Journal of Cell Science 106, 641-648 (1993)
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Inhibition of constitutive protein secretion from lactating mouse mammary epithelial cells by FIL (feedback inhibitor of lactation), a secreted milk protein

Michelle E. Rennison[1], Marian Kerr[2], Caroline V. P. Addey[2], Susan E. Handel[1], Mark D. Turner[1], Colin J. Wilde[2] and Robert D. Burgoyne[1,*]

[1]The Physiological Laboratory, University of Liverpool, P O Box 147, Liverpool L69 3BX, UK
[2]Hannah Research Institute, Ayr, Scotland KA6 5HL, UK

*Author for correspondence

SUMMARY

The effect of a protein feedback inhibitor of lactation (FIL) on casein synthesis and secretion was examined using isolated acini from lactating mouse mammary gland. As previously found, FIL partially inhibited protein synthesis but produced an additional inhibition of constitutive casein secretion. The inhibition of synthesis and secretion showed similar dose-dependency and the inhibition was fully reversible. Constitutive secretion of pre-formed protein was inhibited by FIL in a pulse-chase protocol, indicating that the inhibitor regulated protein secretion by reducing protein movement through the secretory pathway independently of any initial inhibition of synthesis. Regulated exocytosis was not inhibited since casein release due to elevation of cytosolic Ca^{2+} concentration by the ionophore ionomycin was unaffected. Brefeldin A, which is known to block ER-to-Golgi transport, also inhibited both protein synthesis and secretion in mammary cells. The action of FIL on synthesis and secretion and previously described actions on casein degradation would be consistent with a block at an early stage in the secretory pathway. In support of this idea FIL treatment was found to result in vesiculation and swelling of the endoplasmic reticulum. These data provide evidence for a novel control of a constitutive secretory pathway by a physiological extracellular regulatory protein.

Key words: mammary cells, secretion, exocytosis, constitutive secretion

INTRODUCTION

Exocytotic release of proteins can occur via distinct pathways in numerous secretory cell types (Burgess and Kelly, 1987). Secretory proteins may be stored in vesicles or granules and accumulated in the cytoplasm prior to exocytosis regulated by changes in intracellular second messengers, usually a rise in cytosolic calcium concentration. Alternatively, they may be packaged into constitutive secretory vesicles and released immediately following synthesis. In secretory cells these two pathways usually coexist (Burgess and Kelly, 1987). Copius quantities of milk proteins, mainly caseins, are secreted by lactating mammary epithelial cells (Saacke and Heald, 1974; Linzell and Peaker, 1971) corresponding to up to 80% of the total synthesised protein (Turner et al., 1992). Although the mammary gland has been studied extensively, little is known about the factors that regulate milk protein secretion. It has generally been assumed that exocytotic secretion is a constitutive process in lactating cells, and constitutive secretion of milk proteins has been confirmed in lactating mouse mammary epithelial cells in vitro (Turner et al., 1992). In addition, however, it has been found that a portion of newly synthesised casein is stored and may be secreted by a regulated pathway following elevation of cytosolic Ca^{2+} concentration (Turner et al., 1992; Rennison et al., 1992). The physiological signal triggering regulated exocytosis has not yet been identified.

Secreted milk proteins accumulate in the alveolar lumen of the gland, until they are removed by milking or suckling assisted by the milk ejection reflex. It is this extracellular storage that provides an additional local level of control on the rate of milk secretion. Extensive studies in lactating animals (Wilde et al., 1988; Wilde and Peaker, 1990) indicate that the regulatory mechanism involves a chemical inhibitor of milk secretion, and experiments in tissue culture have identified a small whey protein that fulfils this role (Wilde et al., 1987; C. V. P. Addey et al., unpublished observations). The inhibitory protein, termed FIL (feedback inhibitor of lactation), is synthesised by the secretory epithelial cells of the mammary gland and secreted into the alveolar lumen along with other milk constituents. FIL has been purified and N-terminal sequencing

shows it to be a novel protein (C. V. P. Addey et al. unpublished observations). Addition of purified FIL to milk stored in the gland produces a temporary decrease in the rate of milk secretion.

FIL was identified by its ability to inhibit milk protein synthesis in tissue explant cultures. The possibility that FIL may act directly on the secretory pathway to inhibit secretion has not been assessed. Since recent work has shown that disruption of the early secretory pathway leads to inhibition of translation (Kuznetsov et al., 1992), we have used isolated lactating mammary epithelial cells to examine the possibility that FIL may act directly on the secretory pathway to block constitutive secretion. We found that constitutive secretion is indeed inhibited by FIL. It has been shown that constitutive secretion is switched off during mitosis (Featherstone et al., 1985; Kreiner and Moore, 1990) and phosphorylation of as yet unknown proteins regulate intracellular transport from ER to the Golgi (Lucocq et al., 1991; Davidson et al., 1992). So far no indication has been found that constitutive secretion can be modulated in response to extracellular factors apart from the drug brefeldin A (BFA). Our findings from mammary epithelial cells show that the constitutive secretory pathway can be inhibited by a physiological extracellular regulator revealing new possibilities for the control of ER and Golgi function.

