Rat prolactin synthesis by lactating mammary epithelial cells

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Abstract It has previously been suggested that the mammary cell could produce prolactin (PRL). This hypothesis was investigated by incubation with [^{35}S]methionine-cysteine followed by SDS-PAGE, immunoblotting and autoradiography of immunoprecipitated PRL, and by electron microscopic analysis after incubation without or with cycloheximide. Immunoreactive 14-, 23-, 25-, 32- and 36-kDa PRL forms were radioactive. By two-dimensional electrophoresis analysis, immunoreactive and radioactive spots, of about 25 kDa and high molecular weight, were also detected. After incubation of mammary epithelial cells with cycloheximide, immunogold electron microscopy showed a drastic decrease of labelling in organelles involved in synthesis and secretion, compared to those incubated in control medium. These results make it possible to conclude that lactating mammary tissue is able to synthesize PRL.

Key words: Prolactin; Synthesis; Mammary epithelial cell; Lactation; Bromocriptine

1. Introduction

Prolactin (PRL), first discovered in the pituitary gland, is now thought to be produced in a variety of mammalian tissues [1–5]. The PRL mRNA has been observed in several cell types [6–9], including mammary epithelial cells (MEC) of lactating rat, goat, sheep and rabbit [10–13] and of normal and neoplastic tissue [14]. Newly synthesized PRL has been detected in a breast cancer cell line [15]. However, PRL synthesis in lactating mammary tissue has never been reported until now. PRL detected in the milk has been considered a pituitary plasma-borne PRL. This hormone is carried across the MEC [16,17] and transferred into milk in a variety of bioactive and immunoreactive variants [18–20]. In lactating MEC, PRL was located inside the organelles involved in endocytosis and also in synthesis and exocytosis. When rats were injected with bromocriptine, the labeling was shown to increase in the later compartments and to decrease in the endocytic region. Subsequently, it was suggested that PRL located in the MEC could be of two origins [21].

The goal of this study was to elucidate whether lactating MEC synthesize PRL. Two approaches were used. First, newly synthesized proteins were labeled in vitro to detect newly synthesized PRL after immunoprecipitation. Second, mammary tissues were incubated in the absence or presence of cycloheximide, an inhibitor of protein synthesis, then intracellular localization of the hormone was carried out by immunogold electron microscopy.

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2. Materials and methods

2.1. Animal treatments

Female Wistar rats on day 14 of lactation, weighing 180–250 g, originating from our laboratory, were used for all experiments. All animal experiments were conducted in accordance with the highest standards of care, as outlined in the NIH guide for the care and use of laboratory animals.

To compare between control and bromocriptine-treated rats, one group (control) received the drug vehicle only. The second group was injected subcutaneously with 800 μg of bromocriptine (CB 154 from Sandoz, Hanover), twice daily (9 h, 17 h) for 3 days and the 4th day, 30 min before decapitation.

2.2. Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was extracted from the mammary gland, muscle and pituitary gland using a modified version of the guanidinium-phenol method [22].

The sequences of primers actin 1 corresponding to positions 2746–2767 (exon 5): 5′-CATCCGTAAAGACCTCTATGCC-3′ and actin 2 to positions 3019–3039 (exon 6): 5′-TAGAGCCACCAATCCACACAG-3′ were deduced from the rat gene encoding cytoplasmic β-actin (accession number V01217). Primers PRL1: 5′-TGTTCTGGTGGCGACTGCCA-3′ and PRL2: 5′-TATCTTTTCGATCCCTTCGAGAAGCCG-3′ correspond to primers of rPRL-A (exon 2) and rPRL-C (exon 4), respectively, used by [4]. Oligonucleotides were provided by Oligoexpress (Paris, France).

