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The effect of goat milk fractions on synthesis of milk constituents by rabbit mammary explants and on milk yield *in vivo*

Evidence for autocrine control of milk secretion

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1. Lactose and casein synthesis by rabbit mammary explants in organ culture was inhibited when fractions of goat milk were included in the culture medium. Inhibition was dose-dependent, and readily reversed when milk fractions were removed. 2. The pattern of effects obtained with various fractions of milk indicated that inhibition was caused by a protein of 10000–30000 Da, which was present in the milk serum or whey fraction. 3. The inhibitor fraction decreased milk accumulation when injected into lactating rabbit mammary glands via the teat ducts, whereas other milk proteins had no effect. 4. Results are discussed in terms of autocrine regulation of milk synthesis through negative feedback by milk constituents.

INTRODUCTION

Frequency of milking or suckling is a major determinant of the rate of milk secretion by a lactating animal. This control is exerted not only through suckling-stimulated release of galactopoietic hormones [1]: studies with lactating goats indicate that locally active mammary factors are also involved, i.e. milk secretion is also under autocrine regulation. More frequent milking of goats, applied unilaterally either hourly [2,3] or thrice daily [4] instead of twice daily (as in the other gland), increased the rate of milk secretion in the thrice-milked gland only. The increase cannot be due to systemic factors, and is not the results of a decrease in physical distension of the gland [5]. Instead, it appears that this response is due to more frequent removal of a locally active chemical inhibitor, which decreases milk secretion by negative feedback.

Milk contains a number of hormones [6,7] and growth factors [8–11], mostly blood-borne in origin, which regulate mammary growth and/or differentiation in culture [8,12–14]. In this study we demonstrate that a fraction of goat milk serum proteins is capable of rapid reversible inhibition of the synthesis of milk constituents by rabbit mammary explants in organ culture, and that this fraction also inhibits milk secretion by rabbit mammary gland *in vivo*.

METHODS

Preparation of milk fractions

Milk was obtained at the morning milking (except where indicated) from British Saanen goats in mid-lactation, and was defatted by centrifugation (2500 *g*, 15 °C, 20 min) and filtration through glass wool. Defatted milk was dialysed against 40 vol. of 10 mM-Hepes, pH 7.4, for 24 h (dialysed milk) or centrifuged at 80000 *g* for 2 h at 15 °C, yielding a pellet of casein micelles and a clear supernatant containing serum proteins. The casein pellet was dissolved at pH 8.0 and dialysed against 10 mM-Hepes, pH 7.4. Portions of the serum protein fraction were subjected to ultrafiltration with filters with nominal cut-off values of 10000, 30000, 50000 and 300000 Da. In each case, the retentate volume was decreased by 95% and washed with 4 vol. of 10 mM-Hepes, pH 7.4. The filtrate, containing material of < 10000 Da, was concentrated by freeze-drying; other filtrates, and the retentate obtained with the 30000 Da filter, were concentrated by ultrafiltration with a 10000 Da filter. Serum protein fractions of 10000–30000 Da and > 30000 Da were prepared for intra-ductal infusion (see below) by dialysis against 10 mM-Hepes, pH 6.7, containing 0.3 M-sucrose. Fractions were sterilized by γ-irradiation or filter sterilization.

Tissue culture

Mammary explants were prepared from mid-pregnant New Zealand White rabbits and cultured in Medium 199 containing insulin (5 μg/ml), cortisol (100 ng/ml) and prolactin (1 μg/ml), under an atmosphere of air/CO_2 (19:1) for up to 72 h. At 42–48 h groups of 30 explants (three or four groups/treatment) were cultured with fractions of goat milk, and average rates of lactose and casein synthesis over the 6 h period were measured by addition of [U-^{14}C]glucose (0.18 mCi/mmol) and L-[4,5-^{3}H]leucine (2.22 mCi/mmol) respectively to the culture medium. Milk fractions were included at 50% or 100% (v/v) of their original milk concentration. Where necessary, osmotic imbalance, owing to the presence of milk fractions, was avoided by use of a modified culture medium prepared from half-strength Medium 199 supplemented with glucose, essential and non-essential amino acids, vitamins and hormones to obtain normal concentrations of these constituents. Explants in modified medium made iso-osmotic with 0.15 M-NaCl synthesized lactose and casein at average rates (over 6 h) similar to those measured in normal medium. At the end of the 6 h period, explants and culture medium were separated and stored frozen at −75 °C. In other experiments, explants exposed to milk fractions at 42–48 h in the absence of radiolabel were washed and cultured again for 23.5 h in normal medium containing [U-^{14}C]glucose and L-[4,5-^{3}H]leucine.

