Suckling-induced prolactin release potentiates mifepristone-induced lactogenesis in pregnant rats

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Summary. Suckling, starting at 19:00 h on Day 18 of pregnancy, induced a significant increase in serum prolactin concentration at 20:00 h on Day 19 of pregnancy, but no increase in mammary gland casein or lactose content. Mifepristone (2 mg/kg) injection at 08:00 h on Day 19 of pregnancy induced significant increases in casein, but not in lactose, 24 h after administration. Mifepristone alone did not induce prolactin secretion, indicating that lactogenesis was induced by placental lactogen in the absence of progesterone action. When mifepristone was injected into suckling rats, serum prolactin concentrations were higher than in the untreated suckling rats. Casein in these rats increased significantly 12 h after mifepristone administration and lactose at 24 h after. If the suckling mifepristone-treated rats were given two injections of bromocriptine (1.5 mg/kg) at 12:00 h on Days 18 and 19 of pregnancy, serum prolactin concentrations were not increased by suckling, but casein and lactose concentrations in the mammary gland showed values similar to those obtained in the mifepristone-treated non-suckling rats. Mifepristone can therefore potentiate suckling-induced prolactin release in pregnant rats, demonstrating a direct central inhibitory action of progesterone on prolactin secretion. This suckling-induced prolactin secretion, unable to induce casein or lactose synthesis in the presence of progesterone, enhanced significantly synthesis of these milk components in the absence of progesterone action (rats treated with mifepristone). Fatty acid synthase, which is stimulated by the suckling stimulus in lactating rats, was not modified by mifepristone or suckling in pregnant rats.

Keywords: prolactin; suckling; mifepristone; mammary gland; lactogenesis; progesterone

Introduction

Suckling is one of the most powerful stimuli for hormone release. After a latency of a few minutes the stimulus provided by hungry young can increase serum prolactin concentrations more than 20-fold (Amenomori *et al.*, 1970; Deis & Prilusky, 1975). During the second half of pregnancy in rats, prolactin secretion is endogenously inhibited by placental hormones (possibly placental lactogen) (Yogev & Terkel, 1978, 1982; Tonkowicz & Voogt, 1983; Tonkowicz *et al.*, 1983) and progesterone (Jahn *et al.*, 1986) on Days 11–13 and solely by progesterone later on (Jahn *et al.*, 1986). The fall in serum progesterone values during late pregnancy is therefore sufficient to induce prolactin secretion (Vermouth & Deis, 1972, 1974; Nicholas & Hartmann, 1981a, b). In rats with a concurrent pregnancy and lactation, suckling can induce potent prolactin release during the first 5 days *post partum*, but is much less effective on Days 14–19 (which correspond to Days 10 to 15 of normal pregnancy) (Yogev & Terkel, 1978, 1982). This lack of effectiveness of suckling during this period was attributed to the inhibitory influence of placental secretions (Yogev & Terkel, 1978, 1982). Pregnant rats, exhibiting maternal behaviour and allowing sucking by young, also show a low response to the suckling stimulus when it is applied on Days 15 to 22 of pregnancy (Deis, 1984).

Blockade of progesterone receptors by administration of mifepristone (RU 38486; 17 β -hydroxy-11 β -(4-dimethyl-aminophenyl)-17 α -propinyl-oestra-4,9-dien-3-one; Philibert, 1984; Bardon *et al.*, 1985; Philibert *et al.*, 1985) to pregnant rats induces lactogenesis with increases in casein and lactose content of the mammary glands, but there is no prolactin release, in contrast with the effect of removing progesterone (Deis & Bussmann, 1986). The aim of the present study was to determine whether the suckling stimulus applied during late pregnancy is able to induce prolactin secretion and lactogenesis, with or without treatment with mifepristone.

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. The rats were kept in a light (lights on 06:00–20:00 h)- and temperature-controlled room; rat chow (Cargill, Buenos Aires, Argentina) and tap water were available *ad libitum*. Vaginal smears were taken daily.

