Regulation of milk lipid secretion: effects of oxytocin, prolactin and ionomycin on triacylglycerol release from rat mammary gland slices

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A system for the study of the regulation of the release of triacylglycerols by mammary gland slices was developed. By prelabelling the triacylglycerol pool with [³H]oleate measurements of release of both mass of triacylglycerol and of newly synthesized triacylglycerol have been made. Oxytocin and ovine prolactin stimulated release of triacylglycerol and protein, but the former was 40-fold more effective. Recombinant bovine prolactin was even less active than ovine prolactin, suggesting that contamination of the latter with oxytocin and/or vasopressin was partly responsible for its stimulatory effect on release. The findings support the view that the major effect of oxytocin is to stimulate contraction of myoepithelial cells and thus release

secreted lipid stored in the lumen of the mammary gland alveoli. Ionomycin, a Ca²⁺ ionophore, also stimulated lipid release, but probably not by the usual apocrine route. Parathyroid hormone-related protein, a peptide produced by the mammary gland, did not stimulate release or antagonize the effects of oxytocin. Release of lipid was also measured in mammary gland slices from late-pregnant, early- and mid-lactating rats and lactating rats made prolactin-deficient. Hormonal stimulation *in vitro* showed the maturation of response seen *in vivo* on transition from late pregnancy to peak lactation. Prolactin deficiency resulted in decreased release of newly synthesized lipid in response to oxytocin.

INTRODUCTION

Different strategies are employed by the mammary gland for the secretion of the macromolecules in milk. Milk proteins and lactose are synthesized in the endoplasmic reticulum, assembled into vesicles in the Golgi apparatus and secreted by exocytosis [1]. Milk lipids, on the other hand, are secreted by a specialized process, specific to the mammary gland epithelial cells. Lipids of exogenous or endogenous origin are esterified to triacylglycerols in the rough endoplasmic reticulum [2,3] and assembled into droplets that migrate to the apical region of the cell [4,5]. At the apical region they become enveloped in plasmalemma and protrude into the alveolar lumen, and are finally pinched off [6,7].

The stimulatory effect of oxytocin on milk secretion is mainly accounted for by its role in the milk ejection reflex [8]. In the rat, milk ejection reflex occurs regularly at 5–10 min intervals during active suckling [8] and in this species release of oxytocin is important for secretion of milk from the gland [9]. Suckling stimuli activate discharge of oxytocin from the posterior pituitary gland into the circulation. Oxytocin then promotes the contraction of the myoepithelial cells surrounding the mammary gland alveoli and expels the contained milk by extrusion. Oxytocin may have other roles, e.g. acceleration of the transit of synthesized milk components, especially casein, from the cytoplasm of the epithelial cells to the lumen in rabbit mammary gland explants [10]. The presence of oxytocin receptors in the mammary tissue has been demonstrated by various techniques. Radiolabelled oxytocin binds to particulate fractions of plasma membrane [11] and especially to dissociated cell suspensions enriched in myoepithelial cells [12]. The mechanism of post-receptor signalling in mammary gland myoepithelial cells by oxytocin is poorly understood [8], although its dependence on external Ca²⁺ for maximal response is well-defined [11,13,14]. More recent studies have suggested that InsP₃ (inositol 1,4,5-triphosphate) may be involved in the mechanism by which

oxytocin mobilizes intracellular Ca²⁺ and elicits contraction of the myoepithelial cells [15,16].

Prolactin is important in maintenance of milk synthesis and secretion [9]. A direct stimulatory effect of prolactin on the release of milk lipid has been demonstrated *in vitro* in mammary gland tissue fragments from rabbits [17]. Prolactin deficiency *in vivo*, induced with bromocryptine, decreases milk lipid transfer to pups and causes tissue accumulation of milk fat [18].

The present work was undertaken to develop a system for the study of the hormonal regulation of milk lipid release (secretion and/or extrusion) *in vitro* with the eventual aim of investigating the mechanisms and signalling systems involved. Mammary gland slices were chosen in preference to other preparations (acini, epithelial cells) because the morphological structure of the gland is likely to be more intact. Rat mammary gland contains a considerable amount of triacylglycerol (about 15%), much of which is presumably in the cytoplasmic secretory pool. It is therefore necessary to label this pool with radioactive fatty acids so as to measure the release of newly formed triacylglycerols.