Explants were homogenized at 4 °C in 1.0 ml of 10 mM-Tris/HCl, pH 7.0, containing 5 mM-EGTA and

2 mM-phenylmethanesulphonyl fluoride by ten strokes with a glass/Teflon homogenizer, followed by sonication for 30 s (10 μm amplitude; MSE Soniprep 150), and particle-free supernatant was prepared by centrifugation in a micro-centrifuge for 5 min (Eppendorf model 5414). ^{3}H-labelled casein was isolated from the particle-free supernatant by isoelectric precipitation and SDS/polyacrylamide-gel electrophoresis [15]. [^{14}C]Lactose was measured by selective precipitation from explant homogenates and culture medium [16]. Synthesis of fatty acids was measured by incorporation of [U-^{14}C]acetate (2.2 mCi/mmol) into fatty acids extracted from explants and culture medium [17]. Inclusion of milk fractions in the culture medium did not affect the distribution of secreted products between the explant extracellular space and the medium. Culture medium was from Gibco Europe Ltd., analytical reagents were from Sigma, and radiolabels were from Amersham International, Amersham, Bucks., U.K.

Fractions prepared from 15 milk samples were tested in 14 explant preparations. Practical considerations prevented testing of all the milk fractions described in each preparation. Therefore each explant preparation was used to test the effect of a limited set of fractions (e.g. defatted milk, dialysed milk and serum fraction or serum fraction and subfractions thereof) prepared from a single milk sample, or in one case two sets prepared from two samples. In five additional experiments, where dose-dependence and/or recovery from inhibition were studied, only one or two milk fractions were tested; these results were included with data presented in Table 1.

Mammary infusion

Under light anaesthesia (sodium pentobarbitone, 25 mg/kg body wt., intravenously), lactating Dutch rabbits accustomed to a schedule of 1 h suckling and 23 h separation from their litter were infused unilaterally in four glands via the teat duct. Each gland received 1.0–1.25 ml (0.25 ml/duct) of the 10000–30000 Da fraction of milk serum proteins, concentrated 20-fold relative to their milk concentration. Other rabbits received the > 30000 Da fraction or carrier solution. After 23 h the mammary glands were carefully removed, and the total weights of treated and of untreated glands were calculated.

RESULTS

A consistent pattern of inhibition was observed with each set of milk fractions tested, and throughout the explant preparations described in this study. This reproducible pattern of inhibition, obtained with progressively better-defined milk fractions, allowed the inhibitory activity to be located in a particular milk fraction.

Lactose synthesis by mammary explants and, to a lesser extent, casein synthesis was inhibited when defatted goat milk or dialysed milk was included in the culture medium (Table 1). The degree of inhibition was calculated from average rates of synthesis measured over 6 h in culture. However, inhibition was apparent soon after exposure to milk fractions: in the presence of defatted milk, the average rates of lactose and casein synthesis during the first 1 h and over 3 h were approx. 30% and 27% respectively of those in control cultures, approaching values determined after 6 h exposure (Table 1). Synthesis of these two milk constituents was also inhibited, although less effectively, by a milk 'serum' fraction prepared by removal of casein micelles: this fraction was added to culture medium at twice the concentration of dialysed milk, but inhibited casein synthesis to the same extent and had less effect on lactose synthesis (Table 1). When a casein solution was tested on mammary explants, some precipitation of these proteins occurred at high concentrations in culture medium; however, in three experiments no consistent effect was observed (results not shown). Therefore, although the serum fraction was less potent than cruder fractions of milk, the results indicated that inhibitory activity was contained principally in this fraction. Such a view was confirmed when the serum fraction was resolved further by ultrafiltration.

Low-molecular-mass constituents (< 10000 Da) of the serum fraction had no effect on mammary explants (Table 1), but a fraction containing material of

Table 1. Lactose and casein synthesis by rabbit mammary explants cultured with fractions of goat milk

The effect of fractions prepared from 20 milk samples on average rates of lactose and casein synthesis over 6 h were tested in 19 separate explant preparations. A limited set of fractions prepared from a single milk sample was tested in each preparation (see the Methods section). Rates of synthesis were expressed relative to those for groups of explants cultured in unsupplemented medium in the same experiment (three or four groups per treatment). Values shown are means ± S.E.M. of relative rates of synthesis, with the numbers of experiments in parentheses. Statistical significance between actual mean rates of synthesis with and without milk fraction are compared by Wilcoxon Signed rank test or (for four values) by paired t test: $*P < 0.05$; $**P < 0.01$.