Reverse transcription reactions were carried out using 2.5 μg of total RNA of mammary gland or muscle or 0.5 μg of pituitary total RNA in the presence of 1×RT buffer (Gibco-BRL Life Technologies, Cergy-Pontoise, France), 1 mM DTT, 2 mM of each dNTP and 40 units RNasin (Promega, France). Reactions were primed with 10 mM oligo(dT) by incubating for 1 h at 37°C in the presence of 200 units of superScript II reverse transcriptase (Gibco-BRL) which was subsequently inactivated by heating at 95°C for 5 min. The reaction mix was then adjusted to 50 μl with distilled water.

PCR amplification was performed in 50 μl essentially as described [23], with buffer and *Taq* DNA polymerase supplied by Promega. For actin DNA amplification, after the initial denaturing step (94°C for 5 min), 25 cycles of amplification were performed: 40 s at 94°C, 40 s at 60°C and 40 s at 72°C. For PRL DNA amplification, after the initial denaturing step (94°C for 40 s), 35 cycles of amplification were performed: 40 s at 94°C, 40 s at 55°C, 45 s at 72°C. 20 μl of each reaction mix were fractionated in a 3% agarose TBE 1× slab gel in the presence of ethidium bromide (0.5 μl/ml).

2.3. Incubation and metabolic labeling

Mammary fragments were obtained after removing fat and connective tissues (weight of each fragment 0.1–0.2 mg; total weight 2 g). After washing and 30 min of preincubation in Hanks' medium (Gibco-BRL), at 37°C, atmosphere 95% O_2+5% CO_2, the fragments were incubated in methionine and cysteine-free RPMI medium (Gibco-BRL) for 45 min at 37°C, rinsed extensively with the same medium, then labeled for 45 min at 37°C with Pro-Mix [^{35}S] Cell labeling mix (85 μCi per ml, Amersham, UK). Mammary fragments were then extensively washed and homogenized as described below.

In order to deprive the fragments of circulating PRL and to inhibit protein synthesis, fragments were preincubated for 30 min in Hanks' medium, rinsed extensively, then incubated 3 times (30 min each) in RPMI medium or in RPMI containing 0.35 mM cycloheximide (Sigma Chemical Co., St. Louis, MO). After each 30 min incubation, the medium was replaced by a fresh one. At the end of incubation, frag-

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ments were treated for immunogold electron microscopy as described below.

To verify the effect of cycloheximide on protein synthesis, metabolic labeling was carried out in the presence of this inhibitor. Briefly, following a 30 min preincubation in Hanks' medium, fragments were incubated for 45 min with methionine and cysteine-free RPMI medium in the presence or absence of 0.35 mM cycloheximide, then labeled for 45 min with Pro-Mix [^{35}S] (85 μCi per ml) in the presence or absence of 0.35 mM cycloheximide. Radioactivity of trichloracetic acid precipitated proteins was counted as described in [16].

2.4. Preparation of tissue extracts and immunoprecipitation

Each gram of tissue was homogenized in 1 ml of 10 mM HEPES, 150 mM NaCl, pH 7.5, containing a protease inhibitor cocktail (aprotin, pepstatin A, leupeptin, chymostatin and antipain 2 μg/ml final each; Sigma). The homogenate was spun at 600×*g* for 15 min at 4°C. After adding 50 μl of 10% Triton X-100, the supernatants underwent three cycles of freeze-thawing and were centrifuged in a Beckman TL100 Ultracentrifuge at 100 000×*g* for 30 min at 4°C, to eliminate casein micelles which could be pelleted during immunoprecipitation.

Immunoprecipitation was carried out as described in [21]. Briefly, each supernatant supplemented with solubilizing buffer (1 v:1 v) was incubated with 2 μl preimmune rabbit serum and 50 μl protein A sepharose, then the mixture was centrifuged at 15 800×*g*. The supernatant was incubated with 3 μl anti-rPRL antiserum IC-5 (generously supplied by The National Hormone and Pituitary Program, NIDDK) for 12 h at 4°C, then with 40 μl of protein A sepharose for 90 min at room temperature. Beads were washed with appropriate buffers. After centrifugation at 15 800×g for 1 min, proteins attached to beads were solubilized in 50 μl of Laemmli sample buffer and supernatants were pooled. Proteins were analyzed by SDS-PAGE or two-dimensional electrophoresis followed by immunoblotting and autoradiography.

