Original Paper

Neuro
-endocrinology

Neuroendocrinology
DOI: 10.1159/000161113

Received: November 28, 2007
Accepted after revision: July 1, 2008
Published online: October 2, 2008

Pituitary Changes Involved in Prolactin Secretion Induced by Mifepristone and Naloxone during Late Pregnancy

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Key Words
Prolactin · Pregnancy · Pituitary · Opiate peptides · Naloxone · Progesterone · Mifepristone

Abstract
Background/Aims: The antiprogesterone mifepristone facilitates prolactin release, an effect enhanced by administration of the opioid antagonist naloxone. The present study explores ultrastructural changes in lactotropes after mifepristone and naloxone administration, correlating them with the expression of pituitary prolactin. ***Methods/Results:*** Rats were sacrificed at 18:00 h on day 19 of pregnancy. Prolactin immunoelectron microscopy of lactotropes from control rats showed characteristics of quiescent cells with numerous small and spherical secretory granules. Naloxone administration did not modify lactotrope morphology or prolactin expression in terms of mRNA or protein abundances. Mifepristone treatment induced lactotrope activation with development of the rough endoplasmic reticulum and Golgi complex with prolactin immunoreactive small newly formed and large mature secretory granules. Mifepristone increased prolactin mRNA and protein expression. Naloxone administration to mifepristone-treated rats potentiated lactotrope activation compared with mifepristone alone showing exocytotic images of prolactin granules and some cells with evident signs of involution. ***Conclusions:*** (1) Blockade of progesterone action by mifepristone activated the lactotrope, increased significantly prolactin mRNA and protein expression and prepared the pituitary for naloxone action. (2) The high serum prolactin levels induced by mifepristone and naloxone may regulate negatively lactotrope activity as suggested by the presence of regressing cells neighboring the actively secreting cells.

Introduction

Numerous observations obtained from cycling, lactating and ovariectomized steroid-primed rats indicate that endogenous opioid peptides and opiate agonists suppress the activity of tuberoinfundibular (TIDA) neurons [1–3] thereby increasing prolactin secretion [4, 5]. Moreover, it has also been shown that a continuous infusion of naloxone is able to prevent the prolactin surge during early pregnancy and the ante partum prolactin surge and in-

Ricardo Deis passed away on April 26, 2008.

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crease the transcriptional activity of TIDA neurons [6, 7]. However, late pregnancy seems to be a particular endocrine period with respect to the regulation of prolactin secretion by the opioid system. In previous reports [8–10], we demonstrated that the blockade of the central inhibitory action of progesterone on prolactin release by the antiprogesterone mifepristone [11] facilitated prolactin release in rats on day 19 of pregnancy. The effect of mifepristone was enhanced by the administration of the opioid antagonist naloxone, suggesting an inhibitory-neuromodulatory role of the opioid system at the end of pregnancy [8, 9]. Furthermore, we have recently showed that mifepristone is able to inhibit the hypothalamic dopaminergic neuronal system in terms of dopaminergic transmission and tyrosine hydroxylase (TH) expression [12], thus lowering the inhibitory dopaminergic tone upon prolactin secretion.

There is no consensus about how the opioid system regulates prolactin secretion at the end of pregnancy compared with early pregnancy. During the first days of gestation, endogenous opioid peptides are critical stimulatory factors for the diurnal and nocturnal PRL surge [9, 13] and these actions are partially mediated by decreasing TIDA neuronal activity [13, 14]. Moreover, during early pregnancy the existence of alternative pathways by which endogenous opioid peptides may regulate prolactin secretion has been suggested [13]. These authors have shown that at this period of pregnancy, there are more neurons expressing Fos/FRA than Fos/FRA-positive TIDA neurons in the arcuate nucleus. Moreover, it seems that naloxone treatment activates these neurons as well, suggesting an additional pathway by which endogenous opioids may regulate prolactin secretion. Although, at the end of pregnancy there is no evidence of the participation of this neuronal system to control prolactin secretion, the results above may support our recent study [12] which demonstrates that during late pregnancy, naloxone treatment has no direct effect on dopaminergic neurons, either in control oil-treated rats or in mifepristone-pretreated rats. Thus, endogenous opioids may inhibit prolactin secretion during late pregnancy through a nondopaminergic neuronal system without involvement of a direct effect on neuronal DA activity and probably with the participation of as yet undetermined prolactin-releasing factors unmasked by naloxone treatment. Moreover, methodological differences between our results and those of other investigations that have described a direct opioid activity on TIDA neurons [6] include that we used a unique acute i.p. dose of the opioid antagonist vs. a continuous infusion and that we studied rats on day 19 of pregnancy pretreated with mifepristone vs. rats on the last day of pregnancy previously cannulated via the jugular vein. Thus, regulation of prolactin secretion by opioids needs further investigation.

We hypothesize that the fall of dopaminergic tone induced by the antagonism of progesterone action may prepare the lactotrope to respond to naloxone-inducing prolactin secretion. Thus, the major goal of the present study was to explore the mechanisms that mediate the increase of serum prolactin levels after the blockade of progesterone and opioid action by mifepristone and naloxone administration, thus investigating the changes at the pituitary level induced by these treatments. With this aim, we examined the ultrastructural modifications of prolactin cells by immunoelectron microscopy and measured the expression of pituitary prolactin and its mRNA to correlate its variations with the increase in serum prolactin levels.