The questions studied include: (a) the peptide specificity of the effects; (b) whether the stimulatory effects of oxytocin and prolactin on lipid release are similar over the period from late pregnancy to peak lactation; and (c) does prolactin deficiency *in vivo* result in decreased release of lipid *in vitro*? The effects of the two hormones have been compared with that of ionomycin, a calcium ionophore, which increases protein secretion from mammary gland acini and appears to facilitate lipid release from droplets rather than promoting true secretion [19].

MATERIALS AND METHODS

Animals

Albino Wistar rats obtained from the breeding programme in our animal house were used. Female rats were mated between 2 and 2.5 months of age. Room temperature (21 ± 2 °C) and dark/light cycle (12-h cycle, lights on at 08:00 h) were controlled.

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Commercial chow (No. 3; Special Diet Services, Witham Essex, U.K., containing by weight: 52 % carbohydrate, 21 % protein, 4 % fat and a residue of non-digestible material) and tap drinking water were given *ad libitum*. Time-mated pregnant rats were used between 19 and 20 days of pregnancy. Lactating rats, suckling 8–10 pups, were selected at lactation day 2 or 12. Prolactin-deficiency (24 h) *in vivo* was induced in 11-day lactating rats by treatment with bromocryptine mesylate [10 mg/kg body wt.; solution in 10 % (v/v) ethanol containing 1 % (w/v) tartaric acid] [20] injected subcutaneously every 8 h from 09:00 h. Dam and litter weights and food intake were measured 24 h prior to the experiments.

Chemicals

Oleic acid (sodium salt, 99 %), bovine albumin (essentially fatty acid free), ovine luteotropic hormone (prolactin), [Lys⁸]vasopressin (acetate salt), [Arg⁸]vasopressin (acetate salt), [Thr⁴, Gly⁷]oxytocin, angiotensin-II, parathyroid hormone-related peptide (1–34 amide) and ionomycin were purchased from Sigma, St. Louis, MO, U.S.A. Recombinant bovine prolactin was a gift from Dr. F. Adriaens, Monsanto Europe. Oxytocin (Syntocinon) was obtained from Sandoz, Frimley, Surrey, U.K. Bromocryptine mesylate was kindly provided by Dr. D. Römer (Sandoz Pharma, Basle, Switzerland). [9,10(n)-³H]Oleate (5 mCi/ml) was from Amersham International. Triacylglycerol GPO-PAP kit was from Boehringer, Mannheim, Germany. All enzymes and coenzymes were purchased from Boehringer Corp. (London), Lewes, Sussex, U.K.

Radioactive labelling of mammary gland slices

Rats were anaesthetized with pentobarbital (60 mg/kg intraperitoneally) and the abdominal mammary gland was rapidly removed. Trimmed pieces (0.5 cm²) of mammary gland were placed in Krebs/Henseleit saline [21] containing 5 mM glucose and 2.4 mM CaCl₂ (37 °C; gassed O₂/CO₂, 19:1). Mammary gland slices (less than 2 mm thick) were obtained with a razor blade and a glass slide [22]. The slices were washed in fresh saline to remove excess milk at the cut cell surface, blotted dry on tissue paper and a weighed amount (about 1.5 g) transferred to an Erlenmeyer flask containing the labelling medium. The flask was gassed (O₂/CO₂, 19:1) and incubated at 37 °C for 30 min in a Dubnoff shaker. The labelling medium consisted of 19.2 ml of Krebs/Henseleit saline containing 2 % BSA plus 0.8 ml of [³H]oleate-albumin complex (0.62 µmol/ml; 0.84 µCi/ml). The radioactivity of the medium was measured before and after incubation to calculate the amount of [³H]oleate incorporated into the slices.

For the time-course studies 3.0 g of slices were pre-incubated in a [³H]oleate-containing medium (as above) and the volume adjusted to 50 ml. Where appropriate, Ca²⁺-free saline was used. After the labelling step the slices were washed and divided between incubation flasks. Each flask contained 50 ml of Krebs/Henseleit saline [21], approximately 1.5 g of slices. One or two additions of oxytocin (50 ng/ml) or ionomycin (2.5 µg/ml) were made at intervals during the incubation. The flasks were gassed, stoppered and incubated for 90 min in a water bath at 37 °C with agitation. Samples of the incubation medium (1 ml) were obtained at intervals and assayed. At least two experiments were carried out for each condition.

