Autocrine regulation of milk secretion by a protein in milk

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Frequency or completeness of milk removal from the lactating mammary gland regulates the rate of milk secretion by a mechanism which is local, chemical and inhibitory in nature. Screening of goat's milk proteins in rabbit mammary explant cultures identified a single whey protein of M_r 7600 able to inhibit synthesis of milk constituents. The active whey protein, which we term FIL (Feedback Inhibitor of Lactation), also decreased milk secretion temporarily when introduced into a mammary gland of lactating goats. FIL was synthesized by primary cultures of goat mammary epithelial cells, and was secreted vectorially together with other milk proteins. N-terminal amino acid sequencing indicated that it is a hitherto unknown protein. The evidence indicates that local regulation of milk secretion by milk removal is through autocrine feedback inhibition by this milk protein.

INTRODUCTION

The mammary gland is one of the few exocrine glands to store its secretion extracellularly. This extracellular storage, within the lumen of the gland, imposes a local level of control on the rate of milk secretion, in addition to the systemic stimulation exerted by galactopoietic hormones. Removal of milk from the gland by suckling, or milking of dairy animals, regulates milk secretion acutely, within hours, through an intra-mammary mechanism which responds to both the frequency and completeness of milk removal [1,2]. Thus the rate of milk secretion changes unilaterally when one gland of goats or cows is milked more frequently or less frequently than the other [1,3–5]. In the same way, recent studies show that breastfeeding mothers regulate milk secretion in each breast independently, according to the proportion of stored milk taken by the infant at each feed [2]. Other studies in lactating goats have been entirely compatible with the hypothesis [1] that the regulatory mechanism involves a chemical inhibitor of milk secretion and is not related to the physical presence of stored milk. The secretory rate is increased when milk is removed at an extra daily milking, even when the milk is replaced immediately by an inert solution to maintain the gland's distension [6]. On the other hand, dilution of stored milk with an inert isotonic solution increases the rate of milk secretion, an effect compatible with dilution of a chemical inhibitor [7].

Explants of mammary tissue in organ culture have been used to confirm the presence of a putative feedback inhibitor in milk. Milk fractions were tested for inhibition of milk synthesis by using explants from mid-pregnant rabbits, which synthesize milk constituents when cultured in the presence of lactogenic hormones [8]. The tissue-explant bioassay showed initially that goat's milk whey proteins, but not caseins (the most abundant milk proteins), inhibited synthesis of both casein and lactose in a dose-dependent manner [9]. Inhibition was rapid and readily reversible. Ultrafiltered whey fractions with constituents of nominal $M_r > 10000$ all inhibited in the bioassay, except for one containing material of $M_r > 30000$. We have therefore used a M_r-6000–30000 fraction of whey proteins to search further for a milk protein able to inhibit milk synthesis.

EXPERIMENTAL

Animals and materials

Mid-pregnant New Zealand White and Dutch rabbits were from Hi-Line, Lymm, Cheshire, U.K. The sources of radiochemicals, hormones and general reagents were as described previously [9]. Culture media were from Gibco BRL, Paisley, Scotland, U.K. Reconstituted basement membrane (Matrigel) was from Universal Biologicals, London, U.K. Pluronic L121 was from ICI Chemicals, Runcorn, Cheshire, U.K.

Fractionation of milk proteins

Fresh goat's milk was mixed with protease inhibitors [2 mM phenylmethanesulphonyl fluoride (PMSF), 15 mM ϵ-aminohexanoic acid], defatted by centrifugation at 800 *g* for 20 min at 10 °C, and the infranatant was centrifuged at 50000 *g* for 2 h at 10 °C to produce a casein pellet and a whey supernatant. Whey was filter-sterilized and ultrafiltered using a M_r-30000 cut-off membrane (Millipore). The filtrate was dialysed against water (Spectropor-1; Pierce and Warriner) and freeze-dried. The M_r-6000–30000 fraction was redissolved in 20 mM Bistris-propane, pH 7.0, applied to a HR 16/10 Mono Q column (Pharmacia), and bound proteins were eluted with a 0–200 mM gradient of sodium acetate in the same buffer. Eight fractions were collected on the basis of the A_{280} elution profile, dialysed overnight against water, freeze-dried, and stored at −20 °C for testing in explant culture bioassays. The fractions designated 2, 3 and 4 were redissolved at 50–100 μg/ml in 25 mM piperazine/HCl, pH 5.5, and, after filtration through a 0.2 μm-pore filter, were applied individually to a Mono P column (Pharmacia) equilibrated in the same buffer. Bound proteins were eluted with a pH gradient of 5.5–4.0 formed with 10% (v/v) Polybuffer 74, pH 4.0 (Pharmacia). Fractions 3.1–3.3 identified from the A_{280} elution profile were dialysed, freeze-dried and stored at −20 °C until bioassay. Alternatively, anion-exchange fraction 3 was redissolved in Bistris-propane, and anion-exchange chromatography was repeated.

Abbreviations used: PMSF, phenylmethanesulphonyl fluoride; FIL, feedback inhibitor of lactation.

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Chromatofocused peak 3.2 was subjected to reversed-phase h.p.l.c. on a Gilson 302 system using a PLR-S column (Polymer Laboratories, Church Stretton, Shropshire, U.K.) equilibrated in 0.1% (v/v) trifluoroacetic acid. Protein was eluted with a linear 0–35% acetonitrile gradient in 0.1% trifluoroacetic acid, and a single protein peak that was eluted close to the void volume was dried by rotary evaporation and reconstituted in 1% trifluoroacetic acid for gas-phase amino acid sequencing.