Materials and Methods

Animals

Virgin female rats, 3–4 months old (200–220 g), bred in our laboratory and originally of the Wistar strain were used. They were kept in a light- (06:00–20:00 h) and temperature- (22 ± 2°C) controlled room; rat chow (Cargill, Argentina) and tap water were available ad libitum. Vaginal smears were taken daily; virgin rats showing two or three consecutive 4-day cycles were used. Rats were made pregnant by being caged individually with a fertile male on the night of proestrus. Vaginal smears were checked for the presence of spermatozoa on the following morning and that day was considered day 0 of pregnancy. Rats from our colony normally deliver on day 22 of pregnancy. Animal conditions were in compliance with the Guidelines on the Handling and Training of Laboratory Animals published by the Universities Federation for Animal Welfare and the local Institutional Animal Care Committee.

Experimental Procedures

We used the same schedule as previously described [8, 9]. Briefly, mifepristone (RU-486) (17β-hydroxy-11β-[4-dimethylamino-phenyl]-17α-propinyl-estra-4, 9-dien-3-one) (provided by Dr. A.F. Parlow, National Hormone and Pituitary Program) was dissolved in sunflower seed oil and injected s.c. at 08:00 h on day 19 of pregnancy at a dose of 5 mg/kg. Control animals were injected with the respective volume of vehicle. Naloxone (Sigma Chemical Co., St Louis, Mo., USA) was dissolved in 0.9% (w/v) NaCl and injected i.p. at a dose of 2 mg/kg at 17:30 h. Rats were decapitated 30 min after administration of naloxone or saline, trunk blood was recollected and the pituitaries were rapidly removed discarding the neurointermediate lobe. Three groups of control and treated rats were used for immunoelectron microscopic, RT-PCR and Western blot analysis, respectively. Serum samples were obtained for determination of PRL levels by RIA.

Immunoelectron Microscopy

Pituitary gland blocks were fixed in a mixture of 2% (v/v) glutaraldehyde and 4% (w/v) formaldehyde in 0.1 M cacodylate buffer and then treated with 1% osmium tetroxide for 2 h, dehydrated in increasing concentrations of acetone and embedded in Araldite. Some blocks were also included in LR-White (London Resin Corp.), a hydrophilic acrylic resin, omitting osmium fixation and dehydrating in increasing concentrations of ethanol. Thin sections were cut with a diamond knife on a Porter-Blum MT2 and JEOL-BLUM-7 ultramicrotomes and mounted on nickel grids. A total of six sections per group, obtained from a total of three pituitary glands, were evaluated. For immunocytochemistry, araldite sections were pretreated with 1% sodium metaperiodate for 15 min to remove osmium tetroxide, while LR-White sections received no pre-treatment. Araldite and LR-White sections were then incubated overnight on a drop of a rabbit anti-rat PRL (NIH) at a dilution of 1:2,000 or 1:4,000, respectively. To validate the specificity of the immunostaining, the primary antiserum was replaced by 1% bovine serum albumin (BSA) in 0.1 M phosphate buffer, pH 7.3 plus 0.15 M sodium chloride (PBS). Bounded specific IgGs were exposed with a protein A gold complex prepared according to Maldonado and Aoki [15]. Sections were observed and photographed in a Zeiss LEO 906E electron microscope.

Total RNA Extraction and RT-PCR

Total RNA was isolated from the anterior pituitaries obtained after the different treatments with the single-step method based on guanidine isothicyanate/phenol/chloroform extraction using TriZol (Gibco-BRL, Inc.) according to the manufacturer's instructions. RNA concentration was determined by absorbance at 260 nm and its integrity was verified by electrophoresis on 1.5% agarose gel. Reverse transcription (RT) was carried out using 5 μg of total RNA obtained from the hypophysis of each rat. RT was performed at 37°C for 60 min with 200 U of Moloney murine leukemia virus reverse transcriptase (Gibco-BRL, Inc.). Before proceeding with the semiquantitative PCR, the conditions were established for each mRNA such that the amplification of the products was in the exponential phase, and the assay was linear with respect to the amount of input cDNA. For the PCR amplification, specific oligonucleotide primers (0.5 μM each) were incubated with 5 μl of cDNA template in a 35-μl PCR reaction mixture containing 1.5 mM $MgCl_2$, 25 mM KCl, 10 mM Tris-HCl, pH 9, 1 μl deoxynucleotides (1 mM each), and 1 unit Taq polymerase (Invitrogen Life Sciences, Argentina). The sequences of the specific primers for amplification of the PRL mRNA were: sense primer: 5′-CTCCTGCTGATGATGTCAAACCT-3′ and antisense primer: 5′-ATGATAGCATCAGGAGCTTCATG-3′ [16] and for amplification of β-actin were: sense primer 5′-CGTGGGCCGCCCTAGGCACCA-3′ and antisense primer 5′-TTGGCCTTAGGGTTCAGAGGGG-3′ (BC063166). The thermal cycling program for prolactin PCR amplification was as follows: 95°C for 1 min 20 s, 62°C for 1 min 20 s, 72°C for 1 min 30 s for 23 cycles, followed by an elongation step of 5 min at 72°C. The same protocol was used for β-actin amplification with an annealing temperature of 56°C and 30 cycles. Samples from control (n = 8) and treated rats (n = 8) were run and processed simultaneously. RNA samples were assayed for DNA contamination by PCR without prior RT. The amplicons (PRL 380 and β-actin 243 bp) were analyzed on 1.5% agarose gels containing 0.5 mg/ml ethidium bromide and photographed with a Kodak DC-290 camera. Band intensities of the RT-PCR products were quantified using the NIH Image software; relative levels of mRNA were expressed as the ratio of signal intensity for the target genes relative to β-actin cDNA.