2.5. SDS-PAGE and immunoblotting analysis

Samples were resolved by SDS-PAGE in 15% gel under reducing conditions, and transferred to nitrocellulose membrane (Schleicher & Schüll, Keene, NH). Membranes were saturated and incubated for 2 h with anti-rPRL antiserum at 1:1000, then incubated for 60 min with peroxidase-conjugated monoclonal anti-rabbit IgG (γ-chain specific; 1:500; Sigma), after washing. Proteins were revealed using an enhanced chemiluminescence detection kit (Amersham, UK).

2.6. Two-dimensional electrophoresis

Proteins obtained by immunoprecipitation were precipitated with cold acetone, then solubilized in lysis buffer. Samples were analyzed by two-dimensional electrophoresis performed basically according to [24]. They were first subjected to isoelectric focusing in the presence of 9.5 M urea capillary gels containing 2.5% of ampholines pH 3.5–5, 2.5% of ampholines pH 5–7 and 2% of ampholines pH 3.5–9.5. The pH gradient in capillaries showed a range of 4.35–6.95. After isoelectric focusing, capillary gels were incubated in equilibration buffer (0.062 M Tris-HCl pH 6.8; 2.3% SDS; 5% β-mercaptoethanol and 10% glycerol) then subjected to electrophoresis in the second dimension in 12.5% gel.

Nitrocellulose filters from SDS-PAGE and two-dimensional electrophoresis followed by immunoblotting left for one night at 4°C, were washed and dried, then exposed to a BioMax film (Kodak, New York) at −80°C.

2.7. Immunogold electron microscopy

Mammary fragments were fixed in 2% paraformaldehyde–0.1 M sodium cacodylate buffer, dehydrated with ethanol and embedded in Unicryl (Tebu, France). Thin sections were processed for immunogold labeling (see [21]) using anti-rPRL antiserum (1:100) and gold-conjugated anti-rabbit IgG (1:300). Control sections were treated similarly using a nonimmune rabbit serum or with omission of primary antibody.

Mammary tissues from two animals in each group (control and bromocriptine-treated group) were used to quantify the PRL labeling in the MEC. In each experiment, ultrathin sections of standard thickness (~80 nm) from mammary tissue incubated in the absence or presence of cycloheximide were processed in pairwise fashion during immunogold labeling. The number of gold particles (G) and their distribution (%G) were counted on micrographs taken at random at the same magnification (×10 000). At this low magnification, different cellular compartments were illustrated on each micrograph. Forty micrographs (20 from each control rat) and 20 micrographs (10 from each bromocriptine-treated rat) were used. The area of cellular compartments (P) was evaluated by spot numbering [25].

3. Results

3.1. Detection of PRL mRNA in the mammary tissues from control and bromocriptine-treated rats

Total RNA prepared from lactating rat mammary tissue, muscle and pituitary gland was reverse transcribed and amplified using primers complementary to nucleotides of actin and PRL genes. Fig. 1B shows that the signal corresponding to PRL was detected in mammary tissue from control and bromocriptine-treated animals. The size of amplified fragments generated using primers PRL1 and PRL2 was in agreement with the size for the full-length 23 kDa PRL mRNA previously described [4] and was identical to those amplified from the pituitary gland.

A comparison of the amount of amplified fragments from different control and bromocriptine-treated rats is given in Fig. 1. This showed that, while the amount of amplified fragments using actin primers remained identical for all the samples from mammary tissues (Fig. 1A), the amount of amplified fragments corresponding to PRL transcripts was variable depending on the animal (Fig. 1B).