Freeze-dried peak 3, or peak 3 reprocessed by anion-exchange chromatography, was reconstituted in 50 mM Tris/HCl, pH 7.5, containing 100 mM KCl and subjected to gel filtration on a Superose 12 HR 10/30 column. The column was calibrated by using M_r standards, and the M_r of eluted material was calculated from plots of log M_r versus V_e/V_0, where V_0 is the void volume and V_e the elution volume of each protein.

Bioassay of milk proteins

Anion-exchange and chromatofocusing fractions were tested for their ability to inhibit casein and lactose synthesis in a rabbit mammary organ-culture bioassay, as described previously [9]. Briefly, groups of mammary explants (30 explants/group, 3–4 groups/treatment) from mid-pregnant New Zealand White rabbits were cultured in Medium 199 containing 5 μg of insulin/ml, 1 μg of cortisol/ml and 1 μg of prolactin/ml for 48 h, with milk fractions added at once or twice their original milk concentration (v/v) for the final 6 h. Inclusion of milk fractions had a negligible effect on protein concentration in the medium: for example, the concentration of anion-exchange peak 3 in bioassay medium was 0.98 μg/ml. Average rates of lactose and casein synthesis over the 6 h period were measured by incorporation of [U-^{14}C]glucose (0.18 mCi/mmol) and L-[4,5-^{3}H]leucine (2.22 mCi/mmol) respectively. At the end of culture, the medium was removed and the explants were blotted, weighed and stored in liquid N_2 for subsequent analysis. Explants were homogenized in 10 mM Tris/HCl, pH 7.0, containing 5 mM EGTA and 2 mM PMSF by 10 strokes with a glass/Teflon homogenizer, followed by sonication for 30 s (setting 30, Kontes KT50 cell disrupter). ^{3}H-labelled casein was isolated from the homogenate by preparation of a particle-free supernatant (10000 *g*, 5 min, 4 °C), isoelectric precipitation and SDS/PAGE [10]. [^{14}C]Lactose was measured by selective precipitation from culture medium and explant homogenates.

Antiserum production

Polyclonal antisera against anion-exchange peak 3 and chromatographed peak 3.2 were raised in female New Zealand White rabbits by subcutaneous injection of 100 μg of protein dissolved in PBS, pH 7.6, and emulsified with complete Freund's adjuvant. In the case of peak 3.2, protein was conjugated to keyhole-limpet haemocyanin by glutaraldehyde treatment [11]. A second injection of protein in incomplete Freund's adjuvant was given 4 weeks later, and serum collected 7 and 14 days thereafter was tested for antibody titre and specificity. A third polyclonal antiserum was raised against a bovine inhibitor of milk synthesis, which was isolated from cow's milk by anion-exchange f.p.l.c. and chromatofocusing, and identified by mammary-explant bioassay [12]. The inhibitory protein (100 μg) was coupled to keyhole-limpet haemocyanin by glutaraldehyde treatment, emulsified with 2.5% (v/v) Pluronic L121, 5% (w/v) squalene and 0.2% (v/v) Tween 80 in PBS, pH 7.4, and injected subcutaneously into a female Dutch rabbit. The injection was repeated twice at 4-weekly intervals, and serum collected 10 days after each injection was tested by non-competitive e.l.i.s.a. for antibody titre and specificity for goat FIL (Feedback Inhibitor of Lactation).

To test antibody specificity, e.l.i.s.a. plates were coated with 0–1.0 μg of anion-exchange peaks 2, 3 and 4 or chromatofocused peaks 3.1, 3.2 and 3.3, each dissolved in 100 μl of PBS, by incubation overnight at 4 °C. The wells were washed three times with PBS containing 0.1 % Tween 20, blocked with 5% BSA in the same buffer, and, after further washing, incubated at 40 °C for 2 h with 100 μl of antiserum diluted 1:500 in PBS. After repeated washing, binding was determined by sequential incubation with peroxidase-conjugated anti-rabbit IgG (Scottish Antibody Production Unit, Carluke, Scotland, U.K.) and *o*-phenylenediamine substrate (Sigma).

Protein glycosylation

Anion-exchange peak 3 was tested for the presence of oligosaccharide by hexose assay [13] and by detection of bound sialic acid residues using the acidic ninhydrin reaction [14]. Peak 3 was deglycosylated by treatment with trifluoromethanesulphonic acid [15]. Enzymic deglycosylation proved unsuccessful, under conditions [16] in which oligosaccharide was cleaved effectively from α_1-glycoprotein and κ-casein standards.

Protein sequencing

The inhibitory protein in anion-exchange peak 3 (termed FIL) was purified for protein sequencing by re-running on anion-exchange chromatography, and gel filtration on a Superose 12 HR 10/30 column (Pharmacia) to remove minor low-M_r contaminants. The protein was dried on a poly(vinylidene difluoride) (PVDF) membrane using a Prospin sample-preparation cartridge (Applied Biosystems). Sequencing was performed on an Applied Biosystems 471A gas-phase sequencer with on-line phenylthiohydantoin-amino acid analyser.