Treatment	$10^{-3}\times$ Molecular mass (Da)	Concentration (% relative to milk)	Lactose synthesis (% of control)	Casein synthesis (% of control)
No additions	–	–	100.0	100.0
Defatted milk	–	50.0	19.4 ± 5.2 (5)*	60.3 ± 11.7 (5)*
Dialysed milk	–	50.0	45.9 ± 10.2 (7)*	62.0 ± 10.4 (5)*
Serum fraction	–	100.0	72.8 ± 7.1 (9)**	61.5 ± 8.0 (7)*
Serum fraction	< 10	100.0	91.4 ± 18.0 (5)	98.4 ± 12.5 (5)
Serum fraction	10–30	100.0	60.7 ± 3.2 (10)**	53.3 ± 6.4 (10)**
Serum fraction	10–50	100.0	68.0 ± 5.5 (5)*	53.9 ± 14.1 (5)*
Serum fraction	10–300	100.0	47.5 ± 12.4 (4)**	78.5 ± 9.6 (4)
Serum fraction	> 30	100.0	103.2 ± 5.7 (4)	106.0 ± 13.6 (4)

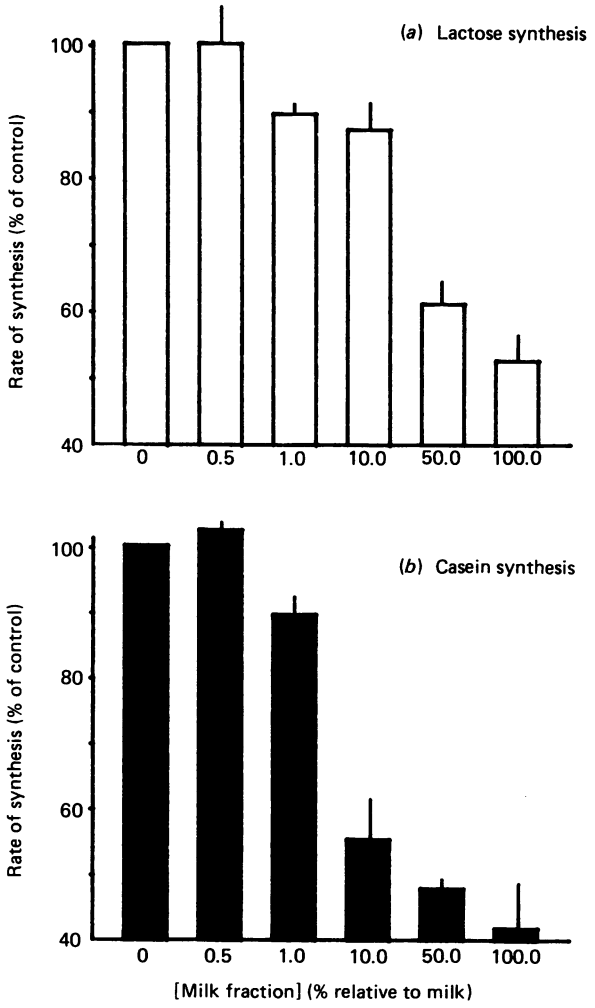

Fig. 1. Inhibition of (*a*) lactose and (*b*) casein synthesis by increasing concentrations of goat milk serum proteins

Lactose and casein synthesis was measured in groups of explants cultured for 6 h with milk serum proteins of 10000–30000 Da. Serum proteins were added to culture medium at concentrations of 0.5–100% relative to their original concentration in milk. Rates of synthesis are expressed relative to control groups of explants (three groups/treatment), with error bars indicating the S.E.M. The results of a representative experiment are shown.

10000–30000 Da did inhibit both lactose and casein synthesis. Inhibition was also observed with fractions containing constituents of 10000–50000 Da and 10000–300000 Da, but serum constituents of > 30000 Da did not themselves affect lactose or casein synthesis. Heat-treatment of inhibitory fractions (75 °C, 15 min) resulted in loss of the capacity to inhibit lactose and casein synthesis (results not shown). Together, these observations indicate that inhibition is caused by serum protein constituent(s) of 10000–30000 Da.

Inhibition of lactose and casein synthesis by the 10000–30000 Da fraction of serum proteins was dose-dependent (Fig. 1). No effect was observed when proteins were present at 0.5% of their original milk concentration, but increasing degrees of inhibition were observed with concentrations of 1%, 10%, 50% and 100% of that in milk.

In contrast with the effects on lactose and casein synthesis, synthesis of fatty acids by mammary explants, measured by incorporation of [U-^{14}C]acetate, was not affected consistently by culture in medium containing milk fractions. In four experiments, rates of fatty acid synthesis in the presence of dialysed milk and serum fraction, expressed as a percentage of rates in control groups of explants, were 88.4 ± 20.5% and 107.7 ± 21.6% respectively (means ± S.E.M.).