3.2. In vitro synthesis of PRL in lactating rat mammary tissue

To verify whether PRL mRNA present in these mammary tissues could be translated to express the protein, mammary fragments were labeled with Pro-Mix [^{35}S], homogenized and immunoprecipitated with anti-rPRL antiserum. As revealed by immunoblotting, immunoreactivity was associated with protein bands of 14, 23, 25, 32 and 42 kDa (Fig. 2A). It must be noted that the immunodetectable band corresponding to the 25-kDa form was always more intense than the 23-kDa band. The 14- and 23-kDa forms were more or less detectable or not detectable, depending on the animal.

Autoradiography performed on the same nitrocellulose filters showed radioactive bands which superimposed exactly onto the 14-, 23-, 25-, 32-, and 42-kDa immunoreactive bands. Radioactivity associated with these forms of PRL strongly suggested that PRL was synthesized during the labeling. Immunoprecipitates were further analyzed by two-dimensional electrophoresis followed by immunoblotting and autoradio-

Table 1
Effect of cycloheximide treatment on the distribution of gold particles, in cellular compartments of MEC from control rats

		1	2	3	4	5	6
A	%P	3	3.4	14.7	16.1	3.9	7.5
	G	20	72	68	204	28	30
	%G	4.7	17	16.1	48.3	6.6	7.1
B	%P	6.3	2.8	15	16.6	7.3	1
	G	17	39	29	33	19	2
	%G	12.2	28	20.8	23.7	13.7	1.4

MEC were incubated in the absence (A) or presence of cycloheximide (B). The distribution of gold particles was counted on 20 micrographs per rat. %P, Percentage of the cell area occupied by each compartment; G, number of gold particles in each compartment; %G, percentage of total grains. 1, Endocytic region; 2, endosomes plus multivesicular bodies; 3, nucleus; 4, rough endoplasmic reticulum; 5, Golgi apparatus; 6, secretory vesicles. Values from two animals.

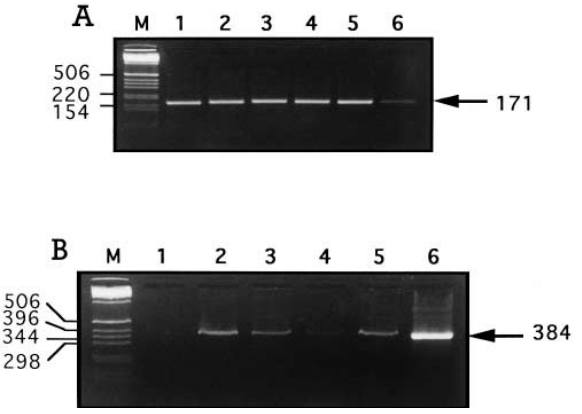

Fig. 1. Detection of PRL transcripts from the mammary gland of lactating rat. Total RNA, extracted from muscle (lane 1), mammary tissue from two control rats (lanes 2 and 3) and two bromocriptine-treated rats (lanes 4 and 5), pituitary gland (lane 6), was subjected to RT-PCR using either primers actin 1 and actin 2 (A), or primers PRL1 and PRL2 (B). Amplified fragments were stained with ethidium bromide. The size of the single fragments (in bp) generated by PCR is indicated on the right and of molecular standards (line M) on the left.

graphy to eliminate the risk of contamination by other newly synthesized proteins. Fig. 3A showed that PRL of about 25 kDa and high molecular weight forms were immunologically detectable. Autoradiography of the nitrocellulose filter revealed that these forms were also radioactive (Fig. 3B). In these conditions the 14- and 23-kDa forms were not detectable. It could be supposed that the very low quantities of these forms of PRL, always smaller than the 25-kDa form, make it impossible to detect them by two-dimensional electrophoresis.

Samples from homogenized and immunoprecipitated tissue previously incubated with cycloheximide did not shown any immunoreactive or radioactive band (not shown).

