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Comparison of primary and secondary stimulation of male rats by estradiol in terms of prolactin synthesis and mRNA accumulation in the pituitary

(steroid hormone action/hybridization/cDNA/acellular translation/immunoprecipitation)

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ABSTRACT **Male rats received acute or chronic primary or acute secondary stimulation with estradiol, and the effects on pituitary prolactin synthesis and its mRNA accumulation were examined. Prolactin synthesis was determined by the *in vitro* incorporation of [^{3}H]leucine into prolactin over a period of 1 hr. Prolactin mRNA was measured both by cell-free translation in a nuclease-treated rabbit reticulocyte lysate and by hybridization to the complementary DNA. The latter two methods gave similar results under all experimental conditions. Acute primary stimulation with estradiol produced a significant increase in pituitary prolactin mRNA accumulation at 12 hr, which further increased by 2- to 3-fold over the next 48 hr. In contrast, no increase in prolactin synthesis was observed during the first 24 hr. Chronic stimulation with estradiol induced increases of both prolactin synthesis and prolactin mRNA that were quantitatively indistinguishable over the period of 1–4 weeks, reaching a plateau at 5-fold the basal values. By the 13th day after withdrawal of therapy both prolactin synthesis and mRNA had returned to the prestimulation levels. When the effects of estradiol on previously unexposed and estrogen withdrawn animals were compared, it was found that secondary stimulation not only produced a more rapid accumulation of the prolactin mRNA but also abolished the lag period of prolactin synthesis observed during the primary estrogen stimulation. These data demonstrate a lag in the endogenous translation of newly accumulated pituitary prolactin mRNA translatable *in vitro* after primary estrogen stimulation of male rats. The mechanism for the abolition of this lag during the secondary stimulation is not known.**

It is well established that estrogens control the expression of specific genes in their target tissues (1, 2). Induction of egg proteins in the liver (3–8) and in the oviduct (9–11) of birds and amphibians has been studied in detail because the magnitude of the response allowed the isolation with relative ease of the specific mRNAs coding for vitellogenin and ovalbumin, and the synthesis of the corresponding complementary DNAs (cDNAs). Quantitation of these mRNAs after estrogen administration has been achieved by cell-free translation and molecular hybridization, revealing in most cases an overall parallelism between the synthesis of the specific proteins and the level of their mRNAs (4–6, 10). When tissues from male or from immature female animals are exposed to estrogen (primary stimulation), specific mRNAs accumulate much more slowly than when the steroid is administered for a second time after a period of withdrawal (secondary stimulation) (4–7).

Prolactin (PRL) synthesis in the pituitary of mammals is controlled by many factors, such as thyrotropin-releasing hormone, dopamine, and estrogens (12–21). Work from several laboratories demonstrated a positive effect of estradiol on PRL mRNA activity (18, 21). Recently, we have succeeded in obtaining PRL mRNA preparations over 80% pure and in synthesizing the corresponding PRL cDNA (unpublished).

In this communication we compare PRL synthesis to PRL mRNA levels in pituitaries of male rats during primary and secondary stimulation with estradiol. Primary exposure to estradiol resulted in a considerable accumulation of PRL mRNA before any stimulation of PRL synthesis could be observed. During secondary stimulation, PRL mRNA and synthesis increased much faster and in almost parallel ways.

MATERIALS AND METHODS

Handling of Rats. Male rats of the C/D strain (250–300 g) were obtained from Charles River Breeding Laboratories. Four rats were used in each experimental group. For primary and secondary stimulation, 17β-estradiol or estradiol valerate in oil was injected subcutaneously at a dose of 80 or 400 μg/100 g of body weight, respectively. For the chronic stimulation estradiol pellets (2 mg per rat, Bartor Pharmaceutical Company, Rye, NY) were implanted subcutaneously. Withdrawal was achieved by removal of the pellets 28 days after implantation. Secondary stimulation was started 13 days after removal of the pellet. Animals were killed by cardiac exsanguination. Serum was separated and stored at −20°C for subsequent estrogen determination (see below). Two 1/8th pieces of pituitary from each animal were rinsed in Krebs–Ringer bicarbonate buffer, pH 7.4, containing 2.56 mg of D-glucose per ml (KRBG) and used for the *in vitro* determination of PRL synthesis, while the remaining 6/8ths were processed for PRL mRNA measurement.

