence of two distinct casein kinases [29]. The membrane-associated intraluminal casein kinases have yet to be purified, and further molecular characterization of these kinases and demonstration of their exact intracellular localization will be required in order to test this interpretation.

In conclusion, Ca^{2+} within the ER is clearly required to maintain the rates of synthesis of secretory proteins in lactating mammary cells. Luminal Ca^{2+} is not required, however, for the constitutive secretion of the caseins. The significance of the role of Ca^{2+} in the maintenance of the casein micellar structure is unclear, but a high luminal Ca^{2+} concentration is not essential for casein transit through the secretory pathway. The active uptake of Ca^{2+} into the Golgi cisternae of lactating mammary cells is also likely to be required to allow accumulation of Ca^{2+} for its secretion, and in addition for a late phase of casein phosphorylation.

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REFERENCES

- 1 Balch, W. E. (1989) *J. Biol. Chem.* **264**, 16965–16968
- 2 Burgoyne, R. D. and Morgan, A. (1993) *Biochem. J.* **293**, 305–316
- 3 Sambrook, J. F. (1990) *Cell* **61**, 197–199
- 4 Berridge, M. J. (1993) *Nature (London)* **361**, 315–325

- 5 Chandra, S., Kable, E. P. W., Morrison, G. H. and Webb, W. W. (1991) *J. Cell Sci.* **100**, 747–752
- 6 Chin, K.-V., Cade, C., Brostrom, C.-O., Galuska, E. M. and Brostrom, M. A. (1987) *J. Biol. Chem.* **262**, 16509–16514
- 7 Wong, W. L., Brostrom, M. A., Kuznetsov, G., Gmitter-Yellen, D. and Brostrom, C. O. (1993) *Biochem. J.* **289**, 71–79
- 8 Prostko, C. R., Dholakia, J. N., Brostrom, M. A. and Brostrom, C. O. (1995) *J. Biol. Chem.* **270**, 6211–6215
- 9 Srivastava, S. P., Davies, M. V. and Kaufmann, R. J. (1995) *J. Biol. Chem.* **270**, 16619–16624
- 10 Wileman, T., Kane, L. P., Carson, G. R. and Terhorst, C. (1991) *J. Biol. Chem.* **266**, 4500–4507
- 11 Booth, C. and Koch, G. L. E. (1989) *Cell* **59**, 729–737
- 12 Ivesa, N. E., De Lemos-Chiarandini, C., Gravotta, D., Salatini, D. and Kriebich, G. (1995) *J. Biol. Chem.* **270**, 25960–25967
- 13 Yoshimoto, A., Nakanishi, K., Anzai, T. and Komine, S. (1990) *Cell Biochem. Struct.* **8**, 191–198
- 14 West, D. W. (1991) *Biochim. Biophys. Acta* **693**, 374–386
- 15 Virk, S. S., Kirk, C. J. and Shears, S. B. (1985) *Biochem. J.* **226**, 741–748
- 16 Bingham, E. W., McGranaghan, M. B., Wickham, E. D., Leung, C. T. and Farrell, H. M. (1992) *Ann. N. Y. Acad. Sci.* **671**, 418–420
- 17 Clegg, R. A. and West, D. W. (1991) *Biochem. Soc. Trans.* **19**, S194
- 18 Aoki, T., Umenda, T. and Kako, Y. (1992) *Biosci. Biotech. Biochem.* **56**, 1878–1879
- 19 Holt, C., Davies, D. T. and Law, A. J. R. (1986) *J. Dairy Res.* **53**, 557–572
- 20 Bingham, E. W., Farrell, H. M. and Basch, J. J. (1972) *J. Biol. Chem.* **247**, 8193–8194
- 21 Capusso, J. M., Keenan, T. W., Abeijon, C. and Hirschberg, C. B. (1989) *J. Biol. Chem.* **264**, 5233–5240
- 22 Turner, M. D., Handel, S. E., Wilde, C. J. and Burgoyne, R. D. (1993) *J. Cell Sci.* **106**, 1221–1226
- 23 West, D. W. and Clegg, R. A. (1984) *Biochem. J.* **219**, 181–187
- 24 Varro, A., Henry, J., Vaillant, C. and Dockray, G. J. (1994) *J. Biol. Chem.* **269**, 20764–20770
- 25 Rosa, P., Mantovani, S., Rosboch, R. and Huttner, W. B. (1992) *J. Biol. Chem.* **267**, 12227–12232
- 26 Bingham, E. W. and Farrell, H. M. (1974) *J. Biol. Chem.* **249**, 3647–3651
- 27 Bingham, E. W. and Groves, M. L. (1979) *J. Biol. Chem.* **254**, 4510–4515
- 28 Turner, M. D., Rennison, M. E., Handel, S. E., Wilde, C. J. and Burgoyne, R. D. (1992) *J. Cell Biol.* **117**, 269–278
- 29 Rennison, M. E., Handel, S. E., Wilde, C. J. and Burgoyne, R. D. (1992) *J. Cell Sci.* **102**, 239–247
- 30 Rennison, M. E., Kerr, M., Addey, C. V. P., Handel, S. E., Turner, M. D., Wilde, C. J. and Burgoyne, R. D. (1993) *J. Cell Sci.* **106**, 641–648
- 31 Thastrup, O., Dawson, A. P., Scharif, O., Foder, B., Cullen, P. J., Probak, B. K., Bjerrum, P. J., Christensen, S. B. and Hanley, M. R. (1989) *Agents Actions* **27**, 17–23
- 32 Moore, G. A., McConkey, D. J., Kass, G. E. M., O'Brien, P. J. and Orrenius, S. (1987) *FEBS Lett.* **224**, 331–336
- 33 Robinson, I. M., Cheek, T. R. and Burgoyne, R. D. (1992) *Biochem. J.* **288**, 457–463
- 34 Takemura, H., Hughes, A. R., Thastrup, O. and Putney, J. W. (1989) *J. Biol. Chem.* **264**, 12266–12271
- 35 Wilde, C. J., Hasan, H. R., White, D. A. and Mayer, R. J. (1981) *Biochem. Biophys. Res. Commun.* **103**, 934–942
- 36 Varro, A. and Dockray, G. J. (1993) *Biochem. J.* **295**, 813–819
- 37 Turner, M. D., Wilde, C. J. and Burgoyne, R. D. (1992) *Biochem. J.* **286**, 13–15
- 38 Brooks, C. L. (1989) *FEBS Lett.* **243**, 385–388