Experimental procedures. To study the effect of the suckling stimulus in pregnant rats it is first necessary to induce maternal behaviour in the animals before pregnancy and to ensure that the maternal response to young will be retained over long periods. Pregnant rats do not develop maternal behaviour before parturition but nearly 90% of virgin rats kept continuously in the presence of young (replaced by recently fed ones every 12 h), will behave maternally within 48 h of the introduction of the young, if the foster young are first presented at 18:00 h on the day of pro-oestrus (Gonzalez & Deis, 1986). Virgin female rats showing regular 4-day cycles were introduced in special maternal cages (Gonzalez & Deis, 1986) on di-oestrus Day 2 at 18:00 h. On the following day (of pro-oestrus) a male and three 1–3-day-old freshly nourished young were introduced at 18:00 h into the maternal cage. The morning after mating was considered as Day 1 of pregnancy if spermatozoa were found in the vaginal smears. The male was removed from the cage and the young replaced by recently fed ones every 12 h, only to the rats which became pregnant. After development of maternal behaviour, the rats were kept with young for 5 days to consolidate maternal experience. After a period of 10–12 days without young, the rats were provided with foster young. They rapidly displayed maternal traits within 15 min of exposure to young. This procedure is quite adequate to provide the stimulus of suckling at any time of pregnancy. For example, when foster young were presented to apply the suckling stimulation, they were immediately retrieved by the pregnant rat. The well developed nipples in the mammary glands at Day 18 of pregnancy facilitate the establishment of suckling. Inspection of the rats indicated that suckling started within 1–2 h after introducing the young into the maternal cage. The young were well attached to the nipples. All the rats used showed evident signs of suckling during the experiment.

Special zinc cages, 40 cm long, 25 cm wide and 10 cm high were used as maternal cages. The cages are divided in two compartments. Each compartment has an individual wire mesh-cover permitting observation of the rat behaviour and the introduction of the rats, young and food. One compartment is smaller than the other. The rats usually build their nest in the small compartment. To facilitate mating, the wire-mesh cover of the bigger compartment was replaced during the night of pro-oestrus by a special wire-mesh cover 20 cm high. The following morning the original cover was reinstalled.

The following groups (6–8 rats/group) were used. Group C contained non-suckled pregnant rats (controls). Group S contained suckling rats: a litter of 8 freshly nourished 1–3-day-old young was introduced into the maternal cage holding pregnant rats at 19:00 h on Day 18 of pregnancy and the young remained with the females for the next 25 or 37 h. The rats were routinely inspected for signs of suckling at the nipples, and only those showing signs of suckling were used. The litters were changed every 12 h. In Group M, pregnant rats were injected s.c. with 2 mg mifepristone/kg (kindly donated by Roussel-Uclaf, Romainville, France) in 0.2 ml sunflower seed oil at 08:00 h on Day 19. For rats in Group SM, young were introduced into the maternal cage at 19:00 h on Day 18 of pregnancy and they were subjected to the same procedures as those in Group S except that mifepristone was injected at 08:00 h on Day 19 as for those in Group M. In Group SMB, rats were injected s.c. at 12:00 h on Day 18 and again on Day 19 of pregnancy, with 1.5 mg bromocriptine/kg (kindly donated by Sandoz, Buenos Aires, Argentina) dissolved in ethanol–saline (0.154 M-NaCl) solution (30:70 v/v) at a concentration of 1.5 g/l. Young were introduced into the maternal cages at 19:00 h on Day 18 of pregnancy and the rats were injected with mifepristone at 08:00 h on Day 19. The rats in Groups C and S were injected with oil at 08:00 h on Day 19 of pregnancy.

Rats were killed by decapitation at 20:00 h on Day 19 or at 08:00 h on Day 20 of pregnancy. A group of control rats was killed at 08:00 h on Day 19 of pregnancy, the moment of injection of mifepristone. Blood samples were collected and allowed to clot at room temperature ($\sim 20^\circ\text{C}$), and the serum was separated and stored frozen at -30°C for prolactin and progesterone determinations. Both inguinal mammary glands were dissected and stored frozen at -70°C until processed for casein and lactose determinations.