A number of observations indicated that inhibition of lactose and casein synthesis were specific effects and not due to toxic effects of milk fractions on the explants. First, inhibition was readily reversed: in three experiments, when explants were cultured for 23.5 h (48.5–72 h in culture) in normal medium after being cultured for 6 h in medium containing milk serum proteins of 10000–30000 Da, lactose synthesis recovered from 57.4 ± 4.3% (mean ± S.E.M.) to 91.2 ± 3.8% of control values, and casein synthesis recovered from 63.8 ± 5.2% to 93.0 ± 8.8% of controls. Second, measurements of lactate dehydrogenase activity in cultured media as an indicator of cellular integrity indicated that inclusion of milk fractions had no deleterious effect (results not shown). Medium containing defatted or dialysed milk contained relatively high concentrations of non-esterified fatty acids (0.17 mM and 0.13 mM respectively), which can have toxic effects in tissue or cell culture. However, non-esterified fatty acids extracted from goat milk and included in culture medium at similar concentrations had no significant effect on lactose or casein synthesis, and in any case non-esterified fatty acid concentrations were considerably lower (27 μM) in culture medium containing serum fractions. Therefore the inhibitory effect was not due to a toxic effect of non-esterified fatty acids. Finally, it is unlikely that inhibition was due to decreased availability of hormones: insulin, cortisol and prolactin were included in considerable excess of the concentrations required by explants for lactose and casein synthesis, and, when measured by radioimmunoassay, these concentrations were found to be unaffected by the presence of milk fractions (results not shown).

Preliminary measurements on the effect of intraductal mammary infusion of goat milk serum fractions in lactating rabbits (each animal treated in four glands unilaterally) support the explant data. The weight difference between treated and untreated glands (69.4 ± 6.2 and 77.8 ± 7.9 g/kg body wt. respectively for eight animals; $P < 0.05$) was considerably greater than that for untreated rabbits, in which left and right sets of glands differed by only 1.0 ± 1.4 g/kg body wt. (12 animals). When milk yield was calculated by using a previous measurement [18] of rabbit mammary weight immediately after suckling, this difference was estimated to represent a 20.5% decrease in milk yield by the serum protein fraction. In contrast, infusion of a 20-fold-concentrated solution of milk serum proteins of > 30000 Da or the carrier solution did not significantly affect milk accumulation.

DISCUSSION

The effects observed in mammary explants in organ culture and in mammary glands *in vivo* indicate that goat milk contains a factor or factors which can inhibit synthesis of milk constituents in a rapid and readily reversible manner. The presence of such a factor offers the

possibility of local acute regulation of milk secretion in the mammary gland, i.e. via an autocrine mechanism. There is substantial evidence from studies *in vivo* that milk secretion is subject to this type of control: unilateral manipulation of milking frequency elicits local changes in milk synthesis attributable directly to more frequent removal of milk constituents [2–5], and this effect is readily reversed when frequent milking is limited to a short period [4]. In the present study, synthesis of lactose and casein was generally inhibited to a similar degree, whereas the same milk fractions had no consistent effect on fatty acid synthesis. On the other hand, milk composition is not affected by frequent milking [3,4], which suggests that local feedback control is a general mechanism regulating synthesis of all milk constituents. This discrepancy between observations *in vivo* and *in vitro* may arise because fatty acid synthesis is under separate local control by (a) milk constituent(s) not present or not active in the fraction that regulates lactose and casein synthesis. Alternatively, milk fat concentration may have been maintained during frequent milking through an increase in the proportion of milk fatty acids derived from the circulation.

The pattern of effects obtained with goat milk fractions indicate that the inhibitor is a protein of 10000–30000 Da, which is contained principally, if not wholly, in the milk serum fraction. As the inhibitor was active when introduced into the teat ducts of lactating rabbits, it appears that it acts on the mammary cell by negative feedback across the apical membrane; whether this occurs via a receptor-mediated event and/or internalization by the cell has yet to be determined. The 10000–30000 Da fraction of goat milk serum proteins also effectively inhibits milk secretion in lactating goats. When introduced unilaterally into the udder by intraductal injection, it produced a substantial but temporary decrease in the rate of milk secretion in the treated gland only (C. Wilde & M. Casey, unpublished work).

The present study provides evidence that milk secretion is indeed under feedback control by constituents of milk during lactation. Further studies are required to identify precisely the milk constituent(s) exerting this control.

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