Western Blot Analyses

After decapitation, the pituitaries were rapidly removed, the neurointermediate lobe was discarded and immediately placed in 1 ml ice-cold 0.25 M sucrose, 20 mM Tris HCl, 2 mM $MgCl_2$ buffer with a mix of protease inhibitors (2 μg/l of aprotinin, leupeptin and pepstatin), homogenized with a glass Teflon homogenizer and centrifuged at 500 rpm for 15 min. The pellets were discarded and the supernatants collected and centrifuged at 5,000 rpm for 30 min at 4°C. All steps were carried out on ice except when otherwise noted. The supernatants were recovered, frozen at –80°C until used for prolactin immunoblots and for measurement of total protein concentration by the Bradford assay on triplicate aliquots. Previously, we tested the best concentration of the antibody and the adequate quantity of protein to obtain a clear and definable band. Because of prolactin pituitary levels are elevated, several sample dilutions were made. We selected the dilution 1:80 corresponding to 40 ng of protein (fig. 8, inset) and an antibody anti-prolactin diluted 1:50,000. At this concentration, no prolactin variants were present on the blot. Using a more diluted prolactin antibody and a high concentration of protein, we obtained a broad and diffuse band. For these reasons, we had to use separate blots run for prolactin and β-actin, rather than stripping and reproving the blots. Briefly, 40 ng (for prolactin Western blots) or 10 μg (for β-actin Western blots) of protein from each homogenate were loaded per lane and separated by 12.5% SDS-PAGE on minigels in parallel with prestained protein molecular weight standards [17]. Electrophoresis was performed at 100 V for 90 min. Proteins were electrotransferred onto 0.2 μm pore diameter nitrocellulose (Hybond C, Amersham Life Science) in a semidry blotter at 1 mA/cm^2 overnight in 20% methanol/40 mM Tris-glycine (pH 9.0). Liquid transfer efficiency and protein loading were monitored by staining the membranes with Ponceau Red. Membranes were then blocked with 0.5% BSA/2% horse serum/0.1% Triton X-100/phosphate-buffered saline (PBS) for 30 min at room temperature, followed by 90 min incubation in primary antibody antiprolactin diluted 1:5,000 (rabbit polyclonal, NIH, Lot # AFP425_10_91) or anti β-actin dilutes 1:10,000 (Clone AC-15 mouse ascites fluid, A 5441, Sigma) in blocking solution at room temperature. Membranes were then washed five times in 0.1% Triton X-100/PBS over 25 min, incubated with a 1:3,000 dilution in blocking solution of the anti-rabbit or anti-mouse horseradish peroxidase-conjugated secondary antibody (DAKO Corporation, Carpinteria, Calif., USA) for 60 min. Secondary antibody was removed by washing five times in 0.1% Triton X-100/PBS over 45 min, then for 15 min in 10 mM phosphate buffer (pH 7.4). Specific protein bands were detected using a chemiluminescence kit (ECL TM, Amersham Pharmacia Biotech, UK) according to the manufacturer's instructions. Multiple exposures of different times were made to bring the exposures within the linear response range of the film (X-OMAT-AR, Kodak) and then the bands were quantified by densitometry using digital image processing and the freeware program NIH Image 1.6/ppc (developed at the US National Institutes of Health and available on internet at http://rsb.info.nih.gov/nih-image/), using the gel plotting macros after background subtraction (2D rolling ball set to the width of the individual gel lanes). No other digital manipulation of the

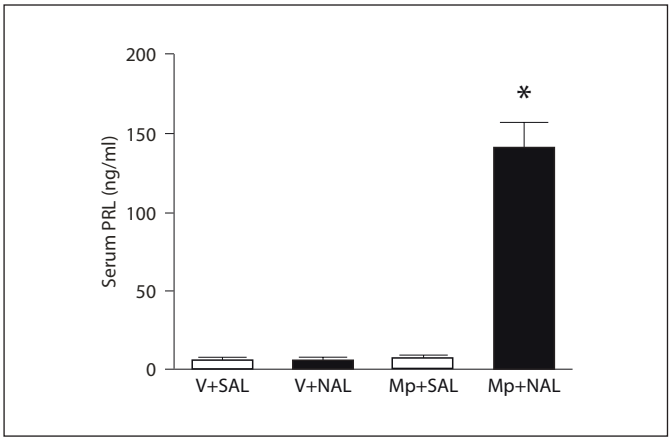

Fig. 1. Effect of saline (SAL) or naloxone (NAL; 2 mg/kg i.p.) on serum PRL concentration on day 19 of pregnancy in rats pretreated with mifepristone (Mp; 5 mg/kg s.c.) or vehicle (V; oil). Results are means ± SEM of 8 animals. * $p < 0.01$ compared with control group.

images was carried out prior to quantification. Gel and film images in the figures had contrast and brightness adjusted for optimum presentation. Samples from control (n = 8) and treated rats (n = 8) were run and processed simultaneously.