For the single time-point values the slices were washed, after the labelling phase, three times with 10 ml of warm Krebs/Henseleit saline to remove excess radioactivity, blotted dry and divided (100–150 mg) between incubation flasks. Each flask contained 4 ml of Krebs/Henseleit saline and the additions

indicated. All flasks were incubated in duplicate, gassed with O₂/CO₂ (19:1, v/v), stoppered and placed in a Dubnoff bath at 37 °C with gentle agitation. Controls had no addition and the other flasks contained 0.1 I.U. (50 ng/ml, 50 nM) oxytocin, 0.64 unit of ovine prolactin (5 µg/ml, 200 nM) or 2.5 µg/ml (3.3 µM) ionomycin. The incubation was terminated after 60 min by removing the slices and blotting them dry on tissue paper. At least five independent experiments were carried out for each of the physiological conditions.

Measurement of release

Secretory products were measured as described below.

(a) The turbidity of the medium was measured by the absorbance at a wavelength of 540 nm. This reflects the amount of milk components released, i.e. fat globules and/or casein micelles [23].

(b) Triacylglycerol released was measured directly in the incubation medium with an assay kit according to the manufacturer's description. The standard curve was prepared with triolein.

(c) The radioactivity released (at least 85 % triacylglycerol) was measured directly in the incubation medium.

(d) A portion of the incubation medium (0.5 ml) was used for protein assay. Casein, the major protein component of rat milk, was precipitated at the isoelectric point with 1 M sodium acetate buffer (pH 4.6). After allowing to stand overnight at 4 °C sedimentation of the protein was obtained by centrifugation (1500 g, 10 min) and the supernatant decanted. The sedimented protein was washed with 1 ml of 0.1 M sodium acetate buffer (pH 4.6). After centrifugation and decantation of the supernatant the precipitated protein was assayed as described by Lowry et al. [24] using BSA as a standard.

Glucose utilization was determined as an index of the metabolic activity of the slices. The incubation medium (50–100 µl) was added directly to spectrophotometer cuvettes and glucose measured enzymically [25].

Separation of the lipid fractions

The slices were transferred (at the end of the incubation) to a screw-capped glass tube containing 5 ml of chloroform/methanol (2:1, v/v; Folch mixture) for lipid extraction [26]. Lipids extracted from the medium and the slices were analysed by TLC [27]. The samples were run with free fatty acid, triacylglycerol and cholesterol standards. After identification of the bands under UV light on a rhodamine-sprayed plate, the fractions were scraped from the plate and the radioactivity in each fraction measured by scintillation counting.

Data presentation and statistics

The results of the single-time-point experiments are presented as means ± S.E.M. Difference analyses between basal incubation (no addition) and treated slices were performed by paired Student's *t* test. Comparisons among the groups were carried out by one-way analysis of variance (ANOVA) and *post hoc* analysis performed with the Tukey test. The level of significance considered was < 5 %.

RESULTS AND DISCUSSION

The first experiments involved determining the characteristics of the system, namely the time course of the hormonal stimulation, the effects of a Ca²⁺-free medium, and the peptide specificity and concentration dependence. Slices obtained from mid-lactating rats (10–12 days) were used and the results are from representative experiments.

Figure 1 Effects of oxytocin on the release of milk components from rat mammary gland slices

(a) Turbidity of the medium at 540 nm; (b) total triacylglycerol; (c) release of radioactivity; and (d) glucose utilization. Additions of oxytocin (50 nM) were at 10 and 40 min (○) and at 40 min (●) after the start of the incubation.

Time course data

After addition of oxytocin (50 nM final concentration) the turbidity of the medium increased approximately 2-fold within 5 min and continued to rise at an increased rate for a further 20 min (Figure 1a). A second challenge with oxytocin at 40 min after the start of the incubation did not cause a similar rapid increase in turbidity (secretion of milk components) (Figure 1a). When addition of oxytocin was delayed until 40 min there was again a 2-fold increase in turbidity (Figure 1a). Release of triacylglycerol and radioactivity to the medium showed a similar response pattern after addition of oxytocin (Figures 1b and 1c). Addition of oxytocin had no effect on the utilization of glucose from the medium (Figure 1d). The lack of oxytocin effect on a second challenge implies that either the myoepithelial cells become desensitized to the hormone, or that the first addition expels milk already secreted to the alveoli lumen. Support for this latter view is the finding that the increment in radioactivity released to the medium subsequent to the addition of oxytocin at 10 min was consistently lower [d.p.m. released/100 mg of tissue: 20357 ± 9221 (10 min), 29283 ± 10984 (20 min) and 34736 ± 11877 (30 min)] than when oxytocin was added at 40 min (comparable values were 34115 ± 9265 , 44720 ± 14027 and 47273 ± 14730 ; mean $\pm$ S.D., $n = 5$). The differences are significant on a paired *t*-test basis ($P = < 0.026$, < 0.005 and < 0.012 respectively). It is noteworthy that the difference in radioactivity at these time points between the two conditions is similar (13758, 15437 and 12537 respectively). We interpret the increased release of radioactivity when addition of oxytocin is delayed for 40 min to indicate rapid release of triacylglycerol which has accumulated in the lumen during the additional