***In Vitro* Determination of the Rate of PRL Synthesis.** Duplicates of four pieces of 1/8th pituitary each from 4 animals in an experimental group were pooled and preincubated for 2 hr in 1 ml of KRBG at 37°C under an atmosphere of 95% O_2/5% CO_2. The medium was then replaced by 1 ml of KRBG containing 25 μCi (1 Ci = 3.7×10^{10} becquerels) of [^{3}H]leucine (Amersham, 61 Ci/mmol). After a 1-hr incubation, the tissue was homogenized in its incubation medium in an all-glass homogenizer at 0°C. Half of the homogenate was saved for protein determination (22) and the other half was made 1% in sodium deoxycholate, 1% in Triton X-100, and 0.1% in L-leucine and centrifuged at 100,000 × *g* for 60 min in a Beckman SW 60 rotor at 2°C. Total protein synthesis and PRL synthesis were determined by precipitation of aliquots of the supernatant with 10% trichloroacetic acid and specific anti-rat PRL serum (21), respectively. Results were expressed as cpm incorporated per mg of protein. For PRL synthesis, the results were corrected by using ^{125}I-labeled rat PRL as an internal standard for recovery (21).

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Abbreviation: PRL, prolactin.

Table 1. Effect of primary estrogen stimulation

Hr	Serum estrogens, pg/ml	Rate of protein synthesis, cpm × 10^{-3}/mg per hr		Total mRNA		PRL mRNA	
		Total	PRL	Translation, cpm × 10^{-3}	Hybridization, cpm	Translation, cpm × 10^{-3}	Hybridization initial slope, (%/ng RNA) × 20
0	41.5 ± 3.4 (100)	1329 ± 200 (100)	94.5 ± 12.2 (100)	85.8 ± 7.6 (100)	419 ± 7 (100)	11.0 ± 0.17 (100)	5.7 ± 0.2 (100)
12	300 ± 123 (722)	1071 ± 131 (81)	76.6 ± 27.7 (81)	97.6 ± 6.4 (114)	515 ± 9 (123)	13.6 ± 0.9 (123)	6.9 ± 0.2 (121)
24	115 ± 38 (277)	1193 ± 196 (90)	112.4 ± 0.7 (119)	109.1 ± 3.0 (127)	510 ± 6 (122)	21.8 ± 0.6 (198)	11.9 ± 0.2 (208)
48	50 ± 25 (120)	1181 ± 6.2 (89)	113.2 ± 0.2 (120)	84.8 ± 3.2 (98)	472 ± 13 (113)	25.0 ± 0.6 (227)	13.7 ± 0.9 (241)

Data are expressed as mean ± range of duplicate determinations for a single experiment, except for serum estrogens, for which data are given as mean ± SD for individual determinations in each of four animals per group. In parentheses appear mean values expressed as percent of the unstimulated control.

Purification of PRL mRNA and Synthesis of $cDNA_{PRL}$. Details of the procedure will be published elsewhere. Briefly, total RNA was prepared from pituitaries of thyroidectomized rats chronically treated with estradiol, under which condition growth hormone mRNA is suppressed to 1/50th and PRL mRNA is stimulated 15-fold. PRL mRNA was then purified by poly(U)-Sepharose chromatography followed by sucrose gradient centrifugation. Purity of the mRNA was at least 80% as judged by translation in a nuclease-treated reticulocyte lysate (23). DNA complementary to PRL mRNA was prepared according to Keller and Taylor (24). It displayed a single peak with sedimentation coefficient of 7 S after centrifugation through an alkaline sucrose gradient. Back hybridization of the cDNA to its template RNA exhibited a single transition [with an equivalent $R_0t_{1/2}$ (product of RNA concentration and time at half hybridization)] and involved 85% of the cDNA. The specificity of the probe was assessed by hybridization with purified growth hormone mRNA and liver poly(A)-RNA.