FIL detection in goat mammary-cell cultures

Mammary cells were prepared by collagenase digestion of tissue from pregnant or lactating goats [17] and fractionated by density-gradient centrifugation [18] to obtain an epithelial-cell-rich fraction. Lactating cells were cultured for 3 h suspended in Ham's F12/Medium 199 (1:1, v/v) containing 5 μg of insulin/ml, 100 ng of cortisol/ml and 1 μg of prolactin/ml. Cells were harvested by centrifugation and lysed by sonication for 30 s in 50 mM Tris/HCl, pH 8.0, containing 150 mM NaCl, 1% (v/v) Nonidet P40, 1 mM EDTA and 1 mM PMSF. A particle-free supernatant prepared by centrifugation (10000 *g*, 4 °C, 10 min) was run on SDS/12%-polyacrylamide gels [19] alongside whey fractions, anion-exchange peak 3 or peak 3 subjected to gel filtration. Cells from late-pregnant goats were cultured for 5 days on Matrigel in medium containing insulin, cortisol and prolactin, with 200 μCi/ml [^{35}S]methionine (1.75 mCi/mmol) present for the final 4 h. Culture medium was collected and cells were treated for 20 min with 2.5 mM EGTA to extract lumenal proteins [20]. Culture medium and EGTA-extracted protein was precipitated with trichloroacetic acid (final concn. 5%, w/v) and equal proportions were run on SDS/PAGE under reducing conditions.

Protein bands were revealed by Coomassie Blue or silver staining and dried for fluorography, or blotted electrophoretically in 25 mM Tris, pH 8.3, containing 192 mM glycine, 20% (v/v) methanol and 0.02% SDS on to PVDF membranes (Immobilon P, Millipore). Blots were blocked overnight with 3% (w/v) BSA in PBS and incubated with rabbit anti-(bovine inhibitor) antiserum diluted 1/500 in the same buffer. Antiserum was pre-incubated with 58 μg of purified goat inhibitor/ml to test for

competitive blocking of immunoreactivity. After rinsing, blots were made visible with anti-(rabbit Ig)–alkaline phosphatase conjugate and bromochloroindolyl phosphate/Nitro Blue Tetrazolium substrate (Bio-Rad) according to the manufacturer's instructions. Protein bands were also processed for amino acid sequencing by blotting on to PVDF membrane in 10 mM CAPS [3-(cyclohexylamino)-1-propanesulphonic acid] buffer, pH 11.0, containing 10% (v/v) methanol. After blotting, the membrane was equilibrated for 10 min in water, stained with 0.1% (w/v) Coomassie Blue in 50% (v/v) methanol for 5 min, and destained in several changes of 50% (v/v) methanol/10% (v/v) acetic acid. Protein bands were then excised, rinsed in water, air-dried and stored at −20 °C until required.

Intra-mammary injection of FIL

Inhibitory protein was prepared as the third peak resolved by anion-exchange chromatography of a M_r-6000–30000 fraction of goat whey proteins. Freeze-dried protein was reconstituted in sterile 10 mM Hepes, pH 6.7, containing 0.3 M sucrose, and used to treat five goats, each on three occasions, with doses of 100, 250, 500 or 750 μg of protein. On each occasion, protein was injected through the test duct in 20 ml of iso-osmotic carrier immediately after the afternoon milking, and the contralateral gland received the same volume of carrier solution. The glands were massaged briefly to encourage distribution of the solution through the gland cistern and alveoli. The goats were in weeks 25–35 of lactation, and were milked twice daily at 08:00 and 16:00 h, with time of milking and individual gland yield being recorded. Results for one injection, which coincided with loss of appetite and milk yield in that goat, were not used in subsequent data analysis.

Data analysis

An ipsilateral effect of injected protein on milk secretion in glands of lactating goats was detected by calculation of a relative milk yield quotient (RMYQ) [1]:

$$\text{RMYQ} = t_2c_1/t_1c_2$$

where t_1 and t_2, c_1 and c_2 are the pre- and post-injection daily yields of protein-treated and carrier-treated glands respectively.

RESULTS

Isolation and identification of FIL

Casein and lactose synthesis were inhibited to a similar extent by a protein fraction which was eluted as the third of seven A_{280} peaks during anion-exchange chromatography of the M_r-6000–30000 whey fraction (Figures 1a and 1b). The preceding and succeeding peaks in this fractionation (peaks 2 and 4 respectively) did not inhibit milk-constituent synthesis, nor did peaks 5–7, which promoted lactose synthesis in rat Golgi vesicles containing galactosyltransferase [21], indicating the presence of several active forms of α-lactalbumin [22]. Therefore, since peak 3 exerted a degree of inhibition similar to that of the equivalent concentration of unresolved whey, this protein fraction accounted for all the inhibitory activity in the M_r-6000–30000 whey fraction. The inhibitory component of peak 3, designated peak 3.2, was purified from two minor contaminants on the basis of their differing isoelectric points by chromatofocusing (Figures 2a and 2b). Chromatofocusing also showed that peak 3.2 was a minor component of anion-exchange peak 2, and was absent from peak 4 (Figure 2a), consistent with the specificity of bioassay activity (Figures 1b and 2b). Specific inhibitory activity of goat whey proteins in the tissue-culture bioassay indicated a 40000-fold