30 min of incubation. The alternative explanation that it has accumulated in a specific secretory pool within the epithelial cells is unlikely because (a) the two rates of release are parallel 10 min after addition of oxytocin, and (b) there is no increased release of radioactivity when the slices are challenged for a second time with oxytocin (Figure 1c). Similar findings were obtained with ovine prolactin.

Prolactin (200 nM) and ionomycin ($3.3 \mu\text{M}$) caused a similar pattern of increase in medium turbidity and the secretion of triacylglycerol to that found with oxytocin (results not shown). In the case of ionomycin, however, the radioactivity secreted to the medium increased to higher levels than with oxytocin or prolactin (see Figure 3c), presumably reflecting preferential release of newly synthesized lipid.

Effects of Ca^{2+}

In a Ca^{2+} -free medium, oxytocin still caused an increase in medium turbidity, and in release of triacylglycerol and radioactivity to the medium (Figures 2a, 2b and 2c), which then tended to plateau (cf. Figures 1 and 2). The magnitudes of the responses, however, were lower than in the Ca^{2+} -containing medium (for controls see Figure 1). Ionomycin was ineffective in Ca^{2+} -free medium (Figures 2a, 2b and 2c), confirming that Ca^{2+} influx is required for its stimulatory action on secretion of milk constituents [19]. It is concluded that Ca^{2+} is required for efficient release of luminal contents by oxytocin, although secretion can be initiated by utilization of the intracellular Ca^{2+} store [16]. These findings are in agreement with the view that oxytocin triggers release of intracellular Ca^{2+} for initiating myosin phosphorylation and that it also stimulates influx of extracellular

Figure 2 Effects of oxytocin (50 nM, ●) or ionomycin (3.3 μM, ■) on the release of milk components from rat mammary gland slices in a Ca²⁺-free medium

(a) Turbidity of the medium at 540 nm; (b) total triacylglycerol; and (c) release of radioactivity. Additions of oxytocin were at 10 and 40 min and ionomycin at 40 min after the start of the incubation.

Ca²⁺, which increases the intracellular Ca²⁺ concentration and controls the duration of the cellular response [14].

Specificity of stimulatory effects

A number of peptides related to oxytocin were tested for their ability to stimulate triacylglycerol release. [Thr⁴, Gly⁷]Oxytocin, [Arg⁸]vasopressin and [Lys⁸]vasopressin at a final concentration

Table 1 [³H]Oleate incorporated into lipid of tissue slices of mammary gland at different stages of pregnancy and lactation

Values are means ± S.E.M. for the number of observations indicated (*n*). The differences among the groups were determined by analysis of variance and group comparison performed by Tukey test. * *P* < 0.05 compared with the pregnant group.

Experimental groups	<i>n</i>	10 ⁻³ × Tissue radioactivity (d.p.m./100 mg)	Lipid content (%)
Pregnant (19–20 days)	5	221.4 ± 12.6	41.2 ± 2.0
Lactation (2 days)	5	401.6 ± 28.8*	18.7 ± 2.5*
Lactation (12 days)	6	415.2 ± 67.2*	12.5 ± 2.2*