MATERIALS AND METHODS

Preparation of mammary epithelial cells

Mammary epithelial acini were prepared as previously described (Turner et al., 1992; Rennison et al., 1992). Briefly, mid-lactation mouse mammary tissue was finely chopped and dissociated by collagenase digestion (0.14%, Worthington) for 90 mins at 37°C in Hanks' balanced salt solution (HBSS) supplemented with insulin (5 μg/ml) and cortisol (0.01 μg/ml). Digestion was terminated by filtering the cell suspension first through 150 μm and 53 μm pore nylon mesh. Cells were harvested by centrifugation (80 ***g*** for 5 minutes) and resuspended in HBSS containing 5 μg/ml insulin, 0.01 μg/ml cortisol, 0.04 mg/ml DNase I (Boehringer) and 0.1 mg/ml trypsin inhibitor. This wash procedure was repeated four times. Cell yield was then estimated by haemocytometer counting. Cells were resuspended in culture medium (50% M199, 50% Ham's F12, 5 μg/ml insulin, 0.01 μg/ml cortisol, 0.1 μg/ml prolactin, 0.01 μg/ml EGF and 0.001 μg/ml T_3) at 6×10^6 cells/ml before equilibrium at 37°C in an atmosphere containing 5% CO_2.

Preparation of FIL

FIL was prepared by anion exchange chromatography from a 10-30 kDa fraction of goat whey proteins. The whey fraction was prepared from goat's milk by centrifugation to remove casein and ultrafiltration as described previously (Wilde et al., 1987), and applied to an FPLC Mono-Q HR 10/10 column equilibrated in 20 mM bis-tris propane, pH 7.0. Proteins were eluted with a 0-1.0 M sodium acetate gradient in the same buffer. FIL was collected as the third major protein peak, which eluted at 50-70 mM sodium acetate. Analysis of this material by gel filtration indicated that it resolved as a single species of molecular mass 7.5 kDa (see UK Patent Application GB 2 238 052 from C. V. P. Addey, M. Peaker and C. J. Wilde, 1991). FIL was dialyzed extensively against distilled water and lyophilized for storage. The lyophilized FIL was reconstituted in 10 mM Hepes (pH 7.4) and used at a protein concentration of 8 μg/ml unless otherwise stated.

Assay of protein synthesis and secretion

Protein synthesis and secretion was determined following incorporation of [^{35}S]methionine utilizing either continuous labelling or pulse-chase protocols. In continuous labelling experiments cells were incubated with 10 μCi/ml L-[^{35}S]methionine (cell labelling grade, specific activity >1300 Ci/mmol, Amersham plc) for various times with or without 8 μg/ml goat inhibitor FIL. For pulse-chase experiments cells were incubated for 1 hour with 20 μCi/ml L-[^{35}S] methionine followed by addition of an equal volume of ice cold culture medium and centrifugation at 80 ***g*** for 5 minutes. The supernatant was discarded and the cell pellet resuspended in fresh culture medium. In all cases culture medium (defined above) was serum free but contained 70 μM cold methionine. Cells were either immediately transferred to 37°C and incubated with or without FIL for various periods or pre-incubated for 1 hour at 4°C before warming to 37°C. In some cases 10 μM ionomycin was present during the last hour of incubation. Incubations were terminated by centrifugation at 2500 ***g*** for 1 minute. The supernatant was removed and duplicate 400 μl aliquots were mixed with equal volumes of 20% TCA. The cell pellets were resuspended in 800 μl of 10% TCA and 100 μl of 1% BSA. Samples were kept on ice for 20 minutes before centrifugation at 2500 ***g*** for 15 seconds. TCA-precipitable pellets were washed twice as above by resuspension in 800 μl of 10% TCA. Incorporation of [^{35}S]methionine was determined by scintillation counting. For analysis of polypeptides by SDS-PAGE and fluorography the cells were labelled with 20 μCi/ml of [^{35}S]methionine in the presence or absence of 8 μg/ml FIL for 3 hours and analysed as described previously (Turner et al., 1992) using video densitometry.

Assay of RNA synthesis and triglyceride secretion

RNA synthesis was determined following incorporation of 5,6-[^{3}H]uridine (5 μCi/ml, 37 Ci/mmol; Amersham plc) during 3 hour incubations in the presence or absence of FIL (8 μg/ml). Cells were harvested by centrifugation (5000 ***g*** for 1 minute) and stored in liquid N_2. The cell pellet was resuspended in 1 ml of ice-cold 10 mM Tris-HCl, pH 7.0, containing 5 mM uridine, and lysed by sonication (Kontes KT50 cell disrupter, setting 30, 30 seconds). Duplicate 250 μl samples were mixed with an equal volume of 4% PCA and kept on ice for 15 minutes before centrifugation at 10,000 ***g*** and 4°C for 5 minutes. The supernatant was removed and the pellet washed twice with 1 ml of cold 2% PCA, and the final pellet was counted for radioactivity. For the assay of triglyceride secretion the cells were incubated with 5 μCi/ml 1-[^{14}C]acetic acid (specific activity 55 mCi/mmol) for 2 hours at 37°C. The cells were then centrifuged at 80 ***g*** for 5 minutes and the supernatant discarded. The cells were incubated for a further hour in the presence or absence of 8 μg/ml FIL and secretion of radiolabelled triglyceride assayed following lipid extraction from the cell supernantant as previously described (Turner et al., 1992).