Extraction of Pituitary RNA for PRL mRNA Determinations. Pituitary tissue not used for the determination of the rate of PRL synthesis was stored at −80°C until RNA extraction. The tissue was homogenized in 0.5 ml of 200 mM Tris·HCl, pH 8.5/25 mM $MgCl_2$/50 mM KCl/200 mM sucrose. After treatment for 10 min with 1% Triton X-100, the homogenate was centrifuged at 2500 rpm (HB4 Sorvall rotor) and the supernatant was diluted with 4 vol of 10 mM Tris·HCl, pH 8.5/100 mM NaCl/10 mM EDTA/1.2% sodium dodecyl sulfate before extraction with 1 vol of phenol/chloroform (1:1, vol/vol). After ethanol precipitation, the RNA was washed with 2 M LiCl and 66% (vol/vol) ethanol containing 0.1 M NaCl.

Determination of Total and PRL mRNA Activity. mRNA activity was assayed in a reticulocyte lysate treated with micrococcal nuclease (23). Total mRNA activity was measured as the radioactivity precipitated with 10% trichloroacetic acid from lysates programmed with fixed amounts of RNA minus the radioactivity in control lysates in the absence of RNA. PRL mRNA activity was measured as the radioactivity specifically immunoprecipitated by anti-rat PRL serum. Details of the double antibody immunoprecipitation method have been published (21, 25, 26). Care has been taken to use RNA concentrations in the range in which the response of the lysate is linear with respect to RNA input (100 μg/ml).

Determination of Total Poly(A)-Rich and PRL mRNA by Hybridization. The amount of total poly(A)-rich RNA was determined by hybridization with ^{125}I-labeled poly(U) as described (27, 28). PRL mRNA was measured by RNA·cDNA hybridization performed at 68°C in capillaries containing 20 μl of 50 mM Tris·HCl at pH 7.5, 500 mM NaCl, 1 mM EDTA, 0.2% sodium dodecyl sulfate, 400 μg of *Escherichia coli* tRNA per ml, 2000 cpm of $cDNA_{PRL}$, and 0–100 ng of total pituitary RNA. Hybrid formation was assayed by resistance to S1 nuclease as described (29). The concentration of PRL mRNA was estimated from the slope of the initial part of the hybridization curve.

Determination of Serum Estrogen Levels. Estrogens were measured without chromatography by radioimmunoassay, using an antiserum to 17β-estradiol kindly supplied by G. E. Abraham. Crossreactions with estrone and estradiol were 25% and 8%, respectively. The sensitivity of the assay was 10 pg/ml of serum.

RESULTS

When 17β-estradiol in oil was administered subcutaneously to male rats (80 μg/100 g of body weight) in a single injection, the concentration of estrogens in serum increased rapidly from the mean basal level of 41.5 pg/ml to a mean peak level of 300 pg/ml at 12 hr (Table 1 and Fig. 1*A*). A slight but significant increase in the PRL mRNA activity was observed at 12 hr (23% above the 100% control value), and the activity reached 227% of the starting value at 48 hr, by which time estrogen levels had returned to the basal level. Surprisingly, PRL synthesis measured by pulse labeling of fragments from the same pituitaries exhibited only a slight increase (maximum of 20% above the control value at 48 hr) (Table 1 and Fig. 1*A*). To determine whether the discrepancy between PRL mRNA activity and PRL synthesis was related to the magnitude or the duration of the stimulation by estradiol, a similar experiment was performed using the long-acting estrogen preparation (estradiol valerate) at 400 μg/100 g of body weight. As shown in Fig. 1*B*, in this case higher serum estrogen levels were achieved (600 pg/ml at 12 hr) and the levels were still elevated at 48 hr. The increase in PRL mRNA activity was not very different from that observed after 17β-estradiol injection, only its onset seemed to be more rapid. Here again, virtually no increase in PRL

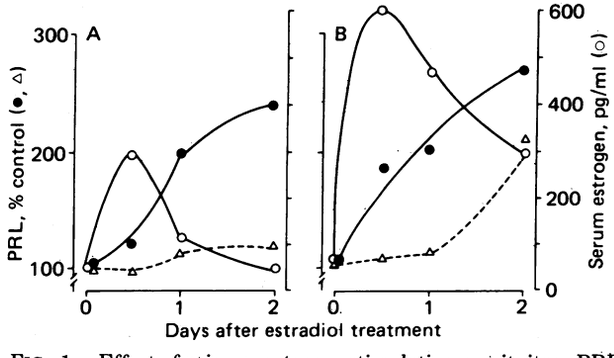

FIG. 1. Effect of primary estrogen stimulation on pituitary PRL in the male rat. Rats were given a single subcutaneous injection of 17β-estradiol at 80 μg/100 g of body weight (*A*) or estradiol valerate at 400 μg/100 g of body weight (*B*). Serum estrogen concentration (○) was measured by radioimmunoassay and PRL synthesis (△) and mRNA (●, translation assay) by methods described in the text.