of 20 nM produced an increase in medium turbidity, radioactivity and triacylglycerol similar to that obtained with oxytocin (results not shown). Parathyroid hormone-related protein (200 nM), a peptide produced by mammary gland [28,29], did not have any significant effect on basal release, nor did it inhibit the stimulatory effect of oxytocin (5 or 0.5 nM) (results not shown). The latter is of interest in view of the report that parathyroid hormone-related peptide can inhibit oxytocin-induced increases in intracellular Ca²⁺ in a myoepithelial cell line derived from human breast [30], which led to the suggestion that parathyroid hormone-related peptide might have an autocrine or paracrine role in the regulation of milk ejection [30]. Comparison of a range of concentrations of oxytocin and prolactin indicated that the former was considerably more effective. The minimum concentration required to cause a 100% increase in turbidity and in radioactivity released was 0.5 nM for oxytocin and 20 nM for ovine prolactin. It is known that ovine prolactin can be contaminated with other pituitary peptides, in particular, vasopressin and oxytocin [31–33]. One preparation of ovine prolactin used in the present experiments contained 0.076% (w/w) of oxytocin by radioimmunoassay [34]. This would mean a concentration of oxytocin of about 0.35 nM when the final concentration of prolactin was 20 nM. A sample of recombinant bovine prolactin (200 nM; containing no detectable oxytocin) produced a significantly smaller increase in medium turbidity or triacylglycerol secretion than the same concentration of ovine prolactin (turbidity: 21.0 ± 10% versus 137 ± 30%, *n* = 4, *P* < 0.01; triacylglycerol release: 2.5 ± 0.5% versus 181 ± 21%, *n* = 4, *P* < 0.001). Assuming there is no species specificity for the hormone, this would suggest that at least part, if not all, of the effects of ovine prolactin might be due to contamination with oxytocin and/or vasopressin [31–33]. Further support for this view is the report that the milk-ejection effect of ovine prolactin preparation was not inhibited by prolactin antibodies [32].

Mammary gland development

To examine whether physiological changes in milk lipid secretion which occur as lactation progresses could be demonstrated *in vitro*, we have studied the pregnant to lactating transition. Mammary gland slices prepared from late pregnant (19–20 days gestation) or lactating rats (2 or 12 days after birth) showed marked differences in the lipid content (Table 1). After the onset of lactation mammary gland lipid content rapidly decreased (55% in 2 days) and then declined more gradually (see also [35]). Similarly, the amount of [³H]oleate incorporated into lipid of mammary gland slices during the labelling period increased by 82% after onset of lactation (Table 1). To compare the release of ³H-labelled lipid in the different reproductive states the following were considered: (a) the difference in pool size of triacylglycerols

Figure 3 Maturation of release

(a) Changes in medium turbidity; (b) triacylglycerol release; (c) release of radioactivity; and (d) protein, in slices from pregnant (□), 2 day-lactating (▨) and 12 day-lactating (▩) rats incubated for 60 min with or without (basal) additions of oxytocin (50 nM), ionomycin (3.3 μM) or prolactin (200 nM) to the medium. Values are means ± S.E.M. for five to six independent experiments in each group. Differences among the groups, tested by ANOVA and Tukey test, are indicated: * $P < 0.05$ from 12 day-lactating group, † $P < 0.05$ from 2 day-lactating group.

present in mammary tissue slices from pregnant and lactating rats; (b) the different amount of [3 H]oleate incorporated into tissue slices between the two conditions; (c) the possibility that newly formed [3 H]triacylglycerol may not enter a homogenous intracellular triacylglycerol population of lipid droplets from the basal membrane (uptake) to the plasma membrane (secretion). In consequence, the values for d.p.m. released are expressed as percentages of total d.p.m. incorporated into the triacylglycerol of the slices in the labelling period.

Basal release of milk components, measured as the turbidity, did not differ in slices obtained from pregnant or lactating rats (Figure 3a). When compared with the respective basal values, slices incubated with oxytocin and ionomycin significantly increased release of milk particles (casein micelles and fat globules) at all stages of mammary gland development. Ovine prolactin was able to increase (76%, $P < 0.013$) turbidity of the medium above basal levels in slices from mid-lactating rats (12 days), while those from pregnant and early-lactating rats did not show a significant increase ($P = 0.23$ and $P = 0.09$, respectively). Pregnant rats are exposed *in vivo* to high levels of progesterone and it is known that this can suppress the lactogenic action of prolactin [36]. Recombinant bovine prolactin was not available for testing in this series of experiments and in any case the aim was to compare the present results with those of other workers who have used ovine prolactin [17,37].

The changes in triacylglycerol release (compare Figure 3b with Figure 3a) paralleled the changes in turbidity. There was no difference in basal triacylglycerol release between the groups. Addition of oxytocin and ionomycin to slices from late-pregnant, early- (2 days) and mid-lactating (12 days) rats increased release

of triacylglycerols above basal levels (Figure 3b, $P \leq 0.03$). In agreement with the changes observed in the turbidity, prolactin only stimulated release of triacylglycerol above basal levels (65%, $P = 0.014$) in the slices from mid-lactating rats (12 days). A stimulatory effect of prolactin (400 nM) on lipid release *in vitro* has been previously shown in mammary gland pieces from lactating rabbits [17].