Specificity of the PRL detection with anti-rPRL IC5 in tissue extracts and immunoprecipitates was checked as previously described [21].

3.3. *In vitro effect of cycloheximide on the localization of PRL in mammary epithelial cells*

As attested by the radioactivity of total tissue precipitable proteins measured after metabolic labeling in the presence or absence of cycloheximide, protein synthesis was inhibited by

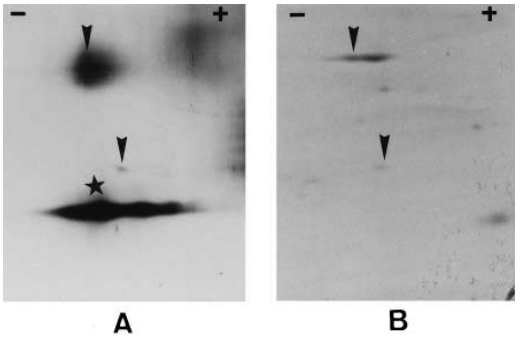

Fig. 3. Two-dimensional electrophoresis analysis of immunoprecipitated rPRL from mammary tissue labeled with Pro-Mix [^{35}S] label. A: Immunoblot with anti-rPRL antiserum. ★: cold PRL added to the sample. B: Autoradiogram of the same nitrocellulose membrane. Arrowhead indicates ^{35}S-labeled spots which are immunoreactive.

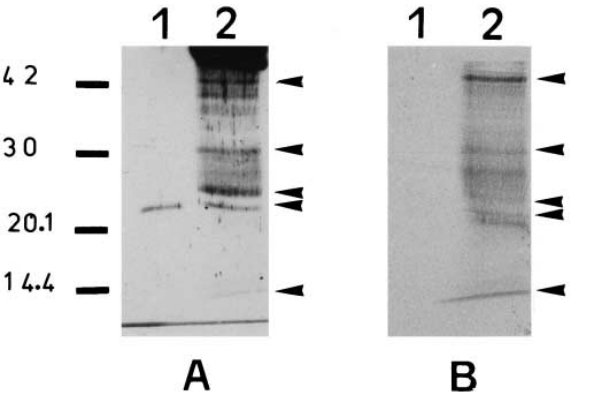

Fig. 2. SDS-PAGE analysis of immunoprecipitated rPRL from mammary tissue labeled with Pro-Mix [^{35}S] label. A: Immunoblot detection with anti-rPRL antiserum. Lane 1: Standard rPRL-B6. Lane 2: Tissue extract. B: Autoradiogram of the same nitrocellulose membrane. Arrowhead indicates ^{35}S-labeled bands which are immunoreactive. Molecular mass markers are indicated on the left.

95% (radioactivity was 132 728 cpm/mg of tissue incubated in control medium and 6400 cpm/mg of tissue incubated in medium containing cycloheximide).

After incubation in the presence of cycloheximide, the morphological aspect of the MEC was modified as attested by the relative volume (% P) of cell compartments (Table 1). Golgi stacks were swollen and very few secretory vesicles were observed. Consequently, the relative volume of these organelles was considerably decreased. This observation revealed that in the absence of protein synthesis, very few secretory vesicles were present in the cell.

Immunogold labeling showed that, after incubation in the absence (Fig. 4a,c) or presence (Fig. 4b,d) of cycloheximide, MEC were faintly labeled. To quantify the intracellular PRL distribution in organelles of MEC, the percentage of the total gold particles was expressed in the following compartments: basolateral membranes and endosomes close to the basolateral membrane considered as the endocytic region; endosomes plus multivesicular bodies; nucleus; rough endoplasmic reticulum (RER); Golgi apparatus and secretory vesicles. It appeared clearly that in MEC incubated in the presence of cycloheximide, the total number of gold particles was lower than in MEC incubated in the control medium (139 versus 422; see Table 1). This phenomenon was particularly noticeable in RER and secretory vesicles but also in nucleus, in Golgi apparatus and in endosomes plus multivesicular bodies. The distribution of gold particles indicated the same decrease of labeling in RER and secretory vesicles and revealed a concentration in basolateral membrane, in endosomes plus multivesicular bodies.