Immunofluorescence and electron microscopy

Lactating mammary cells incubated with or without 8 μg/ml FIL were fixed and processed for immunofluorescence using anti-TGN38 (1:400) or for electron microscopy as previously described (Rennison et al., 1992).

RESULTS

Effect of FIL on protein synthesis and secretion

In lactating mammary epithelial cells up to 80% of newly

synthesised proteins, mainly the caseins, are secreted (Turner et al., 1992). In the present experiments mammary epithelial cells were labelled with [^{35}S]methionine to determine their synthetic and secretory activity, which was assessed by radioactive incorporation into total TCA-precipitable proteins in both cells and medium (Turner et al., 1992). In the present (Fig. 1) and previous studies (Turner et al., 1992; Rennison et al., 1992) we have observed that in these cells protein synthesis occurs linearly whereas secretion into the medium occurs after a 1 hour lag period, which is compatible with constitutive release of a proportion of the proteins immediately after passage through the secretory pathway. The extent of incorporation of [^{35}S]methionine into total protein was inhibited by FIL over a 3 hour period (Fig. 1a). The mean percentage inhibition of synthesis from 4 experiments was 36.5±4.5%. This is consistent with results obtained from rabbit mammary gland explants, which showed that FIL inhibits casein synthesis (Wilde et al., 1987). The nature of the polypeptides synthesised in the presence or absence of FIL was examined (in an experiment in which total protein synthesis was reduced by 40%) by SDS-PAGE, fluorography and densitometric analysis. [^{35}S]methionine incorporation into the major secretory proteins was reduced but FIL did not reduce the incorporation into many minor non-secretory polypeptides (Fig. 2). Release of labelled proteins into the culture medium was substantially inhibited following addition of FIL (Fig. 1b) more so in fact than the extent of synthesis. Examination of the effects of FIL on the percentage of radiolabelled protein that was secreted also clearly showed a reduction (Fig. 1c). After 3 hours the effect on percentage secretion corresponded to an inhibition of 52.0±11.3% compared to control cells, from a series of four separate cell batches. The extent of secretion in control cells (35% of total synthesized proteins) was similar to that we have previously reported (Turner et al., 1992; Rennison et al., 1992). These results indicate that FIL inhibits protein synthesis and secretion, producing a marked inhibition of protein output through the secretory pathway. The finding that FIL reduced the percentage of synthesised protein secreted suggested that FIL has effects on the secretory pathway in addition to any effect on protein synthesis. The degree of inhibition of synthesis and percentage secretion showed similar dose-dependency being half-maximally inhibited at around 2-4 μg/ml of FIL. Even at high doses of FIL only a partial inhibition was observed (Fig. 3). The FIL dose response had essentially plateaued at 5 μg/ml and in additional experiments at higher FIL concentrations (up to 27 μg/ml) the degree of inhibition was unchanged.