Prolactin Determination

Serum concentration of prolactin was measured by a double-antibody radioimmunoassay (RIA) with materials supplied by Dr. A.F. Parlow from the National Hormone and Pituitary Program. Prolactin was radioiodinated using the chloramine T method [18] and purified by passage through Sephadex G-75 and polyacrylamide agarose (ACA 54; LKB, Bromma, Sweden) columns. The assay sensitivity was 1 ng/ml serum and the inter- and intra-assay coefficients of variation were less than 10%. The prolactin antibody does not cross-react with placental lactogen [19].

Statistics

Statistical analysis was performed using analysis of variance followed by the t test for comparison between means. Differences between means were considered significant at the $p < 0.05$ level.

Results

Serum Prolactin Levels after Mifepristone and Naloxone Treatment

As previously described [8], naloxone administration to 19 days' pregnant rats pretreated with mifepristone was able to induce an important increase in serum prolactin levels (fig. 1), meanwhile no change in prolactin level was observed when naloxone was administered alone.

Immunoelectron Microscopy of Anterior Pituitary after Mifepristone and Naloxone Treatment

We studied the ultrastructural changes at the pituitary level produced by the different treatments, identifying lactotrope cells by means of immunoelectron microscopy on thin araldite sections of pituitary glands included following conventional electron microscopy protocols. Figure 2a shows that the most of lactotropic cells belonging to control 19 days' pregnant rats treated with vehicle plus saline have the characteristics of quiescent cells. These lactotropes are characterized by a cytoplasm with poor development of the organelles involved in protein synthesis and numerous small and spherical secretory granules containing prolactin as evidenced by immunogold labeling (fig. 2b). Also, very few or no images of exocytosis or accumulation of immunostained material in the extracellular space were observed (fig. 2b). These results suggest a low rate of hormone release compatible with a reduced biosynthetic activity and a quiescent state. Naloxone administration to rats treated with vehicle did not induce any significant morphological changes, with the lactotropes remaining in a quiescent state.

On the other hand, and in correlation with its effects on prolactin gene expression, mifepristone treatment induced activation of the lactotropes. The cytoplasm showed an important development of organelles involved in protein synthesis, particularly the RER and Golgi complex containing immature secretory granules in formation and the presence of mature secretory prolactin granules labeled with colloidal gold, stored in the cytoplasm (fig. 3a, b). To confirm that the great development of the Golgi complex (fig. 4a) was implicated in the processing and packaging of the PRL into numerous immature secretory granules, we applied immunoelectron microscopy to sections of pituitary gland included in LR White without osmium fixation; a heavy specific labeling for PRL was observed decorating Golgi membranes and the condensing immature granules (fig. 4b).

The administration of naloxone to rats pretreated with mifepristone induced a more important activation of the lactotropes compared with the effect of mifepristone alone as evidenced by the highly developed RER and Golgi complex with numerous granules; in the process of condensation, accumulation of secretory material in the extracellular space and increased numbers of secretory granules undergoing exocytosis next to the plasmatic membrane were also observed (fig. 5, and inset). Interestingly, the presence of some lactotrope cells with signs of involution can also be observed after mifepristone plus naloxone administration. They were characteristic for

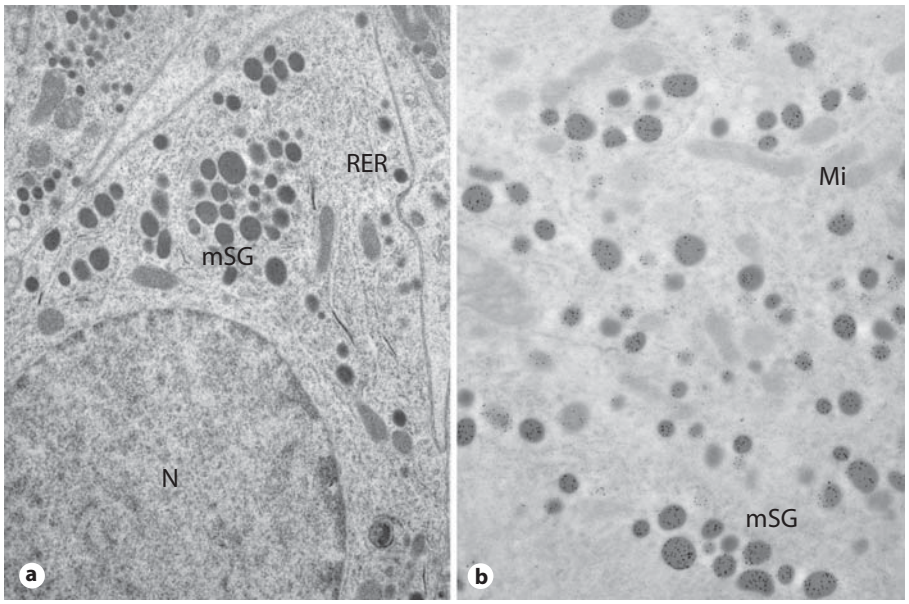

Fig. 2. Electron micrographs of pituitary cells from rats on day 19 of pregnancy treated with vehicle (oil) and saline. **a** A lactotrope with characteristics of a quiescent cell, exhibits numerous round mature secretory granules (mSG), scarce rough endoplasmatic reticulum (RER) and the Golgi complex (GC) is absent. N = Nucleus. ×11,000. **b** Cytoplasmatic portion of a lactotrope cell in quiescent state contains abundant mature secretory granules (mSG) immunolabelled with gold particles. Mi = Mitochondria. ×13,000.