Casein and lactose determination. Mammary tissue (200 mg) was cut into small pieces and homogenized in 2 ml 50 mM-sodium phosphate buffer (150 mM-NaCl, 0.1% Na_3N , 0.1% Triton X-100, pH 7.6) with an Ultra Turrax homogenizer. The homogenate was centrifuged at 600 g for 30 min and the supernatant was used for beta-casein and lactose determination. The RIA procedure for beta-casein was that described by Edery *et al.* (1984). Casein iodination was performed by the method of Greenwood *et al.* (1963) using a low concentration of chloramine T (800 pg) and

5 µg/β-casein. The incubation was allowed to proceed for 90 sec at room temperature. The iodinated hormone was purified on a column of polyacrylamide agarose (ACA 54; IBF LKB, Villeneuve la Garenne, France) (0.9 × 40 cm) and the tubes of the protein peak pooled. All dilutions were made with the buffer used to homogenize the mammary tissue but containing 0.1% bovine serum albumin. The samples were incubated at room temperature for 24 h. Precipitation of bound protein was performed by centrifugation after incubating the samples with the second antibody for 1 h and addition of 0.6 ml of 12.5% polyethyleneglycol (M_r 3000) to aid precipitation. The accuracy of the assay was assessed by the recovery of known amounts of unlabelled β-casein added to mammary gland cytosol preparations. The average recovery was $95 \pm 3.5\%$. Parallelism in immunoreactivity between endogenous casein and β-casein was tested by serial dilution of mammary gland cytosol. For further details about the validation of the assay see Edery *et al.* (1984). Protein was measured by the method of Lowry *et al.* (1951), using bovine serum albumin as the standard.

Lactose concentration was assessed by the method of Kuhn & Lowenstein (1967). A portion (0.5 ml) of the 600 g for 5 min supernatant was precipitated with the same volume of ice-cold 10% perchloric acid, centrifuged and the clear supernatant brought to pH 7.0 with potassium hydroxide; the precipitate was removed by centrifugation. Duplicate aliquants (0.1 ml) were incubated for 90 min at 37°C with 0.25 U β-galactosidase (Sigma Grade XII) in 0.1 ml buffer (0.1 M-phosphate pH 7.2) and the glucose released was measured by the glucose oxidase method (Kit N510 A, Sigma). Correction for endogenous glucose was made by incubating the samples without β-galactosidase. Standard curves for lactose and glucose were run with each batch of experimental samples. All drugs, except when mentioned otherwise, were obtained from Sigma.

Fatty acid synthase measurement. Each abdominal mammary gland was finely minced, washed in saline (0.154 M-NaCl) and suspended in 10 ml 0.1 M-sodium phosphate, pH 7.0, 1 mM-EDTA, 1 mM-dithiothreitol. The tissue was homogenized with an Ultra Turrax homogenizer with two 10-sec strokes. All the operations were performed at 4°C. The homogenates were centrifuged at 100 000 g for 60 min and the cytosols removed by means of a syringe, taking care not to disturb the overlying lipid layer. Enzyme activity was determined spectrophotometrically at 340 nm, measuring the disappearance of NADPH from the reaction mixture (Lynen, 1962). This consisted of 0.1 M-potassium phosphate, pH 6.6, 1 mM-dithiothreitol, 0.15 mM-NADPH, and 0.05 mM-acetyl-CoA. After incubation for 5 min at 30°C the reaction was started by addition of malonyl-CoA at a final concentration of 0.054 mM and measured for 1.5 min. Incubations contained typically 0.2–0.4 mg protein in a total reaction volume of 2 ml. Reaction speed was proportional to protein concentration.

Prolactin determination. Prolactin was measured by a double-antibody radioimmunoassay using materials kindly provided by NIADDK. Prolactin was radioiodinated using the chloramine T method. The results were expressed in terms of the rat prolactin RP-3 standard preparation. Assay sensitivity was 1 µg/l serum and the inter- and intra-assay coefficients of variation were 8 and 3% respectively.

Progesterone determination. Serum progesterone was measured using a radioimmunoassay developed in our laboratory (Bussmann & Deis, 1979) with an antiserum raised in rabbits against progesterone-11-bovine serum albumin conjugate. Assay sensitivity was < 5 µg/l serum (15 nmol/l) and the inter- and intra-assay coefficients of variation were 9 and 4% respectively.