FIL did not have a general inhibitory effect on biosyn-

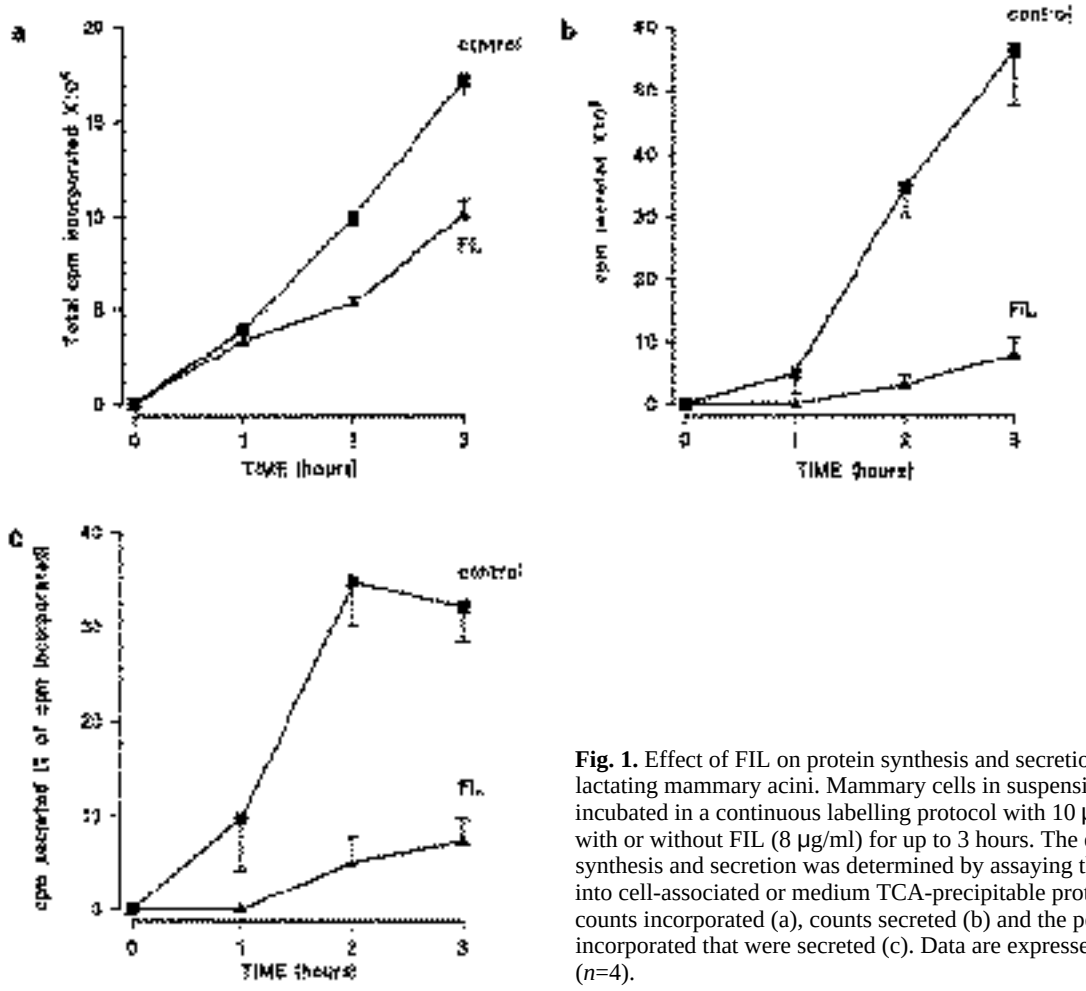

Fig. 1. Effect of FIL on protein synthesis and secretion by dissociated lactating mammary acini. Mammary cells in suspension culture were incubated in a continuous labelling protocol with 10 μCi/ml [^{35}S]methionine with or without FIL (8 μg/ml) for up to 3 hours. The extent of protein synthesis and secretion was determined by assaying the counts incorporated into cell-associated or medium TCA-precipitable protein. The data show total counts incorporated (a), counts secreted (b) and the percentage of total counts incorporated that were secreted (c). Data are expressed as mean ± s.e.m. (n=4).

thesis and secretion. Mammary cells synthesise and secrete large amounts of lipid. Assay of the extent of secretion of radiolabelled triglyceride, a major secreted lipid of mammary cells, following [^{14}C]acetate labelling (Turner et al., 1992) indicated that this was unaffected by FIL under the conditions in which protein synthesis and secretion was inhibited. Over a 1 hour period, labelled triglyceride release was 1,716±229 cpm (n=5) for control cells and 1,854±554 cpm (n=5) for cells treated with 8 μg/ml of FIL. In contrast, the extent of RNA synthesis was reduced by 71% and by 73% over 3 hours in the presence of 8 μg/ml of FIL, in two separate experiments. It is not clear whether this effect was related to the inhibition of protein synthesis. It has been shown recently that inhibition of protein synthesis by Ca^{2+}-depletion from the ER results in a marked fall in actin mRNA levels (Wong et al., 1993). The inhibition of protein synthesis and secretion by FIL was not due to a toxic action of the peptide since its effects, after a 2 hour treatment, were fully reversible (Fig. 4).

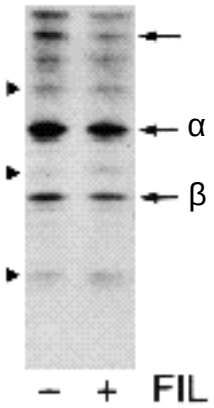

Fig. 2. Characterization of proteins synthesised by lactating mammary acini and the effect of FIL. Mammary cells were labelled with 20 μCi/ml [^{35}S]methionine for 3 hours in the presence (+) or absence (−) of 8 μg/ml FIL and the labelled proteins detected by SDS-PAGE and fluorography. Densitometric analysis showed reduced incorporation into α and β caseins (α, β) in the presence of FIL (59.5% and 52% of control values, respectively) and into another polypeptide (most probably transferrin) indicated by an arrow (41% of control value). On the left of the Figure polypeptides whose labelling was not markedly reduced by FIL are indicated by arrowheads. From the top to the bottom these incorporated to 88, 136 and 124% of control levels, respectively.

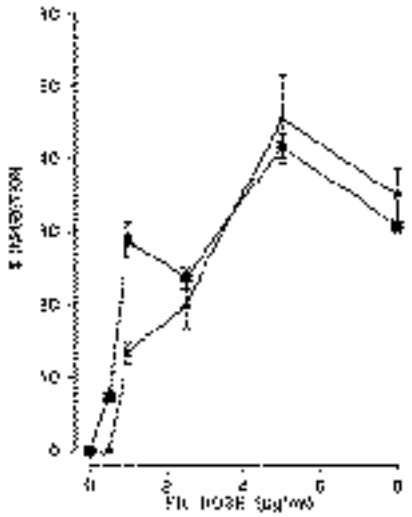

Fig. 3. Dose-response curves of inhibition by FIL of synthesis and secretion in freshly isolated lactating mammary epithelial cells. Cells were incubated with varying doses of FIL for 3 hours in the continuous presence of 10 μCi [^{35}S]methionine and the extent of protein synthesis and secretion determined. The degree of inhibition of synthesis and percentage of synthesised protein secreted at each dose of FIL was calculated and is shown as mean ± s.e.m. (n=4). Triangles, % secretion; squares, synthesis.

In an attempt to determine the mechanism of action of FIL we examined the effects of manipulation of a variety of intracellular signalling pathways. FIL actions were not mimicked by treatment with 100 μM 8-bromo-cAMP, 100 μM 8-bromo-cGMP, 10 μM ionomycin or treating cells with 1 μM okadaic acid, 250 ng/ml pertussis toxin or 75 μg/ml cholera toxin. The inhibitory effect of FIL was not modified by pretreatment of the cells for 2 hours with pertussis toxin or cholera toxin and in experiments using the tyrosine kinase inhibitor genistein (100 μM), the drug itself produced a marked inhibition of protein synthesis.