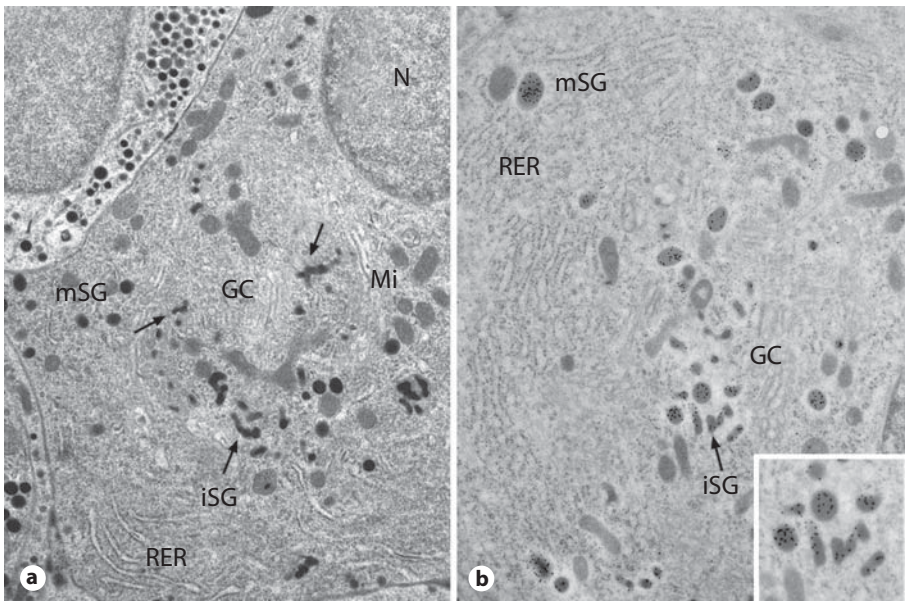

Fig. 3. Electron micrographs of pituitary cells from rats on day 19 of pregnancy treated with mifepristone and saline. **a** An active lactotrope cell can be seen exhibiting an important development of the Golgi complex (GC) and small immature secretory granules in formation (iSG); some mature secretory granules (mSG) can be seen near the plasma membrane. ×8,400. Rough endoplasmatic reticulum (RER) is abundant in this experimental condition. N = Nucleus. **b** A similar active cell is identified as lactotrope by the gold particles specifically labeling iSG and mSG. Mi = Mitochondria. ×12,000. Inset: ×18,000.

the high electron density of the cytoplasmatic matrix, nuclear pycnosis, swelling of mitochondria and dilated RER and Golgi complex (fig. 6). Exocitosis inhibition probably induces accumulation of secretory granules immunopositive for prolactin (fig. 6, inset), which confirms that lactotropes are in these cells.

Prolactin Expression in the Pituitary after Mifepristone and Naloxone Treatment

To correlate the increase of serum prolactin levels obtained after mifepristone and naloxone treatment with changes at the pituitary level, we measured the expression of prolactin mRNA and prolactin protein in the pituitary. Naloxone treatment did not modify prolactin mRNA (fig. 7) or protein (fig. 8) expression when administered alone, which was coincident with the maintenance of quiescent lactotropes. Interestingly, the administration of mifepristone alone significantly increased the levels of expression of prolactin mRNA and protein in correlation with the deep changes found in lactotropes. The administration of naloxone to rats pretreated with oil or mifepristone did not significantly modify the expression of

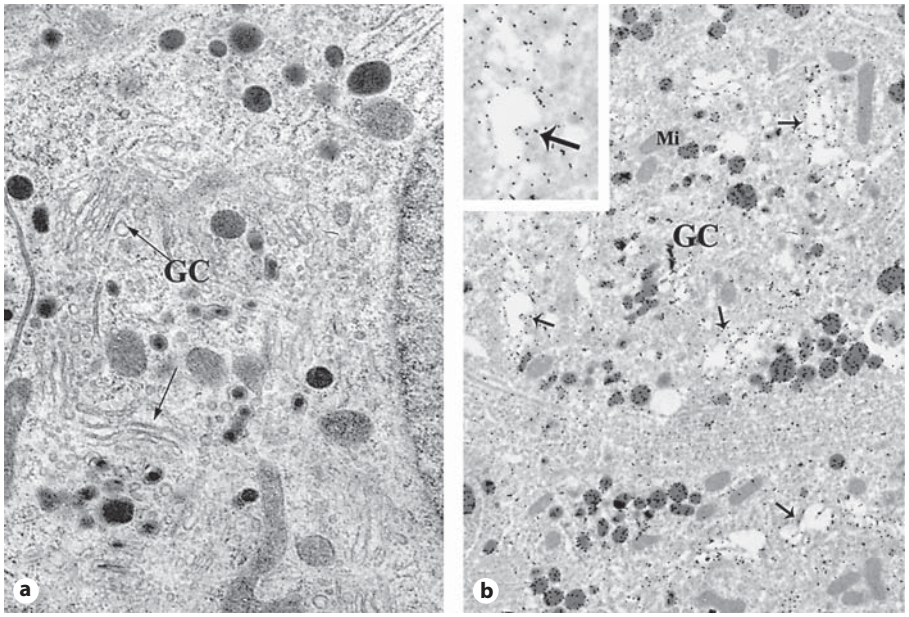

Fig. 4. a Electron micrograph of a well-developed Golgi complex (GC) in a lactotrope from rat on day 19 of pregnancy treated with mifepristone and saline. ×17,000. **b** Immunoelectron microscopy to detect PRL on the Golgi complex (GC) of lactotropes from pituitary glands included in LR-White resin without osmium. Note the intense gold labeling decorating Golgi membranes in lactotropes from rat on day 19 of pregnancy treated with mifepristone and saline. Mi = Mitochondria. ×10,000. Inset: ×25,000.