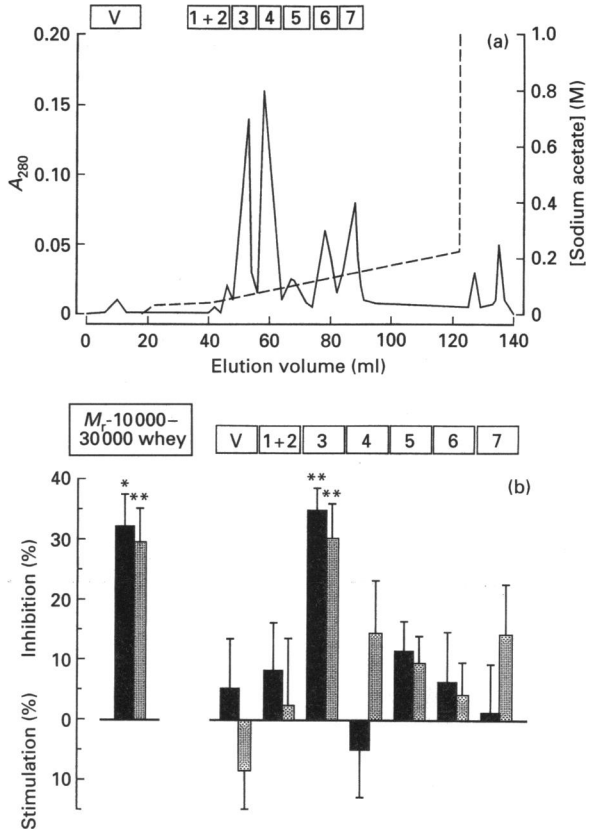

Figure 1 Fractionation and bioassay of goat's milk whey proteins

A M_r-6000–30000 fraction of goat's milk whey proteins was separated by anion-exchange chromatography and tested for their ability to inhibit casein and lactose synthesis in a rabbit mammary-explant culture bioassay. (**a**) Elution profile of a M_r-6000–30000 fraction of goat whey proteins separated by f.p.l.c. (Pharmacia) anion-exchange chromatography using a Mono Q column and sodium acetate gradient. ——, A_{280} elution profile; – – – –, salt gradient. Fractions designated V (void volume) and 1–7 are shown by open boxes. Material eluted at 1 M salt was not present consistently, and if so, contained no protein. (**b**) Bioassay of anion-exchange fractions. Fractions were tested for inhibition of milk-constituent synthesis in rabbit mammary tissue cultures. Effects on casein synthesis and lactose synthesis are shown by black and stippled bars respectively as the percentage inhibition or stimulation produced by these fractions at a concentration equivalent to that in milk. Data are means ± S.E.M. for 9–10 determinations, except fraction 7 ($n = 5$), with statistical significance determined by Student's paired t test and indicated by asterisks: * $P < 0.05$; ** $P < 0.01$.

purification of chromatofocusing peak 3.2 over unfractionated whey (Table 1).

Reversed-phase h.p.l.c. resolved chromatofocused peak 3.2 as a single protein species that was eluted close to the void volume (Figure 3). The position and identity of the eluted protein were confirmed by Bradford protein assay [23] and SDS/PAGE respectively (results not shown). However, chromatofocusing with or without subsequent reversed-phase chromatography appeared to render the protein resistant to conventional gas-phase amino acid sequencing, possibly due to the presence of residual ampholines. Therefore, in order to purify the inhibitory protein for sequence analysis, an alternative strategy was adopted, which involved re-processing of peak 3 by anion-exchange chromatography (Figure 4a), and gel filtration to remove low-M_r contaminants (Figure 4b). This effectively resolved the inactive components of peak 3 (chromatofocused peaks 3.1 and 3.3) which, on the basis of their elution in chromatofocusing, arose from cross-contamination by elements

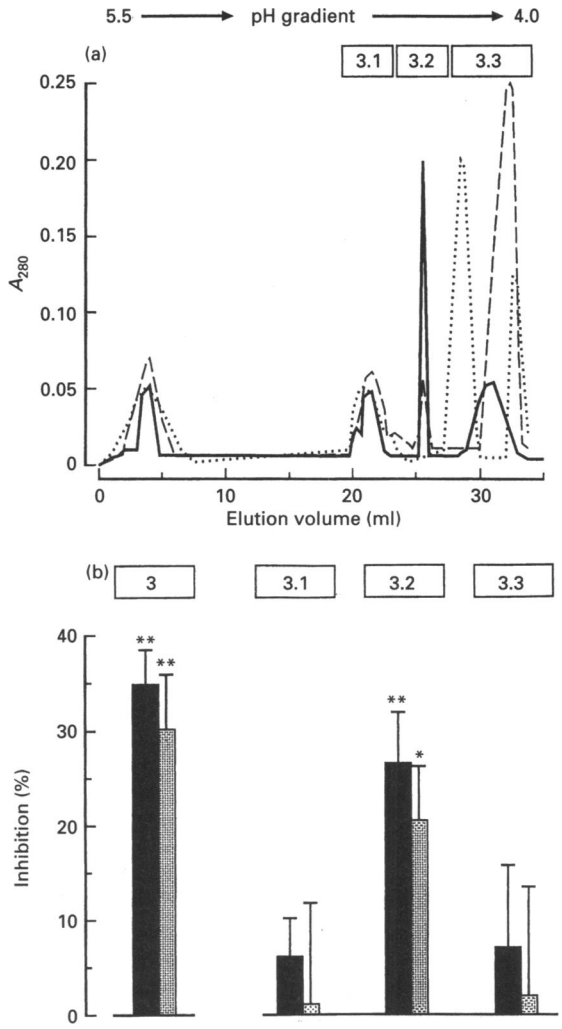

Figure 2 Chromatofocusing and bioassay of anion-exchange-peak 3 components

Anion-exchange peaks 2, 3 and 4 were separated by chromatofocusing, and fractions identified according to the A_{280} elution profile were tested for their ability to inhibit milk-constituent synthesis in a rabbit mammary-explant bioassay. (**a**) Inhibitory fractions 2, 3 and 4 were run on a Mono P column (Pharmacia), and proteins were eluted with a pH gradient of 5.5–4.0. The A_{280} elution profile of peak 3 is shown by ——, and its components, designated 3.1–3.3, are indicated by open boxes. The elution profiles of fractions 2 and 4 are shown by – – – – and · · · · · · respectively. (**b**) Bioassay of peak-3 chromatofocusing fractions. Data are shown as means ± S.E.M. ($n = 7$) and are expressed as the percentage inhibition of casein synthesis (black bars) and lactose synthesis (stippled bars) by fractions 3.1–3.3 added to culture medium at twice their milk concentration. Statistical significance by t test: * $P < 0.05$; ** $P < 0.01$.