FIL inhibits constitutive secretion of preformed proteins

To test directly whether FIL inhibited secretion of preformed caseins and to avoid problems of possible interference with protein synthesis we adopted a pulse-chase approach, which allowed us to observe effects on secretion of pre-labelled packaged proteins as they are transported through the later stages of the secretory pathway to the plasma membrane. Cells in suspension were incubated for 1 hour in the presence of [^{35}S]methionine (20 μCi/ml) to allow labelling of newly synthesized proteins and some packaging into secretory vesicles. Over this time very little

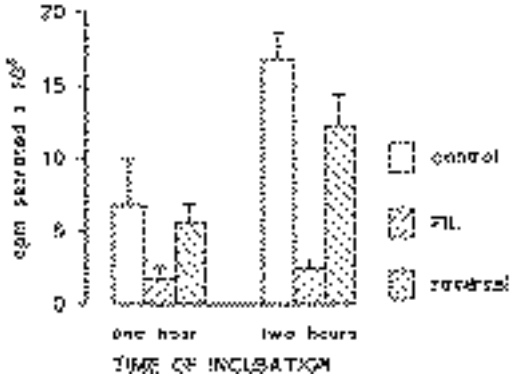

Fig. 4. The inhibition of protein secretion by FIL is reversible. Mammary cells were incubated with or without 8 μg/ml FIL for 2 hours. A portion of the FIL-treated cells were washed and incubated in its absence (reversal). Protein secretion over the following 1 or 2 hours was determined by labelling with [^{35}S]methionine and measurement of medium TCA-precipitable counts. Data is shown as mean ± s.e.m. (n=4).

labelled protein is secreted due to the 1 hour lag. Cells were then washed with ice-cold buffer and incubated with or without FIL (8 μg/ml) for 3 hours at 37°C. Secretion of pulse-labelled protein occured mainly within the first hour of the chase with no lag in control cells as expected. The effect of FIL was to reduce the rate of protein secretion (Fig. 5a). The extent of this inhibition from a series of four cell preparations was 50.3% ± 6.3 at the 3 hour time point. One explanation for there being a partial inhibition of secretion in this protocol could be that the effects of FIL on the cells had a slow onset so that much of the labelled proteins would have passed through the secretory pathway and been released into the medium before FIL could take effect. In an attempt to overcome such a potential problem the cells were pulsed with [^{35}S]methionine for 1 hour to pre-label throughout the secretory pathway, and then preincubated at 4°C for 1 hour to allow time for binding of FIL to the cells whilst slowing secretion to a minimal rate. Cells were then warmed back to 37°C for 2 hours. In this type of protocol incubation at 4°C produced some microtubule dissassembly (Rennison et al., 1992) and may explain why control secretion at 37°C was slower than expected and not complete within 1 hour as for straightforward pulse-chase experiments (Fig. 5a). The results again indicated partial inhibition of secretion by FIL (Fig. 5b).

Regulated exocytosis is unaffected by FIL

In order to test FIL's effect on regulated exocytosis we exploited our previous finding that some of the synthesized caseins can rapidly be released 2-3 hours after synthesis via a Ca^{2+}-regulated pathway (Turner et al., 1992). Treatment of pulse-labelled cells in a protocol similar to that for Fig. 5 was adopted. Secretion of [^{35}S]methionine-labelled proteins was complete within 1 hour after pulse-labelling. Therefore, we examined the extent of secretion between 2 and 3 hours after pulse-labelling. Over this period we would not expect any further constitutive secretion from control cells and this was indeed the case (Fig. 6). In contrast, addition of the Ca^{2+} ionophore ionomycin over this period should stimulate a burst of regulated secretion (Turner et al., 1992). Ionomycin did increase protein secretion from both control and FIL-treated cells (Fig. 6). There was no significant difference between control and FIL-treated cells in the absence or presence of ionomycin and therefore FIL did not affect regulated protein secretion.

Relationship between inhibition of synthesis and secretion

The inhibition of both synthesis and secretion by FIL could be due to independent or related actions of the peptide. The effect of cycloheximide was examined to test whether the inhibition of protein secretion during the pulse-chase protocol could have occurred due to inhibition of synthesis of proteins required for continued transport. A dose of cycloheximide (25 μg/ml) found to inhibit protein synthesis by around 70% had no effect on secretion of [^{35}S]methionine-labelled protein when present during the chase period (34.9±0.9% and 35.9±1.1% of total incorporated cpm released from control and cycloheximide-treated cells, respectively). Therefore, as found for other cell types (Brion et al., 1992; Wieland et al., 1987) continued protein synthesis is not required for constitutive secretion. We also examined the possibility that the inhibition of synthesis may have occurred as a consequence of a block in protein transport through the secretory pathway. The drug brefeldin A (BFA) is known to block transport from the ER to the Golgi (Misumi et al., 1986; Lippincott-Schwartz et al., 1989) and it was found that 5 μg/ml BFA inhibited both the extent of secretion from mammary cells and protein synthesis by 83±3.9% and 54±1.4% (n=4), respectively, apparently mimicking the effects of FIL. An inhibitory effect of BFA on protein synthesis has also been reported for other cell types (Fishman and Curran, 1992).