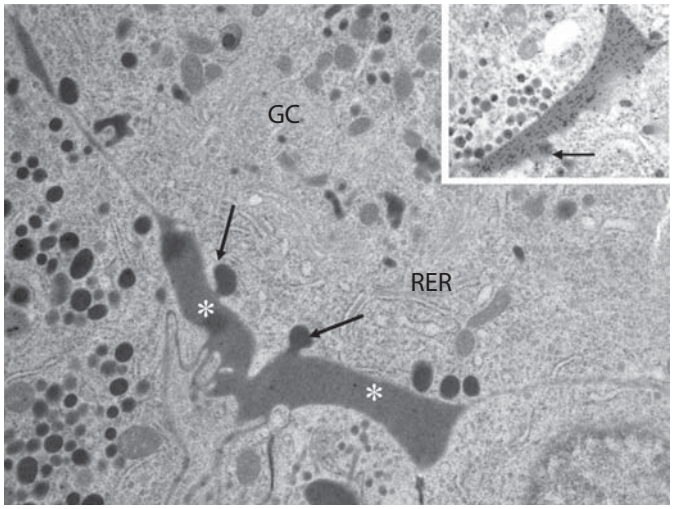

Fig. 5. Electron micrograph of pituitary cells from rats on day 19 of pregnancy treated with mifepristone and naloxone. Cytoplasmic portion of a lactotrope exhibiting an important development of the Golgi complex (CG) and cisternae of RER and accumulation of secretory material in the extracellular space (*) where two mature secretory granules (→) discharge their hormonal content. ×10,000. Inset: Accumulated extracellular material immunolabelled for prolactin (*); a secretory granule in exocytosis can be seen (→). ×16,000.

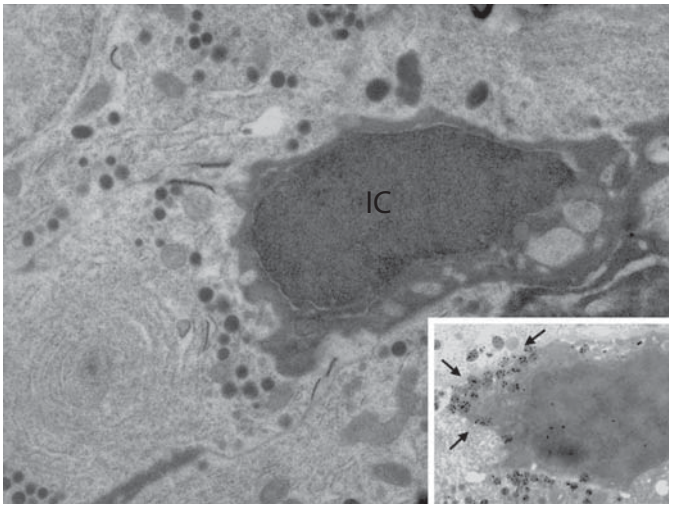

Fig. 6. Electron micrograph of pituitary cells from rats on day 19 of pregnancy treated with mifepristone and naloxone. An involuting cell (IC) can be recognized for the high electron density and nuclear pycnosis. ×10,000. Inset: A similar regressing cell exhibits PRL immunogold labeling on the secretory granules that appear in the periphery (→). ×13,000.

prolactin mRNA (fig. 7) or the protein level (fig. 8); although the mean levels of prolactin mRNA after naloxone administration were slightly lower compared with the group treated with mifepristone alone, this difference did not achieve statistical significance (fig. 7).

Discussion

Accumulated evidence indicates a clear correlation between the decrease of serum progesterone concentrations and the ante partum prolactin surge [20, 21]. Pro-