Table 1 Specific activity of the inhibitory protein in goat whey fractions

Goat's milk protein fractions were tested for their ability to inhibit milk-constituent synthesis in rabbit mammary-explant tissue cultures. Each fraction inhibited casein synthesis and lactose synthesis to the same degree, so specific inhibitory activity is expressed as a calculated mean of the effect on these two parameters.

Milk fraction	Specific activity (% inhibition/μg of protein)	Purification (fold)
Whey: total	0.002	1
Whey: M_r-6000–30000 fraction	0.138	69
Peak 3	34.2	16847
Peak 3.2	80.1	39458

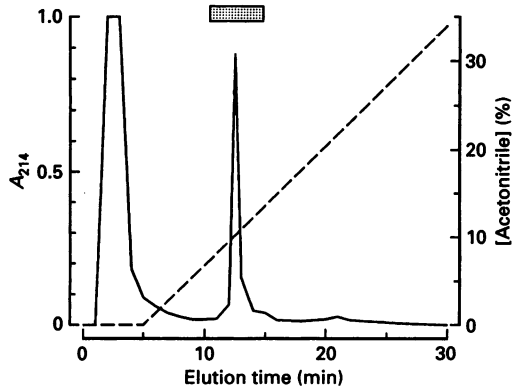

Figure 3 Reversed-phase h.p.l.c. of chromatofocused peak 3.2

Chromatofocused peak 3.2 was subjected to reversed-phase h.p.l.c. as described in the Experimental section. ——, A_{214} elution profile; – – – –, acetonitrile gradient. Eluted material was tested for protein by the Bradford method, and its elution position is shown by a stippled bar.

of peaks 2 and 4 (Figure 2a). N-terminal analysis of the inhibitory protein produced the consensus amino acid sequence:

Ala-Pro-Pro-Phe-Glu-Arg-Asn-Ser-Pro-Gly-Arg-Leu-

A 50–60% yield of phenylthiohydantoin-amino acid in initial sequencing cycles indicated that the sequence represented that of the principal protein constituent. However, deteriorating signal strength in subsequent cycles prevented firm identification of additional amino acid residues. Searching of Swissprot and OWL protein-sequence databases with this amino acid sequence revealed no homology with other known milk proteins, or with any known protein. On this basis, and in view of its bioassay activity and inhibition of milk secretion *in vivo* (see below), the protein has been termed a feedback inhibitor of lactation (FIL).

Chromatofocused peak 3.2, like peak 3, was eluted on gel filtration as a single protein species of M_r 7600 (Figure 4b). In contrast, peak 3 and gel-filtered peak 3 migrated on SDS/PAGE under reducing conditions with an apparent M_r of 66000 (Figure 5). N-terminal sequencing confirmed that this high-M_r protein band was indeed the inhibitory protein, a conclusion supported by immunoblotting using specific antiserum (see below). This anomalous behaviour on SDS/PAGE may be due to the protein's oligosaccharide content. Assays for hexose and sialic acid showed that FIL is a glycoprotein, and chemical deglycosylation of peak 3 with trifluoromethanesulphonic acid [15] before SDS/PAGE under reducing conditions eliminated the high-M_r band, so that the protein migrated as a diffuse band of $M_r \sim 7000$ (Figure 5). Deglycosylation of FIL by trifluoromethanesulphonic acid treatment was monitored by elimination of erythroglutinin PHA-E lectin binding [24] and hexose-positive reaction, and by a decrease in its M_r on gel filtration of approx. 1000 (Figure 4b). Other milk glycoproteins also exhibit anomalous behaviour on SDS/PAGE. Murine and bovine κ-casein migrate as a single band at twice their M_r as predicted from amino acid analysis [25,26]. The milk-fat-globule membrane acidic glycoprotein butyrophilin migrates in purified form as a series of anomalously high M_r bands on SDS/PAGE [27], although whether this is attributable to the protein's oligosaccharide content or to the presence of tightly

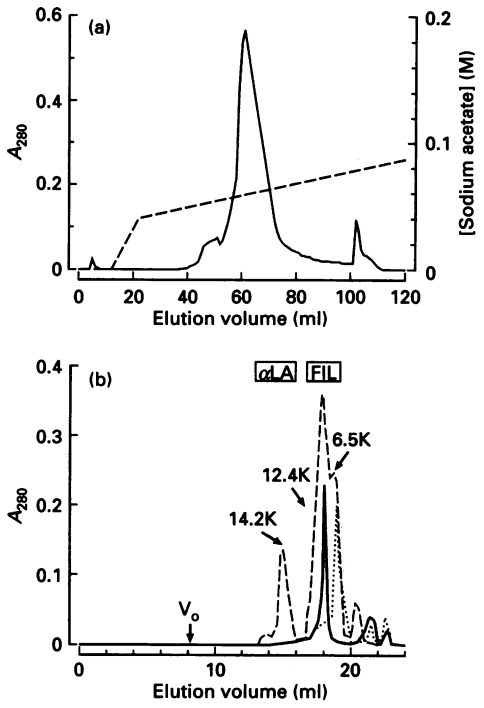

Figure 4 Re-processing of anion-exchange peak 3 for protein sequencing

Freeze-dried anion-exchange peak 3 was re-processed by anion-exchange chromatography using a Mono Q column and sodium acetate gradient, and subjected to gel filtration on a Superose 12 HR column for protein sequencing as described in the Experimental section. (**a**) Anion-exchange chromatography of peak 3. ——, A_{280} elution profile; – – –, salt gradient. (**b**) Gel filtration of reprocessed peak 3. The column was calibrated by using M_r standards (e.g. 6.5 K = M_r 6500), indicated by arrows. ——, Elution profile of reprocessed peak 3; – – –, that of M_r-6000–30000 whey; · · · · · ·, that of deglycosylated peak 3. Whey peaks corresponding to α-lactalbumin (αLA) and the inhibitory protein (FIL) are shown in open boxes.