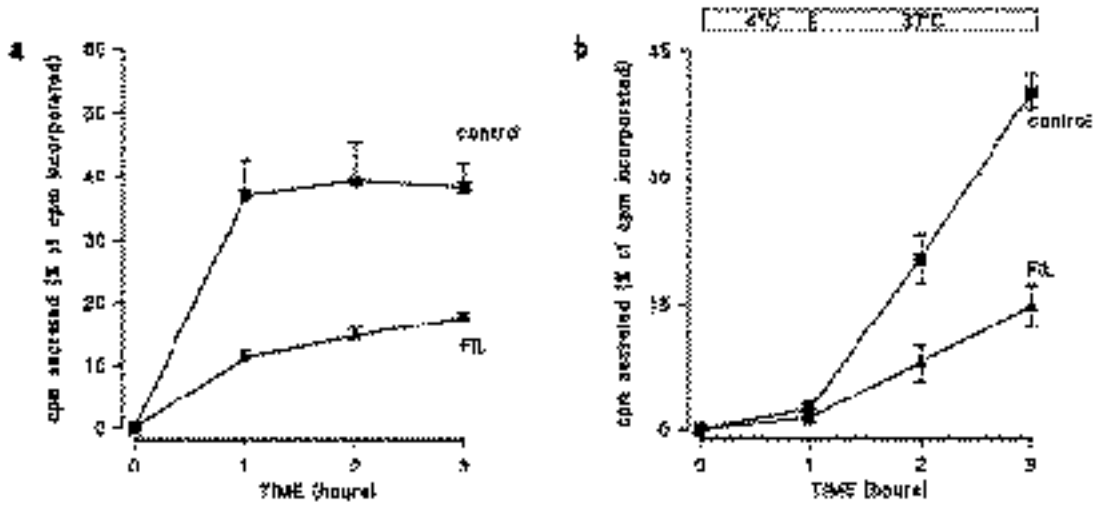

Fig. 5. Effect of FIL on secretion of preformed protein in a pulse-chase protocol. Mammary cells were pulse labelled with [^{35}S]methionine for 1 hour, then washed and either incubated with or without FIL (8 μg/ml) at 37°C (a) or preincubated for 1 hour at 4°C with or without FIL before warming to 37°C (b). The extent of protein secretion was determined expressed as the percentage of synthesized protein secreted and shown as mean ± s.e.m. (a), n=4; (b), n=5.

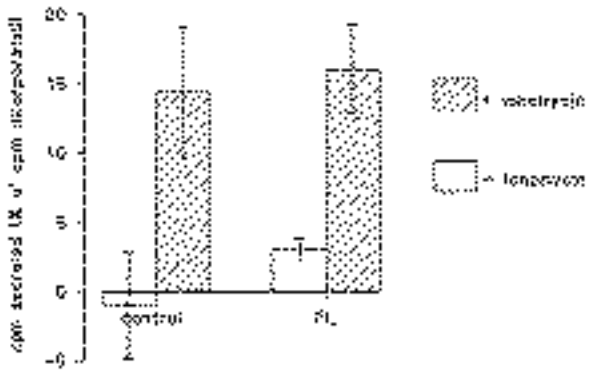

Fig. 6. Effect of FIL on regulated exocytosis of labelled protein. Mammary cells were pulse-labelled with [^{35}S]methionine for 1 hour at 37°C and then incubated with or without 8 μg/ml FIL. Constitutive secretion had terminated after a further 2 hour incubation and the data shows secreted label between 2 and 3 hours after pulse-labelling. Essentially no secretion was seen in the absence of ionomycin. Some batches of cells were treated with ionomycin (10 μM) for the last hour of incubation to stimulate a burst of regulated secretion. The percentage of total synthesized protein secreted over the last hour in control and ionomycin-treated cells was determined and shown as mean ± s.e.m. (n=4).

FIL treatment results in morphological changes in the ER and TGN

If FIL acts by disruption of movement through the secretory pathway at the level of ER-to-Golgi or intra-Golgi traffic then FIL treatment should result in morphological changes in the ER and/or the Golgi complex. Many of the available antibodies against Golgi proteins do not work on mouse cells but using the *trans*-Golgi network (TGN) marker anti-TGN38 (Luzio et al., 1990) we observed that FIL resulted in dispersal of the TGN from its normal perinuclear localisation (Fig. 7). At the electron microscope level it was clear that FIL treatment resulted in a marked change in the morphology of the ER. Lactating mammary cells contain extensive amounts of regularly arranged ER (Fig. 8a). After FIL treatment the ER was clearly modified in almost all cells examined. The regular parallel arrangement of ER elements was lost and individual ER elements appeared vesiculated or swollen (Fig. 8b). We saw similar changes in the ER after blocking transport through the secretory pathway by treatment with 5 μg/ml BFA for 2 hours whereas another inhibitor of the secretory pathway, monensin, was without effect (not shown). Swelling of the ER has previously been seen in BFA-treated cells and suggested to be due to accumulation of secretory proteins in the ER (Hendricks et al., 1992; Hidalgo et al., 1992). Changes in the ER were detectable within 1 hour of treatment with FIL. A similar situation was seen in cells examined from two separate cell preparations and these morphological changes were fully reversible.