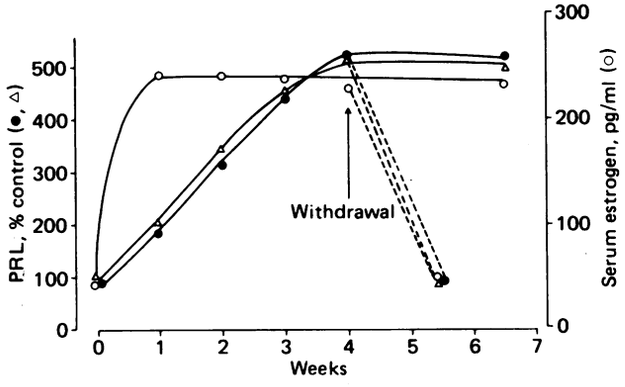

FIG. 2. Effect of chronic estrogen stimulation and withdrawal on pituitary PRL in the male rat. Rats were implanted subcutaneously with 2-mg estradiol pellets, which were removed surgically at the indicated time (broken lines after removal, solid lines for continued hormone). All animals were of the same age at the time of termination of the experiment. This was achieved by staggering the initiation of treatment. ●, PRL mRNA (translation assay); △, PRL synthesis; ○, serum estrogen.

synthesis could be observed during the first 24 hr. Nevertheless, in this case, stimulation of PRL synthesis was evident after 48 hr (Fig. 1*B*). In this experiment, as shown for the former (Table 1), total protein synthesis, total RNA recovery, total poly(A)-rich RNA, and total mRNA activity did not change significantly after estradiol administration. Changes in the amounts of PRL mRNA as measured by hybridization with $cDNA_{PRL}$ were parallel to those of PRL mRNA activity (Table 1).

In order to determine whether the estradiol-induced increase in PRL mRNA accumulation could be quantitatively correlated to PRL synthesis under the conditions of chronic stimulation, male rats were implanted subcutaneously with estradiol pellets. This treatment resulted in the maintenance of a fairly constant serum concentration of estrogen, averaging 240 pg/ml over a period of at least 6 weeks (Fig. 2). Measurements were made of the PRL mRNA concentration by hybridization with $cDNA_{PRL}$ and by cell-free translation together with PRL synthesis at weekly intervals. A perfect parallelism between these parameters was demonstrated, displaying a gradual increase, with stabilization at 500% of the control value 4 weeks after pellet implantation (Fig. 2). The estradiol pellets were removed from some rats at week 4. Thirteen days later, serum estrogen levels together with PRL mRNA concentration and PRL synthesis rate had returned to the basal values (Fig. 2). It must be noted that 4 weeks of exposure to estradiol resulted in a 2- to 3-fold increase in pituitary weight and a corresponding increase in total RNA recovery. The protein-to-RNA ratios remained constant.

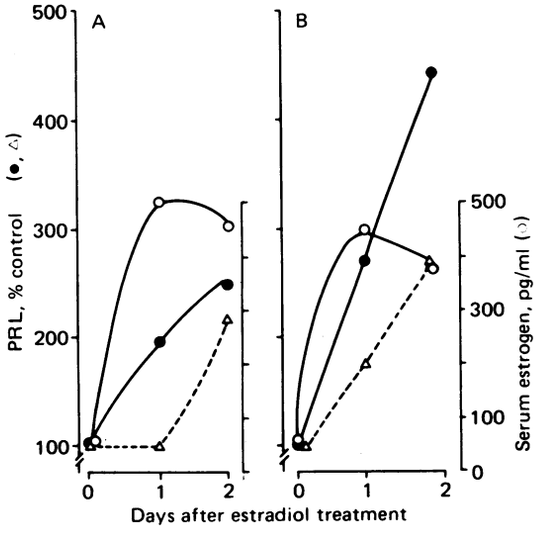

FIG. 3. Effects of primary and secondary estrogen stimulation on pituitary prolactin in the male rat. (*A*) Primary stimulation of animals never treated with estrogen; (*B*) secondary stimulation of animals 13 days after the withdrawal of chronic estrogen treatment. Both groups of rats were age matched. ●, PRL mRNA (translation assay); △, PRL synthesis; ○, serum estrogen.