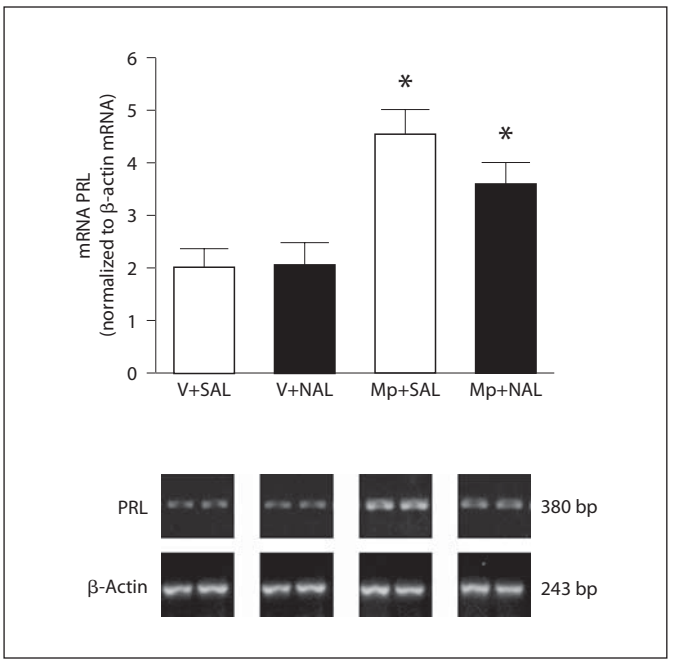

Fig. 7. RT-PCR for PRL in anterior pituitary extracts on day 19 of pregnancy in rats pre-treated with mifepristone (Mp; 5 mg/kg s.c.) or vehicle (V; oil) and/or saline (SAL) or naloxone (NAL; 2 mg/kg i.p.). Representative photos of the PCR gels for each group studied, and densitometry quantification of the bands relative to β-actin. See 'Materials and Methods' for further details. Values represent the means ± SEM of groups of 8 rats. * $p < 0.05$ compared with control animals treated with oil.

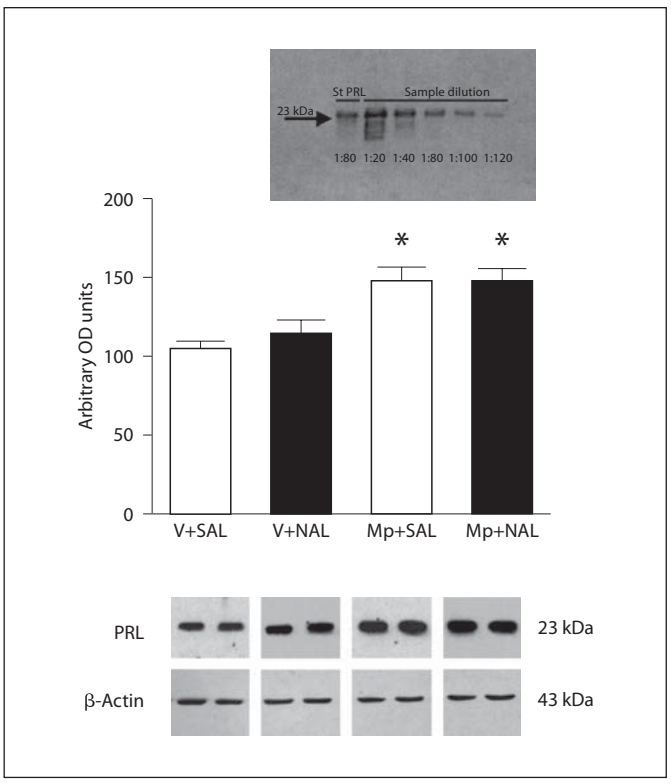

Fig. 8. Western blot for PRL in anterior pituitary extracts on day 19 of pregnancy in rats pretreated with mifepristone (Mp; 5 mg/kg s.c.) or vehicle (V; oil) and/or saline (SAL) or naloxone (NAL; 2 mg/kg i.p.). Representative photos of Western blots for each group studied, and densitometry quantification of the bands relative to β-actin. Blots were probed for β-actin to control for loading of samples. Inset: Photo showing the bands obtained after different dilutions of one control sample compared with a PRL standard preparation (StPRL). See 'Materials and Methods' for further details. Values represent the means ± SEM of groups of 8 rats. * $p < 0.05$ compared with control animals treated with oil.

lactin increases during late pregnancy after removal of the corpora lutea [22], by induction of luteolysis after prostaglandin F2α administration [23] or by estradiol treatment after ovariectomy [7]. In the present study, we demonstrate that the low PRL levels registered at the end of pregnancy correlate with the presence of quiescent lactotropes in the pituitary gland. Quiescent lactotropes have been described in male rats and in female ovariectomized rats, both under conditions of low PRL demand [15]. Besides, we describe that the blockade of progesterone action by mifepristone treatment on day 19 of pregnancy is able to induce lactotrope activation and stimulation of prolactin synthesis, but with a low rate of release of secretory granules and therefore no increment in serum prolactin. Thus, the presence of cytoplasmatic organelles actively involved in protein synthesis, particularly the RER and Golgi complex containing small secretory granules and big mature secretory prolactin granules, suggest such activation. In correlation with the morphological changes, an important increase on prolactin expression was also observed after mifepristone administration. These results confirm previous studies and may provide the major evidence of the inhibitory action of progesterone on prolactin synthesis and secretion during late pregnancy.

One of the mechanisms by which mifepristone could facilitate prolactin synthesis certainly involves inhibition of the hypothalamic dopaminergic neuronal system in terms of dopaminergic transmission and tyrosine hydroxylase (TH) expression. In fact, we have recently demonstrated that mifepristone treatment to rats on day 19 of pregnancy induced a fall in the DOPAC/DA ratio in MBH, a diminution of the expression of TH measured in protein extracts of MBH as well as a decrease in the in-

tensity of TH immunoreactivity in the arcuate nuclei, periventricular nuclei and the nerve terminals of the median eminence [12]. These results complement and confirm previous work showing that progesterone increases hypothalamic dopaminergic tone and TH expression [24, 25]. Based on these results, it is tempting to speculate that after mifepristone treatment, a lesser quantity of DA arrives to the pituitary, thereby lifting at least some of the inhibitory tone that controls prolactin, a potential mechanism by which neurogenic stimuli can induce the release of prolactin.