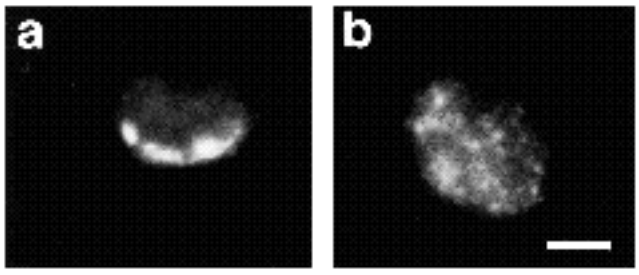

Fig. 7. FIL treatment results in a dispersal of TGN. Mammary cells were incubated for 3 hours in control conditions (a) or with 8 μg/ml FIL (b), fixed and stained with anti-TGN38. The TGN became fragmented and dispersed after treatment with FIL. Bar, 10 μm.

DISCUSSION

Earlier work identified a secreted milk protein (FIL) that acts as a feedback inhibitor of lactation (Wilde and Peaker, 1990; Wilde et al., 1987, 1988, 1989, 1990). FIL is synthesised in mammary epithelial cells (C. J. Wilde et al., unpublished observations), inhibits synthesis of casein and lactose in explant cultures (Wilde et al., 1987), reduces milk output in vitro (Wilde et al., 1987, 1989) and in vivo (Wilde et al., 1988; Wilde and Peaker, 1990) and stimulates the intracellular degradation of newly synthesised casein (Wilde et al., 1989). FIL has been purified, so far, from goat and bovine milk and antibody neutralization experiments have shown that it functions as a feedback inhibitor in vivo (C. J. Wilde et al., unpublished observations). We have determined whether purified FIL has any effect on constitutive or regulated exocytotic release of milk proteins. The data presented here show that FIL inhibits protein synthesis and also directly inhibits constitutive secretion. Exocytosis was not inhibited since the Ca^{2+} ionophore ionomycin still elicited a burst of regulated secretion. In addition, lipid (triglyceride) secretion was unaffected. One interpretation of these data is that FIL blocks an early stage of the secretory pathway necessary for constitutive secretion. FIL was identified and isolated on the basis of its ability to inhibit protein synthesis in mammary tissue and the range of cell types sensitive to FIL is yet to be determined.

FIL has several effects on mammary cells including inhibition of protein synthesis and secretion and morphological changes of the TGN and ER. One important consideration is whether FIL acts at multiple sites in mammary epithelial cells or whether its various effects result from a single site of action. One possibility is that it acts primarily on membrane traffic necessary for transport of protein from the ER to the Golgi and through the Golgi complex. Such an effect could explain the inhibition of protein synthesis. Disruption of transport from the ER by a variety of treatments including depletion of ER Ca^{2+} using ionomycin, results in inhibition of translation, suggesting that accumulation of protein in the ER is able to directly block protein synthesis (Kuznetsov et al., 1992). This interpretation would explain the inhibition of both constitutive secretion and protein synthesis by FIL. It could explain the increase in casein degradation in explants treated with FIL (Wilde et al., 1989) since depletion of ER Ca^{2+} and inhibition of protein transport from the ER is known to result in increased protein degradation in the ER (Wileman et al., 1991). An effect of FIL on vesicular transport could also explain its morphological effects on the TGN and ER. It is