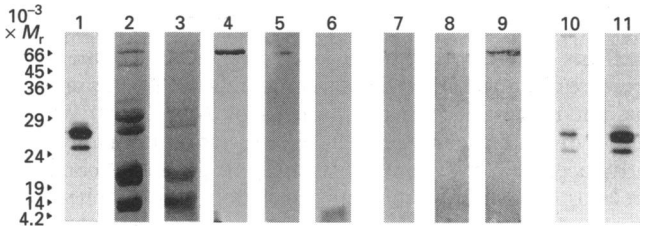

Figure 5 Synthesis of inhibitory protein (FIL) by goat mammary epithelial cells

FIL was detected in lactating-goat mammary epithelial cell suspensions and pregnant-goat mammary cells cultured on reconstituted basement membrane by immunoblotting using a specific polyclonal antiserum. The antiserum was raised in rabbits against a protein with similar inhibitory activity identified in cow's milk [11], and cross-reacted specifically with goat FIL in whey fractions and purified preparations. Lanes 1–6, SDS/PAGE of goat caseins (lane 1), whey proteins (lane 2), M_r-6000–30000 whey protein fraction (lane 3), anion-exchange peak 3 (lane 4) and gel-filtered peak 3 (lane 5) and deglycosylated gel-filtered peak 3 (lane 6). Lanes 7–9, immunoblot detection of inhibitory protein in M_r-6000–30000 whey fraction (lane 7), in proteins secreted into culture medium by goat mammary epithelial cells on reconstituted basement membrane (lane 8), and in an EGTA extract of cells *in situ* on reconstituted basement membrane (lane 9). Lanes 10 and 11, fluorography of [^{35}S]methionine-labelled proteins secreted into culture medium of cells on basement membrane (lane 10) and in an EGTA extract of cells *in situ* (lane 11).

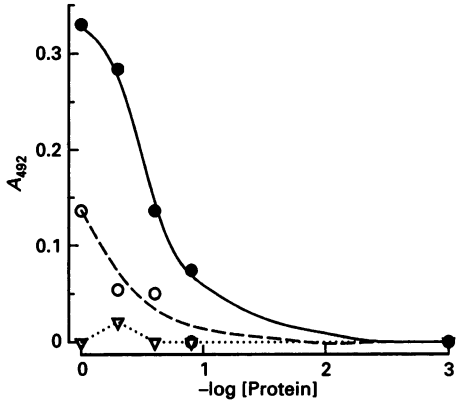

Figure 6 Non-competitive e.l.i.s.a. of antiserum raised against an inhibitory bovine milk protein for binding to goat whey proteins

A rabbit polyclonal antiserum against a bioassay-positive cow's milk protein [11] was tested for binding to goat anion-exchange peaks 2, 3 and 4. Peroxidase-conjugated anti-rabbit IgG and *o*-phenylenediamine substrate was used to measure antiserum binding to immobilized peak 2 (○), peak 3 (●) and peak 4 (▽).

bound lipid is not clear. The present data do not exclude the possibility that post-translational modification other than glycosylation may contribute to FIL's anomalous behaviour on SDS/PAGE and be eliminated by treatment with trifluoromethanesulphonic acid.

Synthesis of FIL by mammary epithelial cells

Polyclonal antiserum raised against peak 3 partly reversed peak-3 inhibition of lactose synthesis (from 19% to 9%) and casein synthesis (from 39% to 21%) when included at an arbitrary concentration in two culture experiments, indicating that it recognized the active protein species subsequently purified as chromatofocused peak 3.2. However, this polyclonal antiserum also cross-reacted with anion-exchange peaks 2 and 4 in non-competitive e.l.i.s.a. (results not shown). One of the chromatofocused components of peak 3 (3.1) appeared to be present in peaks 2 and 4 (as 2.1 and 4.1 respectively), which may in part explain this cross-reactivity (Figure 2a). On the other hand, peaks 2 and 4 were also recognized in e.l.i.s.a. by polyclonal antiserum raised against chromatofocused peak 3.2. The antiserum recognized only component 3.2 of peak 3, but nevertheless bound constituents of peaks 3 and 4. This, and the elution of peaks 2 and 4 with M_r 7600 on gel filtration (results not shown), similar to that of peak 3, suggests that these peaks may contain structurally related but inactive forms of the inhibitory protein.