Statistical analysis. This was performed using one- or two-way analysis of variance followed by the least significant difference between means test when more than two experimental groups were compared (Snedecor & Cochran, 1967).

Results

Effect of suckling and mifepristone on serum prolactin and progesterone concentrations

Serum prolactin concentration was significantly higher 24 (20:00 h on Day 19) and 36 h (08:00 h on Day 20 of pregnancy) in Group-S than in Group-C rats. Serum prolactin concentration was significantly lower on the morning of Day 20 with respect to values on the evening of Day 19 in Group-S rats ($P < 0.02$) (Fig. 1a). Serum progesterone concentrations did not change significantly (Fig. 1b).

Mifepristone (Group M) had no significant effect on serum prolactin or progesterone concentrations (Fig. 1a). Serum prolactin concentrations were significantly higher in Group-SM rats than in Group-S rats ($P < 0.01$) at 20:00 h on Day 19 of pregnancy, but not at 08:00 h on Day 20. Serum progesterone concentrations were not significantly different in Group-M and Group-C rats (Fig. 1b).

Effect of suckling and mifepristone on casein and lactose concentrations and fatty acid synthase activity in mammary tissue

After 24 or 36 h of suckling, mammary gland casein or lactose concentrations were not modified in Group C rats (Figs 1c and d), but in Group M mammary casein content was increased at 08:00 h

Fig. 1. Effect of suckling and/or mifepristone treatment on serum prolactin (a) and progesterone (b) concentrations, and mammary gland content of casein (c) and lactose (d) in rats at 20:00 h on Day 19 and 08:00 h on Day 20 of pregnancy.

○, Group C (control, untreated rats); ●, Group S (suckling rats); □, Group M (rats treated with mifepristone); ■, Group SM (suckling rats treated with mifepristone); ×, Group SMB (suckling rats treated with mifepristone and bromocriptine). Values are means $\pm$ s.e.m. of groups of 6–8 animals. * $P < 0.05$ compared with Group C; † $P < 0.05$ compared with Group M.

on Day 20 of pregnancy (24 h after its administration), and lactose levels were unaltered. In Group-SM rats, casein content was significantly increased by 20:00 h on Day 19 (12 h after administration) but values at 08:00 h on Day 20 did not differ from those in Group-M rats. Mammary lactose concentration was significantly increased with respect to the other groups at 08:00 h on Day 20 of pregnancy. In 5 out of 7 Group-SM rats blood was present in the vagina, indicating the imminence of delivery. None of the rats in Groups S and M showed blood in the vagina. In our colony rats usually deliver on Day 23.

Table 1. Effect of suckling or suckling and mifepristone administration on mammary gland fatty acid synthase activity in rats on Days 19 and 20 of pregnancy

Group	Treatment	Fatty acid synthase (U/mg protein)	
		Day 19, 20:00 h	Day 20, 08:00 h
C	Controls	—	13.2 ± 1.2 (6)
S	Suckling	18.7 ± 2.8 (6)	17.6 ± 2.3 (6)
SM	Suckling + mifepristone	16.1 ± 0.9 (6)	18.3 ± 1.5 (8)

Values are mean ± s.e.m. for the no. of rats in parentheses.

Fatty acid synthase activity in the mammary gland was not significantly modified in rats in Groups S and SM (Table 1).

Effect of bromocriptine administration on serum prolactin and progesterone and mammary gland casein and lactose concentrations in suckled, mifepristone-treated rats

Serum prolactin concentrations in Group-SMB rats were reduced to values similar to those of Group-C rats (Fig. 1a) but serum progesterone concentrations were not affected (Fig. 1b). Casein and lactose concentrations in the mammary glands of these rats were similar to the values in Group-M rats, and so the additional increase induced by suckling was abolished. However, at 08:00 h on Day 20 only casein values were similar to those obtained in Group SM (Figs 1c and d).