The relative triacylglycerol release tended to increase with oxytocin ($P = 0.08$) and prolactin ($P = 0.05$) in slices from mid-lactating rats when compared with slices from 2 day-lactating or late-pregnant rats. Ionomycin increased the release of triacylglycerols to the medium (about 100% above basal levels for pregnant and 12 day-lactating rats) but there was no significant difference between the three stages of lactation ($P = 0.18$) due to the large variability. 14 C-labelled triacylglycerol release in the presence of ionomycin has been reported to increase 103% in a preparation of acini from mouse lactating mammary gland [19]. These workers also presented electron microscopy evidence that ionomycin-treated cells release their lipid content by a non-budding mechanism [19]. This is in contrast to the widely reported mode of milk lipid secretion [1,4–6], re-enforced by a recent ultrastructural study of lipid apocrine secretion in mouse mammary tissue where lipid droplets are shown in the process of 'budding off' from the cytoplasmic membrane [7]. Others have reported a non-apocrine secretion in mammary epithelial cells [38], but the study was carried out in cultured tissue and this system may not necessarily reflect normal lipid secretion of lactating epithelial cells.

The amount of radioactivity released to the medium was significantly increased at all stages by oxytocin and prolactin

Figure 4 Effects of prolactin deficiency *in vivo* on release of milk components

(a) Turbidity; (b) triacylglycerol; (c) radioactivity (d.p.m.); (d) protein, in mammary gland slices from 12 day-lactating (□) and 12 day-lactating plus prolactin-deficiency (▨) rats incubated for 60 min with or without (basal) additions of oxytocin (50 nM), ionomycin (3.3 μM) or prolactin (200 nM) to the medium. Values are means ± S.E.M. for five to six independent experiments in each group. Differences between the lactating and the prolactin-deficient group by Student *t*-test are shown: * *P* < 0.05.

(Figure 3c; pregnant: 63 %, *P* = 0.004 and 43 % *P* = 0.006; 2 day-lactating: 150 %, *P* = 0.01 and 92 % *P* = 0.012; 12 day-lactating: 163 %, *P* = 0.001 and 93 %, *P* = 0.008). Ionomycin had a much greater effect on release of radioactivity in all cases (244 %, *P* = 0.003; 419 %, *P* = 0.002; 492 %, *P* = 0.001; above basal levels in pregnant, 2 day-lactating and 12 day-lactating rats, respectively). TLC of the lipid extracted from the medium indicated that at least 85 % of the radioactivity was present as triacylglycerol. Analogous to the findings with protein release (Figure 3d) the slices from lactating rats (2 days or 12 days) released more newly synthesized lipid (³H-labelled) than slices from the pregnant rats when stimulated with oxytocin (Figure 3c). After the onset of lactation the mammary tissue acquires the ability to incorporate and release lipid more efficiently and this is maintained in the tissue slices (see also [2]).

Addition of oxytocin and prolactin to the incubation medium did not significantly increase release of protein above the low basal values in slices from pregnant and 2 day-lactating rats. However, these hormones significantly stimulated release of protein in slices from 12 day-lactating rats (oxytocin 60 %, *P* = 0.002 and prolactin 45 %, *P* = 0.03) when compared with basal release (Figure 3d). In contrast, protein release was significantly increased when compared with basal values on addition of ionomycin at all stages of mammary gland development (pregnant 233 %, *P* = 0.04; lactation 2 days 170 %, *P* = 0.005 and lactation 12 days 140 %, *P* = 0.004). Turner et al. [19] have shown an increase in the proportion of newly synthesized protein released in the presence of ionomycin in a mammary gland acini preparation from mid-lactating (10 days) mice. However,

the calcium ionophore markedly inhibited protein synthesis [19]. In the present experiments ionomycin inhibited glucose utilization by mammary gland slices (12 day-lactating controls: 4.04 ± 0.27 μmol/100 mg of tissue per h, *n* = 24; oxytocin: 3.84 ± 0.27 μmol/100 mg of tissue per h, *n* = 23; 1.89 ± 0.21 μmol/100 mg of tissue, *n* = 6, *P* < 0.05). Thus there is evidence that ionomycin alters cellular metabolism as well as acting as an apparent stimulator of secretion.