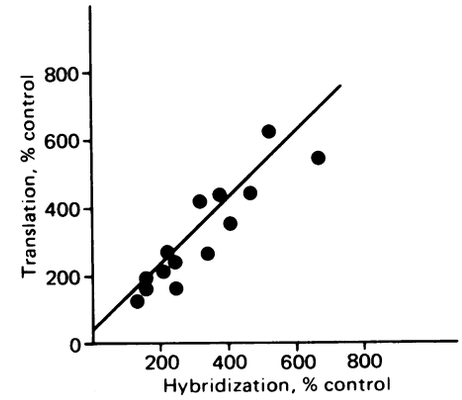

FIG. 4. Correlation of measurements of PRL mRNA by cell-free translation and by hybridization to $cDNA_{PRL}$. Data shown represent changes in treated animals compared to results in untreated control rats expressed as 100%. The regression line follows the equation $y = 0.9x + 50$. Correlation coefficient = 0.9090 ($P < 0.001$).

To investigate possible differences between primary and secondary stimulation by estradiol, groups of age-matched animals were used. A single intramuscular injection of 400 μg of estradiol valerate per 100 g of body weight was administered to rats not previously exposed to the hormone (primary stimulation group) and to rats that had been implanted with estradiol pellets for 4 weeks but were withdrawn for 13 days (secondary stimulation group). The patterns of estrogen concentration in serum were almost identical in both animal groups (Fig. 3). In spite of this, PRL mRNA accumulation was greater and more rapid after secondary than after primary stimulation. While the 24-hr lag period before stimulation of PRL synthesis was confirmed in the primary stimulation (Fig. 3*A*), no such lag was observed after secondary stimulation (Fig. 3*B*).

In the various experiments reported here, variations of both PRL mRNA activity and PRL mRNA concentration were measured simultaneously by cell-free translation and by cDNA·RNA hybridization, respectively. When all the data were plotted as shown in Fig. 4, it is clear that the two variables are highly correlated, suggesting that PRL mRNA accumulated under the influence of estrogen stimulation is translatable even if it is not necessarily translated in the pituitary cells.

DISCUSSION

Our results are in agreement with those of Stone *et al.* (18), leaving little doubt that estradiol promotes the accumulation of PRL mRNA in the pituitary. Moreover, comparisons of PRL synthesis and mRNA concentration indicate that the well-documented (16, 17) increase of PRL production after estradiol administration can be entirely accounted for by the increase of translatable PRL mRNA (Fig. 2). Although this conclusion is in agreement with the current dogma that steroid hormones control the expression of specific genes at the level of transcription (1), we cannot infer from our *in vivo* experiments that

the estrogen acts directly on the pituitary. Arguments in favor of a direct effect include the demonstration of estradiol receptors in the pituitary (30, 31), the persistence of the estradiol effect on PRL production by pituitaries transplanted under the kidney capsule of hypophysectomized rats (15), and the stimulation of PRL synthesis in a pituitary tumor cell line after addition of estradiol to the medium (32). On the contrary, regulation by estrogens of the tonic inhibition of PRL by the hypothalamus could also be involved, because PRL mRNA levels are modulated positively by dopamine antagonists (18) and negatively by dopamine agonists (unpublished data). Also, it has recently been demonstrated (33) that the hypothalamic factor thyrotropin-releasing hormone stimulated PRL production in a clonal pituitary cell line by increasing PRL mRNA levels. Unfortunately, interpretation of data from investigations on the direct action of estrogen on the clonal pituitary tumor cell line *in vitro* are complicated by the variable and occasionally paradoxical response of these cells (34).