Discussion

It has been clearly demonstrated that withdrawal of progesterone action by ovariectomy, removal of corpora lutea or treatment with prostaglandin F-2 α induces prolactin release (Vermouth & Deis, 1972, 1974; Bussmann & Deis, 1979; Bussmann *et al.*, 1983). As previously shown (Deis & Bussmann, 1986) administration of mifepristone to pregnant rats induced casein synthesis in the mammary glands 24 h after treatment. At this time there was no significant increase in lactose concentration, since the increase in this sugar is seen 28 h after mifepristone treatment (Deis & Bussmann, 1986). Blockade of progesterone receptors by mifepristone at the mammary gland level therefore allowed the circulating concentrations of placental lactogen to induce lactogenesis (Bussman *et al.*, 1983; Bussman & Deis, 1984), since there was no significant increase in serum prolactin values after mifepristone administration. Suckling, which induced prolactin release, significantly enhanced lactogenesis, increasing both mammary gland casein and lactose content in rats treated with mifepristone. This increase was produced by the suckling-induced prolactin increase, since bromocriptine administration, which blocked prolactin release, restored both milk components to the values observed in mifepristone-treated rats without young. These results show that, although the circulating concentrations of placental lactogen in late pregnant rats are sufficient to induce lactogenesis in the absence of the inhibitory progesterone action, this increase is not maximal, and prolactin secretion induced by suckling will stimulate casein and lactose further. Conversely, since in suckling rats the main effect was the advancement of lactogenesis, the action of prolactin upon the mammary gland may be more rapidly established than that of placental lactogen. The prolactin

release induced by suckling in control, untreated rats was not able to induce casein or lactose synthesis when progesterone was still acting at the mammary level.

The suckling stimulus is a potent inducer of fatty acid synthase activity in lactating rats (Martyn & Hansen, 1981; Carrizo, 1984). On the other hand, it has been shown that induction of lactogenesis in pregnant rats does not modify the activity of this enzyme (Martyn & Hansen, 1981; Carrizo, 1984). In the present study, no increase in fatty acid synthase activity was observed in the mammary gland of suckling rats, regardless of whether they were treated with mifepristone. Suckling is therefore effective in increasing fatty acid synthase activity only during lactation.

The peripheral antiprogestosterone action of mifepristone on mammary gland and uterus seems to be more pronounced than its central action. It was able to mimic the lactogenic action of progesterone withdrawal in the mammary gland, inducing lactogenesis, but not at central levels, since there was no increase in serum prolactin concentration. On the other hand, mifepristone was able to potentiate the stimulatory effect of suckling on prolactin release. This result gives rise to two conclusions. (1) The central antiprogestosterone action of mifepristone is not strong enough to induce prolactin secretion alone, but is sufficient to potentiate the effect of other prolactin releasers such as suckling. (2) The inhibitory role for progesterone in the suckling-induced prolactin release during pregnancy is confirmed (Deis, 1984). Yogeve & Terkel (1982) found a similar endogenous inhibition of suckling-induced prolactin release in pregnant-lactating rats, but they concluded that placental lactogen was responsible for the inhibition. Our results also show the involvement of progesterone in this inhibition. Placental secretions do not participate in inhibition of prolactin release after Day 16 of pregnancy, although they are active earlier (Jahn *et al.*, 1986). Suckling in intact and in mifepristone-treated rats induced a significant prolactin release when measured at 20:00 h, but by the next day at 08:00 h serum prolactin concentrations were only slightly but significantly higher than control values. The light period is known to be a time of maternal nursing (Ader & Grota, 1970; Lee & Williams, 1977) and young in the litters gain in body weight, an indicator of the amount of milk ingested, during the light period (Levine & Stern, 1975). We can therefore attribute the decline of prolactin values in the morning of Day 20 to a decrease in suckling during the preceding night. A rhythm of maternal nursing exists also during pregnancy in rats with maternal behaviour.

In conclusion, the suckling stimulus, through prolactin release, is capable of inducing lactogenesis, lactose and casein synthesis in the mammary gland of pregnant rats treated with mifepristone. Casein seems to be synthesized earlier than lactose. It has been shown that mifepristone binds to progestagen-specific receptors in the nervous system (Etgen & Barfield, 1986). Our results may represent a direct demonstration that progesterone prevents centrally the release of prolactin, as previously suggested (Vermouth & Deis, 1972, 1974), and that casein synthesis is initiated in the presence of prolactin and in the absence of progesterone at the level of mammary gland receptors.

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