Effects of prolactin deficiency

The aim of these experiments was to see whether the decreased secretion of milk products observed in lactating rats made prolactin-deficient with bromocriptine [18] could be reproduced *in vitro*. Slices from lactating and prolactin-deficient lactating rats showed similar basal release in all parameters studied, namely turbidity, release of triacylglycerols, radioactivity and protein (Figures 4a–4d). Addition of oxytocin, ionomycin and prolactin to the incubation medium causes increases above basal levels in turbidity, and release of triacylglycerol and radioactivity (d.p.m.). However, protein release did not increase significantly in the prolactin-deficient group on addition of oxytocin (*P* = 0.13) or prolactin (*P* = 0.08), which contrasts with the higher protein release observed in normal lactating rats in similar conditions (*P* = 0.002 and *P* = 0.03, respectively). Slices from prolactin-deficient rats showed a 21 % smaller increase (*P* = 0.024) in the medium turbidity on addition of oxytocin. The lower increase in turbidity measured in the prolactin-deficient group was due to the decrease in the amount of protein released

rather than to the total triacylglycerol (compare Figures 4b and 4d). There was a 35% decrease in radioactivity (d.p.m.) released in the prolactin-deficient group in the presence of oxytocin (Figure 4c) and tendency for a decrease with ionomycin ($P = 0.09$) when compared with normal lactating rats. These results suggest that release of newly synthesized lipids is inhibited by prolactin deficiency, although the *in vitro* findings are not so marked as those *in vivo* [18].

Concluding remarks

A question of interest is the comparison of the present rates of lipid release *in vitro* with those that occur *in vivo*. The rate of triacylglycerol secretion from a normal lactating rat fed on chow diet, producing 30 ml of milk/day [39], can be estimated to be 7.1 μmol of triacylglycerol/g of mammary tissue per h. The slices from 12 day-lactating rats released 5.2 μmol of triacylglycerol/g of mammary tissue per h in the presence of oxytocin. The rate of secretion of the mammary gland slices is thus comparable with that *in vivo* and has proved to be sensitive enough to reflect differences in secretory rate in the stages of mammary gland development. The rate of very-low-density lipoprotein-triacylglycerol secretion *in vitro* in the presence of non-esterified fatty acids from cultured hepatocytes is 0.038 μmol of triacylglycerol/g of liver per h (i.e. at least 100-fold lower than in mammary gland slices) [40]. Presumably this difference is in part due to the more complex mechanism for lipid secretion in liver [40].

The present experiments suggest that Ca^{2+} is required for maximal release from epithelial cells and that at least part of the action of oxytocin can be explained by stimulation of myo-epithelial cells to release milk products already secreted. It is not known whether myoepithelial cells possess receptors for prolactin, but it is reasonable to speculate that some of the effects of prolactin (which require a 40-fold higher concentration for maximum effect) in increasing the release of milk components might be due to contamination of the preparation of ovine prolactin with oxytocin and/or vasopressin. The use of comparatively high amounts of prolactin (10 $\mu\text{g}/\text{ml}$; 800 nM) in experiments where release (casein) has been measured [10,37] suggests caution in the interpretation of the results. Alternatively, prolactin may be acting directly on secretory cells to promote secretion, but the sensitivity of this effect is clearly low. It must be emphasized that the present experiments do not rule out a direct stimulatory effect of oxytocin on the epithelial cells.

Prolactin deficiency mainly affected release of newly synthesized lipid from the slices and the total amount of triacylglycerol released after oxytocin and prolactin stimulation was not altered. This implies that the 'old' droplets accumulated in the cytoplasm can be more easily released by hormonal stimulation *in vitro*, whereas 'new' droplets, which are transferred at a lower rate under these circumstances, are retained inside the epithelial cell. If the rate of exocytosis of other milk components is coupled with lipid apocrine secretion in the epithelial mammary cell [6,7] and a flux of membrane material is important for the formation of milk fat globule membrane, then a decrease in the amount of protein contributing to its formation in prolactin-deficiency might limit the process.

We are only aware of one other study where the effects of manipulation *in vivo* on the hormonal (prolactin) stimulation of release (casein) from mammary tissue *in vitro* have been examined [37]. The present experiments suggest that it is a valid approach

and it should provide a model for studying signalling systems involved in the regulation of milk lipid secretion and extrusion.

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