In view of the cross-reactivity of anti-goat antibodies, FIL was detected in goat mammary cell cultures by immunoblotting using antiserum raised against a protein from cow's milk which has similar activity [12]. This antiserum was specific for goat anion-exchange peak 3 when tested in non-competitive e.l.i.s.a. (Figure 6). It also detected a single protein band in immunoblots of unfractionated whey (Figure 5), a reaction which was competed by addition of peak 3 during antibody incubation (results not shown). In culture medium and cell extracts, as with unfractionated whey (Figure 5) and purified FIL, immunoblotting detected the goat inhibitor as a band migrating with an apparent M_r of 66000, consistent with Coomassie Blue and silver staining of purified FIL on SDS/PAGE (Figure 5). This antiserum also detected deglycosylated FIL on SDS/PAGE as a low-M_r band (Figure 5), indicating that the antiserum is specific for the

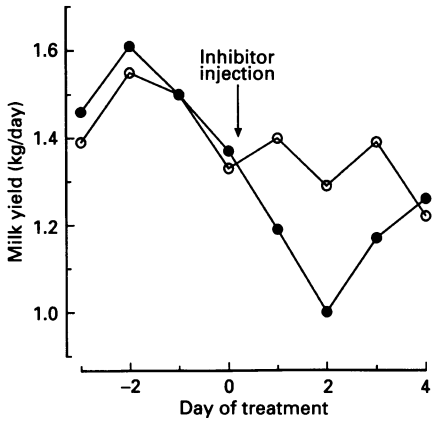

Figure 7 Inhibition of milk secretion in lactating goats by intra-ductal FIL injection

Lactating goats milked twice daily were treated in one gland with 100–750 μg of FIL after the afternoon milking. FIL was prepared by anion-exchange chromatography of a M_r-6000–30000 whey protein fraction (see Figure 1a) and reconstituted in sterile 10 mM Hepes containing 0.3 M sucrose. The Figure shows the daily milk yield of one goat treated with 500 μg of FIL in one gland (●) and the same volume of carrier solution in the contralateral gland (○).

Table 2 Concentration-dependent effect of FIL on milk yield in lactating goats

Change in individual gland milk yield was compared between 3-day periods before and after intra-ductal injection with 0–750 μg of FIL. The decrease in milk yield is expressed relative to the pre-treatment yield in that gland. Values are means ($n = 2$) or means ± S.E.M. ($n \geqslant 3$).

FIL dose (μg)	Milk yield (% inhibition)
0	0.2 ± 1.9
100	0.9 ± 3.4
250	4.0 ± 3.4
500	12.2 ± 4.7
750	17.4

polypeptide rather than the oligosaccharide moiety of the protein. However, the antiserum showed no reaction with standard preparations of serum albumin, nor with growth factors, including epidermal growth factor and transforming growth factor β, known to influence mammary development and function.

The inhibitory protein was detected by immunoblotting in culture medium and in an EGTA extract of goat mammary epithelial cells cultured on a reconstituted basement membrane. Mammary cells form polarized multicellular structures termed mammospheres in this system [20]. Vectorial secretion of protein by cells within mammospheres results in accumulation of basally secreted proteins in culture medium and apically secreted milk proteins in a closed lumen, from which they can be extracted by EGTA treatment. Immunoblotting showed the inhibitory protein to be principally in an EGTA extract of cells *in situ* (Figure 5), suggesting that it is secreted mainly, if not wholly, into milk. Synthesis and vectorial secretion of milk proteins *in vitro* was confirmed by [^{35}S]methionine labelling: fluorography identified radiolabelled casein in the lumenal extract of mammosphere cultures rather than in culture medium (Figure 5). A band of similar M_r was also detected by immunoblotting of lactating mammary cell lysates after short-term culture (results not shown), confirming that the protein is a normal product of lactating goat mammary epithelial cells.

FIL inhibition of milk secretion in lactating goats

The inhibitory milk protein identified by tissue-culture bioassay also decreased the rate of milk secretion in lactating goats. FIL prepared by anion-exchange chromatography (Figure 1a) depressed milk yield ipsilaterally when injected into one gland through the teat canal (Figure 7). The yield of the contralateral gland treated with the same volume of carrier solution was unchanged (Figure 7, Table 2), confirming that the injection itself had no adverse effect. The effect of inhibitor injection was dose-dependent (Table 2), and persisted for up to 3 days at the higher doses tested.

The unilateral response to FIL treatment is demonstrated by calculation of a relative milk yield quotient (RMYQ [1]). RMYQ compares the change in test gland milk yield on consecutive days before and after FIL injection with that in the control gland over the same period, and so eliminates the influence of bilateral day-to-day variations in milk yield caused by nutrition or husbandry. Thus RMYQ values of < 1 indicate that FIL injection had caused a unilateral decrease in milk yield. RMYQ values of 0.93 ± 0.01 and 0.94 ± 0.02 respectively ($P < 0.05$ compared with 1.00) after treatment with 100 μg and 250 μg of protein showed that milk yield was decreased ipsilaterally within 24 h. Doses of 500 μg (RMYQ 0.85 ± 0.03, $P < 0.02$) and 750 μg of protein (RMYQ 0.89) had a more pronounced short-term effect, as well as being more persistent.

FIL decreased the rate of milk secretion without affecting gross milk composition. Measurement of total milk protein, fat and lactose concentrations in control and treated glands before and after FIL injection showed no effect at any of the doses tested (results not shown). SDS/PAGE also showed no qualitative change in milk protein composition as a result of FIL treatment.

DISCUSSION

In this study we have identified a milk protein which inhibits milk-constituent synthesis in mammary tissue explants, and decreases milk yield in lactating goats in a dose-dependent and reversible manner. Inhibition by a M_r-7600 whey protein in tissue explant cultures confirmed an earlier study which showed that synthesis of milk constituents was decreased by culture in the presence of a goat's milk fraction containing small whey proteins, but not by caseins or whey proteins of $M_r > 30000$ [9]. The ability of this protein to inhibit milk secretion reversibly when introduced into the gland of a lactating goat also confirmed previous observations that milk secretion was inhibited temporarily by the M_r-6000–30000 whey fraction from which it is prepared, but not by another milk fraction containing most of the whey proteins [28].