Moreover, we must consider the role of estrogen in the effects of mifepristone. It is known that estrogen is a potent stimulator of prolactin secretion, acting at two levels. On pituitary lactotropes, estradiol directly regulates transcription of the prolactin gene [26, 27], acts as a potent antidopaminergic agent [28] and modifies the response of the lactotrope to physiological stimulators of prolactin secretion [29]. At hypothalamic levels, estrogen decreases TIDA neuronal activity [24, 25, 30, 31]. During early pregnancy in the rat, serum estradiol levels are low, but start to increase gradually on day 15–16 of pregnancy until reaching the surge that precedes parturition. However, in spite of the increasing influence of estrogen, it seems clear that the high progesterone levels at the end of pregnancy curtail any stimulatory action of estradiol on prolactin secretion. Consistent with this, we have previously demonstrated that the low prolactin levels were not modified by estrogen treatment on days 16 and 19 of pregnancy [9]. By contrast, the decrease of circulating progesterone on day 16 produced by excision of the corpora lutea or by ovariectomy followed by estradiol treatment significantly increased serum prolactin on day 17 of pregnancy [32]. Based on this evidence and considering that progesterone can inhibit estrogen-induced prolactin gene expression [33], we can speculate that the antiprogesterone effect of mifepristone treatment allows estrogen to act freely to stimulate the mechanisms that regulate prolactin secretion. Although progesterone receptors (PR) are not present in rat lactotropes [34] both the A and B isoforms of PR are expressed and regulated by estrogens in other cell types of the anterior pituitary of female rats [35] specifically on gonadotropes [36–38] that, in turn, may be affecting lactotrope function. Thus, we cannot discard that after mifepristone treatment additional paracrine mechanisms from neighboring pituitary cells may influence lactotrope function. Although under mifepristone treatment alone there is lactotrope activation and stimulation of prolactin synthesis, no rise in serum prolactin was observed. Moreover, in previous studies we have not observed increases in serum prolactin 12 h [8, 9] after mifepristone treatment. Our hypothesis is that after the prolonged and important pituitary inhibition observed during the second half of pregnancy, the lack of progesterone action induced by mifepristone treatment normalizes the pituitary and prepares the lactotrope cell to receive a subsequent stimulus that finally promotes prolactin release. This conclusion is evident after naloxone administration. Interestingly, the opioid antagonist is not able to induce prolactin secretion when administered alone, but after mifepristone treatment, naloxone activates the lactotrope further, producing accumulation of secretory material in the extracellular space and an increase in the number of secretory granules undergoing exocytosis next to the plasmatic membrane. In contrast with this activation, simultaneously and probably as a consequence of the serum prolactin increase, naloxone administration seems to initiate the inhibition of lactotrope activity, as suggested by the appearance of some regressing lactotropes that contrast with other ones that are still activated. Evidence suggests a direct action of released prolactin on pituitary cells. Prolactin receptors have been detected on rat anterior pituitary cells [39, 40] and more specifically on rat lactotropes themselves [41, 42] supporting this autocrine effect. Although a direct effect of the opioid at the level of the anterior pituitary gland cannot be ruled out completely [43, 44], a lot of evidence suggests the hypothalamus as the site of opioid action in relation to pituitary hormone secretion [45–47]. Moreover, in previous results, we have demonstrated that naloxone acts at hypothalamic levels to induce prolactin secretion because no effect was obtained after the administration of naloxone-methobromide [8], an agent known to have only peripheral action [48]. The existence of a delayed inhibitory action of naloxone on prolactin synthesis and release triggered after the acute stimulatory effect observed on our experimental model is suggested by the aforementioned observation of regressing lactotropes and the tendency to decrease prolactin mRNA content. These observations may resolve the discrepancy between our results, showing an acute stimulatory action of naloxone 30 min after its administration, and the inhibitory action demonstrated by others [49] after continuous infusion of the opioid antagonist, observed at a timing when the delayed phase of inhibition of prolactin synthesis and release may have been established.

In conclusion, the absence of progesterone action induced by mifepristone treatment produces an important activation of the lactotropes, increasing the expression of prolactin mRNA and priming the pituitary for an ulte-

rior stimulatory action of naloxone mediated perhaps through an undetermined PRF. The high serum prolactin levels induced by the blockade of progesterone and opioid action may, in turn, initiate the short loop inhibition of the lactotropes as suggested by the presence of some cells in regression along with the still activated cells. We consider that the present results may help clarify the controversial results existing about the role of the opioid system on prolactin during late pregnancy, particularly in relation to progesterone action.

Acknowledgements

This work was supported by PMT-PICT 06877/99 from the Agencia de Promoción Científica y Tecnológica, Argentina and PIP No. 02596/03 and PIP 5795/05 from CONICET. M.S., C.M. and R.P.D. are members of CONICET and C.V.G. and M.E. have fellowships from CONICET. The authors are grateful to Mr. J. Rosales for his skilful technical assistance and to Dr. G. Jahn and to Dr. R. Carón for revision of the manuscript. This paper is dedicated to the memory of Dr. Ricardo P. Deis, died on April 26, 2008, who was a pioneer on the study of the regulation of PRL release and lactation physiology, and whose counsel and suggestions greatly enriched this work.

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