Although the primary and secondary stimulations result in similar serum estradiol levels (Fig. 3), it is evident that the rate of PRL mRNA accumulation was faster in animals previously exposed to estrogens. Similar differences in the early kinetics of mRNA accumulation between primary and secondary stimulation have been described in the case of vitellogenin induction in the liver of toads (5, 6) and cockerels (4, 7). In the latter studies, this difference could not be attributed to an estrogen-induced proliferation of vitellogenin-producing cells (35). Proposed mechanisms have included permanent alterations of the chromatin in estrogen-primed cells and rapid stabilization of the message during secondary stimulation (7, 11). In the present study, while estradiol does not promote such dramatic histological remodeling as is the case in the chicken oviduct (36), a wave of mitotic activity has been demonstrated after a single dose of the steroid (37, 38). It presumably involves PRL-secreting cells (37). Also, the weight of pituitaries from male rats implanted with estradiol for 4 weeks was 2- to 3-fold higher than that of untreated age-matched controls. Therefore, it may well be that all or part of the observed difference in the kinetics of PRL mRNA accumulation between primary and secondary stimulation is due to modification of the number of cells able to respond to estradiol by increasing their PRL mRNA content.

The most interesting finding in the present work is the discrepancy between PRL mRNA accumulation and PRL synthesis rate during the first 24 hr after primary stimulation by estradiol (Figs. 1 and 3*A*). PRL mRNA activity was significantly increased 12 hr after primary stimulation and had doubled after 24 hr, while PRL synthesis showed little if any change (Figs. 1 and 3*A*). When estradiol was administered in such a way that elevation of serum estrogen levels was transient, PRL synthesis did not show more than a 20% increase above the base line, even after 48 hr, when PRL mRNA had increased by 140% (Fig. 1*A*). Only in situations in which primary stimulation elevated estrogen levels for at least 48 hr could an increase in PRL synthesis be detected by that time (Figs. 1*B* and 3*A*). After secondary stimulation, no such lag in PRL synthesis was observed, although the increase in PRL synthesis was proportionally smaller than that of PRL mRNA (Fig. 3*B*). Thus, it seems that estradiol is always able to promote the very rapid accumulation of PRL mRNA but that primary stimulation of PRL synthesis requires longer exposure of the animals to the steroid.

In most cases in which the effects of estrogens have been studied simultaneously on the synthesis of a specific protein and on the accumulation of the corresponding mRNA, it has been found that both quantities increase in a parallel fashion. These studies include secondary stimulation of ovalbumin synthesis in the chicken oviduct (10), as well as primary and secondary stimulation of vitellogenin synthesis in the livers of chicken (4) and *Xenopus* (6). However, in an elegant study, Farmer *et al.* (8) demonstrated that after primary injection of estradiol into male *Xenopus* there was a lag period of about 2 days before the newly accumulated vitellogenin mRNA became actively translated in polysomes. In the latter case, one could argue that the delay allows the cell to adapt to the synthesis of large amounts of a completely new protein, because vitellogenin mRNA is not detectable in the hepatocyte of the male *Xenopus* (7). This hypothesis certainly does not apply to the situation described in the present study, because prolactin synthesis is definitely taking place in the pituitary of the male rat. The good correlation between the alteration of PRL mRNA levels measured by translation and hybridization (Table 1, Fig. 4) suggests that the accumulation of a nontranslatable form of PRL mRNA may not be involved. Nevertheless, activation of a putative inactive PRL mRNA by the reticulocyte lysate assay system cannot be ruled out. It is tempting to speculate that this imprinting phenomenon of target cells after a first exposure to estradiol involves a complex permanent modification of the whole cellular protein translational machinery. In comparison, the "opening" of the vitellogenin and prolactin genes could represent a relatively simple, rapid, and reversible phenomenon.

The wave of mitotic activity observed in the pituitary after estradiol administration should also be considered as a possible cause of the lag period. Indeed, cells entering the cell cycle experience important alterations of their pattern of both RNA and protein synthesis (39). However, the influence of cell division on the specific processes of PRL mRNA production and of translation is unknown in normal pituitary cells. It will be of great interest to investigate whether the new PRL cells originate from cells previously producing PRL or from a pool of nondifferentiated cells. Histochemical techniques combining immunoreactions and *in situ* hydridization with PRL cDNA should help to answer this question.

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