Reversible concentration-dependent inhibition by FIL *in vivo* (Figure 7, Table 2) and *in vitro* [9] suggests that it is a physiological regulator of the rate of milk secretion, and mediates the effect of milk removal on the rate of milk secretion. Synthesis of FIL by the secretory cells on which it acts further suggests that this feedback inhibition of milk secretion is an autocrine mechanism. Autocrine control is a term applied commonly in relation to control of growth, but several instances of autocrine control of secretion have been reported in endocrine cells [29,30]. Autocrine feedback regulation in an exocrine gland is also not without precedent: 'milking' the venom glands (glands in which the secretion is also stored extracellularly) of the carpet viper *Echis carinatus* increases expression of the genes encoding venom

products [31]. Feedback inhibition of secretion by extracellular product has also been demonstrated in the flowers of *Blandiflora nobilis*, where daily hand-removal of nectar stimulated the plants' nectar production [32].

Concentration-dependence of autocrine inhibition *in vivo* suggests a mechanism in which the concentration of FIL in milk increases as milk accumulates, and is decreased by milk removal. In this way, frequent milking would stimulate milk secretion by limiting the accumulation of inhibitory protein, whereas infrequent milking would decrease it by increasing the amplitude of changes in FIL's concentration. Intra-ductal injection of FIL, and equilibration of injected protein through milk stored in the cistern and alveolar lumen of the gland, should then mimic the effect of infrequent milking. Indeed, based on the response elicited, the increase in FIL concentration produced by intraductal injection appeared to be broadly within the physiological range during once-daily milking. An injection of 500 μg, which should have increased FIL's concentration by 0.47 μg/ml at the next milking, decreased milk yield to an extent comparable with the difference between milking once and twice daily (22% on the day after injection, compared with 26% in goats milked once and twice daily in their two glands) [33]. The persistent effect of a single injection of FIL is likely to be due to the retention of alveolar milk containing injected FIL at subsequent milkings [7,34]. Completeness of milk removal is an important determinant of the rate of milk secretion in lactating goats [35] and breastfeeding mothers [2], presumably because, according to the predicted kinetics of autocrine inhibition, residual milk contains a high concentration of FIL. This would be increased by the presence of exogenous FIL, with consequent effect on milk secretion during the next period of milk accumulation.

Clearly, the high concentration of FIL normally present in residual milk cannot persist indefinitely, otherwise it would prevent cyclical changes of autocrine inhibition and milk secretion with milk accumulation and removal. It is not yet known how autocrine inhibition by FIL in residual milk is relieved, nor how changes in inhibitor concentration are achieved during normal milk accumulation and removal. However, it is unlikely that they occur through a change in the rate of inhibitor secretion, since this would require that it be regulated independently of other milk constituents. An alternative possibility is that FIL is the result of, or is susceptible to, processing after secretion. In either case, first-order processing in the alveolar lumen would bring about an increasing concentration during milk accumulation, even though the inhibitor was being secreted at a constant rate relative to other milk constituents. This may explain the presence in anion-exchange peaks 2 and 4 of proteins structurally related to FIL (based on recognition by antiserum otherwise specific for inhibitory peak 3.2), but inactive in the bioassay.

If FIL is the product of, or subject to, first-order metabolism after secretion, this would also act to dilute or neutralize inhibitor in residual milk, re-establishing an optimal rate of milk secretion. Again, and whatever the mechanism involved, this also would be affected by the presence of exogenous FIL, possibly to the extent that it could render the process ineffective. It was notable that higher doses of FIL were more effective on day 2 after injection. The end product of intra-ductal injection should therefore be a sustained period of elevated autocrine inhibition, a condition which has been shown to decrease mammary epithelial-cell differentiation. For example, once-daily milking [33] and incomplete milking [35] decreased secretory-cell differentiation in lactating goats. More significantly, partly purified FIL decreased cellular differentiation in rabbit mammary glands after intraductal injection [36] and inhibited differentiation of mouse mammary cells on floating collagen gels [37]. This effect on cellular differentiation, secondary in chronological terms to FIL's acute regulation of milk secretion, may be due to down-regulation of galactopoietic hormone receptors [38], and a consequence of FIL's blockade of membrane trafficking in mammary epithelial cells [39].

FIL decreased the rate of milk secretion without affecting gross milk composition (protein, fat and lactose concentrations). A general effect on non-lipid milk constituents could be achieved by regulation of the Golgi secretory pathway in mammary epithelial cells. Most milk components are secreted by exocytosis of secretory vesicles derived from the Golgi apparatus, and initial experiments indicate that FIL may block an early stage in this pathway [39] in a manner analogous to that of the fungal drug Brefeldin A in other cell types [40,41]. However, milk lipid is secreted by a different route [42], and a feedback mechanism involving medium-chain fatty acids has been suggested to control milk lipid synthesis *in vivo* [43]. Experiments with lactating mouse mammary epithelial cells have found no effect of FIL on lipid synthesis *de novo* and secretion, suggesting that this milk constituent is indeed regulated by another mechanism [39]. On the other hand, milk fat content did not differ significantly in the treated gland before and after injection, or between treated and control glands on the day after injection of inhibitor [5.2 ± 0.3 and 5.1 ± 0.2% (v/v) in treated and untreated glands respectively]. Therefore it appears that FIL is competent to control secretion of all milk constituents co-ordinately. This ability to regulate secretion of individual milk constituents in a co-ordinated manner is consistent with its proposed role as mediator of the response to changes in milking frequency, which also affects milk secretion but not milk composition [5].

In conclusion, we have identified a milk protein able to exert reversible concentration-dependent autocrine inhibition on milk secretion in mammary tissue culture and in the lactating animal. Studies in other species, including man [44] and a macropod marsupial [45], suggest strongly that autocrine control by FIL, or an homologous protein is, like lactation itself, ubiquitous in mammals.

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