When MEC from bromocriptine-treated rats were incubated in the absence or presence of cycloheximide, a large decrease of the relative volume of secretory vesicles containing casein micelles was observed. In addition, vesicles empty of any electron dense material accumulated in the cell (Fig. 5a,b). These were counted separately and designated 'non-identified vesicles'. There was no difference in the total number of gold particles with or without cycloheximide (Table 2). However, the number of gold particles counted in MEC in the presence of the inhibitor was decreased in RER, secretory vesicles, nucleus and Golgi apparatus and was increased in endosomes plus multivesicular bodies. The category of 'non-

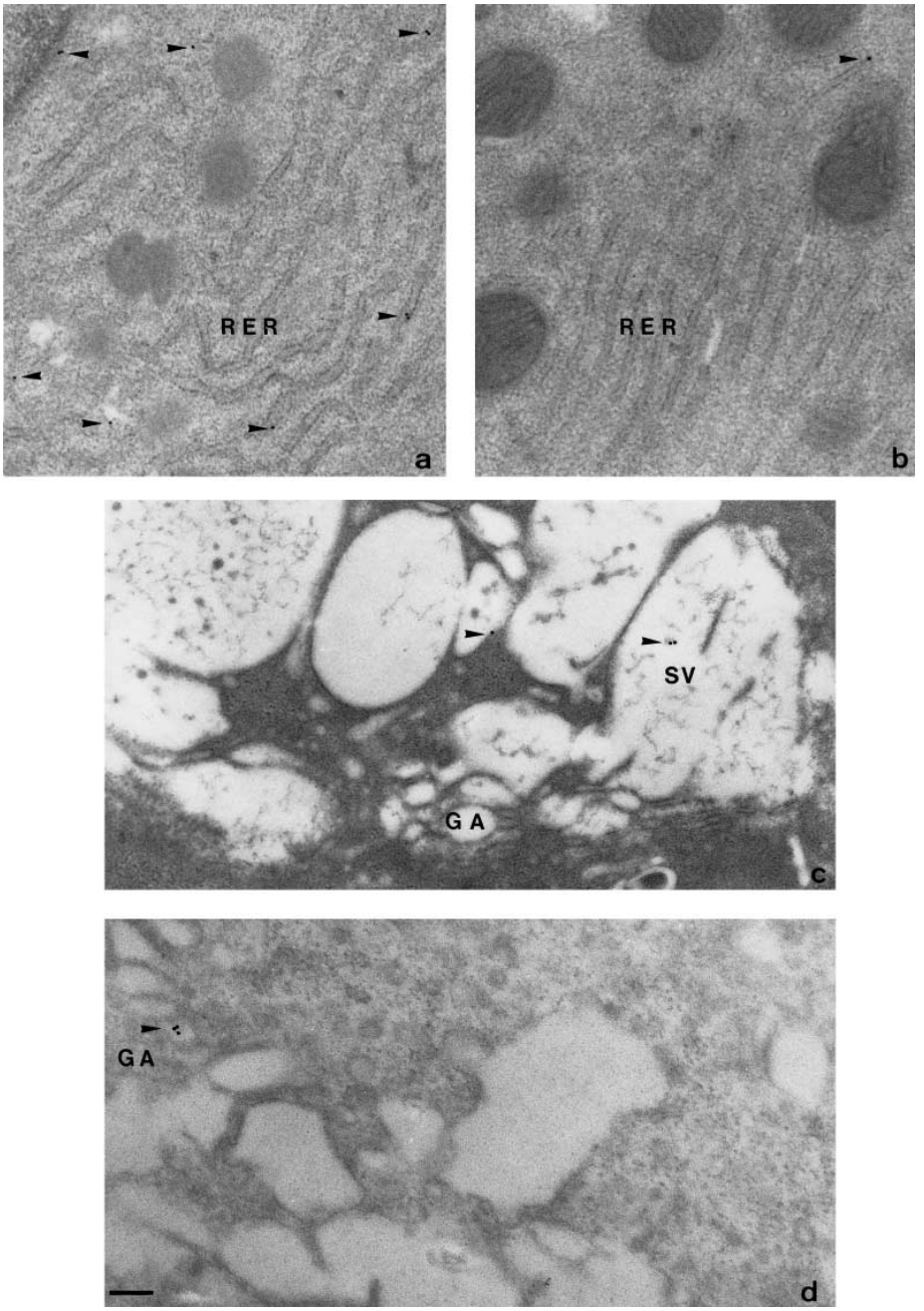

Fig. 4. Immunogold electron microscopy localization of rPRL in MEC from control rats, incubated in the absence (a and c) or presence of cycloheximide (b and d). a: Gold particles are located in RER (arrowhead). b: After incubation with cycloheximide, very few gold particles are located in RER. c: Secretory vesicles, containing filamentous material and casein micelles, are labeled. d: After incubation with cycloheximide, Golgi stacks are swollen, Golgi vesicles are faintly labeled. RER, rough endoplasmic reticulum; GA, Golgi apparatus; SV, secretory vesicle. Bar = 0.2 μm.

identified vesicles' was also labeled. The distribution of gold particles confirmed the decrease of labeling of RER and secretory vesicles when protein synthesis was inhibited. Unexpectedly, distribution of labeling increased in endosomes plus multivesicular bodies but was not modified in the basolateral region.

4. Discussion