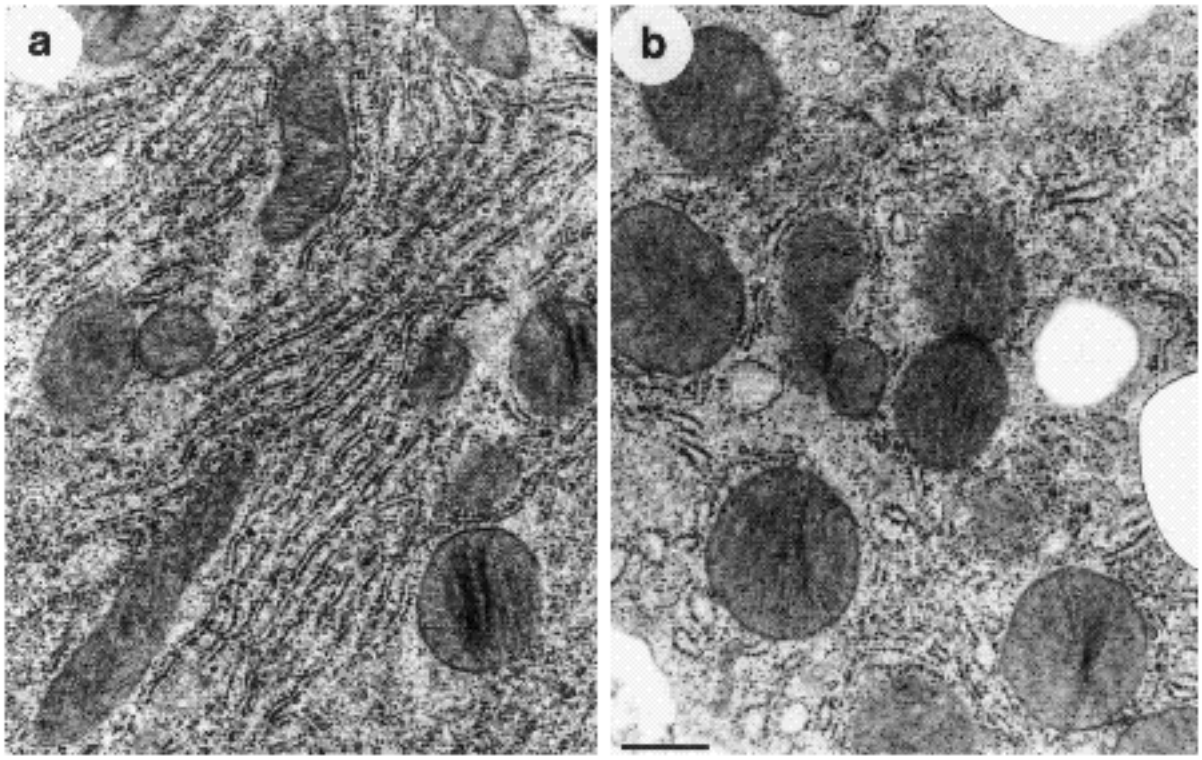

Fig. 8. FIL treatment modifies the morphology of the ER in mammary cells. Mammary cells were incubated without (a) or with (b) 8 μg/ml FIL for 3 hours. In control cells the extensive ER network formed regular parallel arrays. In cells treated with FIL the regular arrangement of the ER was lost and ER elements appeared swollen or vesiculated. Bar, 400 nm.

also possible that FIL disrupts several aspects of vesicular traffic since, in addition to the effects reported here, treatment with FIL reduced the number of prolactin receptors on the surface of isolated mammary cells (Bennett et al., 1990; Wilde et al., 1990) consistent with an inhibition of receptor recycling to the cell surface. In support of the idea that FIL inhibits protein synthesis as a consequence of a block on protein transport we found that BFA, which blocks ER-to-Golgi transport (Misumi et al., 1986; Lippincott-Schwartz et al., 1989; Orci et al., 1991), inhibits protein synthesis in mammary cells as well as secretion and mimicked the effect of FIL on the morphology of the ER. Since FIL also inhibited RNA synthesis we cannot rule out the possibility that it has a number of distinct effects on mammary cell function.

An additional explanation for the inhibition of constitutive secretion by FIL that can be eliminated is that its primary effect is on protein synthesis and that secretion required the continued synthesis of essential proteins during movement through the secretory pathway. In HepG2, CHO (Wieland et al., 1987) and AtT-20 (Brion et al., 1992) cells, continued protein synthesis is not required for constitutive secretion and a similar conclusion can be reached for mammary cells from the use of the protein synthesis inhibitor cycloheximide, which did not reduce secretion when present during a 1 hour chase period after labelling.

In the experiments reported here FIL never gave more than a partial inhibition of synthesis and secretion. The reasons for this are unclear. One possibility is that FIL disrupts some aspect of the secretory pathway that does not result in a complete block but allows some protein to move through the pathway. Consistent with this idea is the finding that, after allowing enough time for proteins to accumulate in regulated casein secretory vesicles, secretion in response to ionomycin was unimpaired in FIL-treated cells. The possibility that the partial inhibition may reflect some heterogeneity in the responsiveness of the isolated cells in the population to FIL could be ruled out due to its consistent effect on ER morphology seen by electron microscopy. In some experiments we preincubated cells with FIL at 4°C in an attempt to prebind the peptide to improve any subsequent inhibitory effect on secretion. We cannot be certain, however, that FIL binding was effective at this temperature. An additional explanation for the partial inhibition by FIL arises from the possibility that intra-Golgi transport can result from alternative vesicular or non-vesicular pathways (Orci et al., 1991). FIL could be acting by inhibiting only one of these pathways.

Constitutive secretion occurs, by definition, in the absence of any stimulatory extracellular or intracellular stimulus. The data presented here and other recent works question the concept of a fully constitutive pathway and suggest that the constitutive pathway may be regulated in some circumstances. Movement of secretory molecules through the constitutive pathway is inhibited by GTPγS (Melancon et al., 1987; Helms et al., 1990; Miller and Moore, 1991) and is regulated by trimeric G-proteins (Barr et al., 1991; Stow et al., 1991; Donaldson et al., 1991) and protein phosphorylation (Lucocq et al., 1991; Davidson et al., 1992). The machinery thus exists in the constitutive

pathway for negative regulation of secretion. The pathway by which FIL acts is unknown but does not appear to involve pertussis or cholera toxin-sensitive G-proteins. With the exception of mitosis when constitutive secretion and other vesicular transport events are inhibited (Featherstone et al., 1985; Kreiner and Moore, 1990) there has been little evidence for situations in which the constitutive pathway is regulated. Our data on mammary epithelial cells provide an example of physiologically relevant inhibitory control of the constitutive pathway for protein secretion.

This work was supported by a Link Research Grant from the Agricultural and Food Research Council to R.D.B. and C.J.W. and in part by the Scottish Office of the Agricultural and Fisheries Department (C.J.W). M.D.T. was supported by a research studentship from the Agricultural and Food Research Council. We thank Dr J. P. Luzio for the gift of anti-TGN38.

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(Received 19 May 1993 - Accepted 18 June 1993)