This study strongly supports the hypothesis that the MEC are able to synthesize PRL during lactation. Detection of PRL mRNA in mammary tissue, from control or bromocriptine-treated rats, is in accordance with previous reports [10–13]. Although the RT-PCR realized were not quantitative, results obtained with amplified PRL and actin fragments, taken together, suggest an individual variability. Subsequently, no relationship between the level of circulating PRL and the amount of PRL mRNA could be established. This variability was also reported in other species [12,13].

Detection of radioactive proteins related to PRL immunoprecipitated after metabolic labeling revealed a synthesis of PRL. This immunoreactive and radioactive PRL was detected

in 14-, 23-, and 25-kDa forms, in addition to some high molecular weight forms. Molecular variants of PRL have been widely reported in pituitary, serum and milk (for review see [26]). The pituitary 25-kDa form has been described as a glycosylated variant [27,28]. The newly synthesized PRL reported in breast cancer cell lines has been described as multiple glycosylated species [15]. It can be postulated that lactating MEC are also able to synthesize a glycosylated form. Moreover, synthesis of PRL as a 23-kDa form by lymphoid cells has been reported [5]. In the hypothalamic-neurohypophyseal system, synthesis of the 23-kDa form of PRL and its processing into a 14-kDa fragment has also been described [4]. Since mammary tissue is able to cleave plasma-borne PRL [29,30], the present results suggest that a similar processing of newly synthesized PRL occurs in MEC.

High molecular weight forms could correspond to oligomeric PRL complexes, which have been widely reported among species (for review see [26]). Mena et al. [31] studying phosphorylated PRL have observed a 45-kDa form in two-dimensional electrophoresis under reducing conditions. In lymphoid cells, in addition to the 23-kDa form, a 36-kDa form has been described as newly synthesized PRL [5]. However, in these cells as in present work, radioactive molecules not immunologically related to PRL were also observed after immunoprecipitation. It could correspond to no specific adsorption of proteins.

After incubation, few PRL remained immunocytochemically detectable in these cells (10% of the hormone detected in lactating cells without incubation [21]). Cycloheximide led to a drastic decrease of labeling in organelles involved in synthesis and exocytosis, in both MEC from control and bromocriptine-treated rats, confirming the role of the MEC in the synthesis of the hormone. Unexpectedly, persisting labeling remained on the basal membrane.

As shown by morphological observation of mammary tissues, after incubation in the presence of cycloheximide, the number of secretory vesicles was strongly decreased. Continuous secretory protein synthesis could be very important for the formation of secretory vesicles. In mammary tissues from bromocriptine-treated rats inhibition of protein synthesis induced morphological modifications resulting in the accumulation of numerous empty vesicles. Since in these experimental conditions plasma-borne PRL was strongly reduced by bromocriptine and since no PRL cannot be synthesized by the cell because of the inhibition of protein synthesis, we may wonder whether these morphological modifications are related to the very low level of PRL.

Table 2
Effect of cycloheximide treatment on the distribution of gold-labeled PRL, in cellular compartments of MEC from bromocriptine-treated rats

		1	2	3	4	5	6	7
A	%P	6.1	2.6	11.4	16.8	2.6	4.6	–
	G	55	73	91	233	34	26	–
	%G	10.7	14.2	17.8	45.5	6.6	5	–
B	%P	5.3	3.4	8.3	13.2	2.2	0.8	9
	G	47	145	55	117	25	3	25
	%G	11.3	34.8	13.2	28	6	0.7	6

MEC were incubated in the absence (A) or presence of cycloheximide (B). The distribution of gold particles was counted on 10 micrographs per rat. P, G and cellular compartments as in Table 1. In addition, 7, non-identified vesicles. Values from two animals.

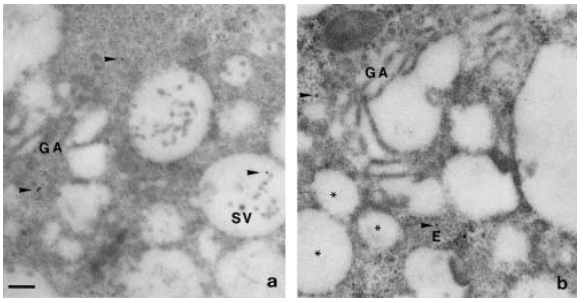

Fig. 5. Immunogold electron microscopy localization of PRL in MEC from bromocriptine-treated rats, incubated in the absence (a) or presence of cycloheximide (b). a: After incubation in control medium, secretory vesicles containing filamentous material and casein micelles and small Golgi vesicles are labeled. b: After incubation in the presence of cycloheximide, vesicles empty of electron dense material accumulate (*). Small vesicles in the Golgi apparatus region were labeled. E, endosome-like vesicle; GA, Golgi apparatus; SV, secretory vesicle. Bar = 0.2 μm.

The synthesis of PRL by MEC raises questions about the functional implication of this production. It is well known that MEC is a target for PRL which regulates transcription of milk protein genes [32], and exerts a secretagogue effect [33]. In these conditions, how could production of PRL by the MEC be of importance for the physiology of the cell? Autocrine or 'intracrine' effects of PRL regarded as a local growth factor have been hypothesized since proliferation of mammary cell line was inhibited by neutralization of newly synthesized PRL [34]. It was reported that the glycosylated form of PRL has the same or less bioactive potency than the standard form (for review see [26]). Moreover, the 14-kDa form of PRL synthesized by the hypothalamus presents mitogenic and lactogenic potencies [29] and anti-angiogenic effects [35]. The question whether processing of PRL in the MEC can convert the hormone into bioactive products is raised. Such a phenomenon has been described in the case of the proparathyroid hormone related peptide which is processed intracellularly to a more active product [36].

On the other hand, a role of the maternal PRL, carried by the milk, on the development of the neonate has been suggested [37]. The ability of the MEC to produce PRL in various forms may be a way of ensuring a supply of hormone in milk to the neonate when the maternal plasma-borne PRL level is very low.

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