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PROGESTINS SPECIFICALLY SUPPRESS α-LACTALBUMIN SYNTHESIS AND SECRETION

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Summary—Mammary gland explants from pregnant (day 12–15) rats were cultured with insulin and prolactin, and their content and secretion of α-lactalbumin determined after exposure to a wide range of doses (0.01–300 nM) of the specific synthetic progestin (ORG2058), alone or with a maximally stimulatory dose of the highly specific glucocorticoid RU26988. ORG2058 alone suppressed α-lactalbumin synthesis below baseline, with a half-maximal effect at a concentration of <0.1 nM; RU26988-stimulated secretion was similarly abrogated by ORG2058, similarly with a half maximally effective dose of <0.1 nM. We interpret these data as suggesting that (i) given the specificity and doses of the steroids used the effect of progestins on αlactalbumin synthesis is directly via progesterone receptor occupancy, and not by competing with glucocorticoids for glucocorticoid receptors and (ii) given the shift to the left in the α-lactalbumin response (half maximal <0.1 nM ORG2058) compared with receptor binding (K_d (37°C) > 1 nM), one possible model for such sensitivity is that of multiple, independent regulatory elements on the chromatin controlling α-lactalbumin gene expression, occupancy of any one of which by an activated progesterone receptor is sufficient to abrogate transcription.

INTRODUCTION

Like other milk components, α-LA (part of the enzyme complex lactose synthetase, responsible for lactose production) is under the hormonal control of adrenal steroids, prolactin and insulin [1–7]. The antagonistic effect of progesterone on lactogenesis is in marked contrast to the effects of adrenal steroid hormones. During pregnancy, the high circulating levels of progesterone suppress the onset of lactogenesis [8]. Ovariectomy during late pregnancy leads to the onset of milk production, as judged by the appearance of casein or lactose in the gland [9–15], which is prevented by the administration of progesterone [11,12,14,15]. In rat mammary gland explants, progesterone prevents the increased transcription of casein mRNA and/or casein synthesis which occur when insulin, prolactin and glucocorticoids are present [15, 16].

Whereas glucocorticoids show classical dose–response curves for casein synthesis, cortisol has a biphasic effect on α-LA production in mouse explants [17]. In studies at a single dose of cortisol, and over single dose or restricted range of progesterone concentrations, progesterone inhibited the onset of α-LA or lactose synthetase activity in explants from mice, rats and cows, in line with its known effects on the onset of lactogenesis. In these studies, concentrations of glucocorticoids and progesterone were at or approaching pharmacological doses [18–20]. Since progesterone has considerable affinity for glucocorticoid receptors [21–25], the question arises as to whether the antagonist effects of progesterone on α-LA production are via occupancy of glucocorticoid receptors [24, 26], as suggested in this and other systems by various authors [16, 22, 23, 27, 28], or whether the effect occurs via agonist occupancy of progesterone receptors [29, 30].

The present studies examine the effect on α-LA levels in explants from mid-pregnant rats of the synthetic, specific progestogen ORG2058, the preferred progesterone receptor ligand in this tissue [29]. A wide range of doses (0.01–300 nM) of ORG2058 was used, both alone and in combination with a maximally stimulating dose (3 nM) of the highly specific Type II (glucocorticoid) ligand, RU26988 [31].

EXPERIMENTAL

Materials

ORG2058 was obtained from Amersham Australia, Sydney. RU26988 was the gift of Roussel–Uclaf (Romainville, France). Rat prolactin (rPRL) (NIH-B4) was obtained through the National Hormone and Pituitary Program from NIADDK. Porcine insulin was Actrapid M.C. (100 U/ml) from Novo, Parramatta, N.S.W., Australia. Rat α-LA was generously provided by Dr Kevin Nicholas (NIH) and Dr Franklyn Bolander (University of South Carolina), and was a highly purified preparation showing a single band on polyacrylamide gel electrophoresis. Purified rabbit anti-rat α-LA IgG was the gift of Dr

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Abbreviations: ORG2058: 16α-ethyl-21-hydroxy-19-norpregn-4-ene-3,20-dione. RU26988: 11β,17β-dihydroxy-17α(1-propynyl)androsta,1,4,6-trien-3-one α-LA: α-lactalbumin

Peter Hartmann (University of Western Australia, Perth). Donkey anti-rabbit gamma globulin was purchased from Wellcome Reagents Ltd, Northcote, Victoria, Australia.

Animals

Pregnant rats at a known stage of gestation were obtained from the Monash University Animal house, and housed from day 7 of gestation in the Prince Henry's Hospital Animal House. Rat chow and water were provided *ad libitum*, with a 12 h light–dark regime.

Methods

Explants were prepared from abdominal mammary glands of pregnant rats at days 13–16 of gestation according to the method of Topper *et al.*[32]. One animal was used per experiment. Incubation of explants was carried out in 6-well plates (Linbro Cat No. 76-058-05, Flow Laboratories, South Yarra, Victoria, Australia) [3.5 cm dia per well]. Eight explants (8–16 mg total) per well were placed on stainless steel grids, bathed by 2 ml of incubation medium containing insulin (5 μg/ml), rPRL (1 μg/ml) and steroids as stated. Incubation medium was Dulbecco's Modified Eagle's Medium (Flow Laboratories) containing 20 mM Hepes, 10 mM $NaHCO_3$, penicillin (50 U/ml), and streptomycin (50 μg/ml). Explants were incubated at 37°C under 95% air–5% CO_2 (v/v). After 24 h the medium was replaced and incubation continued for a further 24 h. At the end of the experiment, the medium from the second 24 h incubation was removed and stored at −20°C until analysis. Explants were weighed, frozen and stored at −20°C until analysis.

For α-LA assay, explants were thawed and homogenized in 0.75 ml 50 mM phosphate buffer–0.1% v/v Triton X-100, pH 7.4, with 20–30 hand strokes in a Potter–Elvejhem homogenizer at 4°C. The homogenate was centrifuged in a Beckman microfuge at 12,000 g_{max} for 15 min, and the supernatant was aspirated and frozen at −20°C until analysis.

Iodination of α-LA was performed by the Iodogen method [33]. Iodogen (1.6 μg in 40 μl dichloromethane) was dried down in an Eppendorf microfuge tube, and 4 μg-LA in 20 μl 0.1 M phosphate buffer, pH 7.4, was added, together with 15 μl 0.5 M phosphate buffer, pH 7.4. Iodination was started by the addition of 0.5 mCi $Na^{125}I$ (13–17 mCi/μg). After 15 min at room temperature, the reaction was stopped by addition of 500 μl PBS (0.01 M phosphate buffer, 0.15 M NaCl, 0.1% w/v sodium azide, pH 7.6) and the mixture loaded onto a Pharmacia PD10 G25 column (Pharmacia (South Seas) Ltd, Carlton, Victoria, Australia; 5.5 × 1.5 cm, bed vol 9 ml) previously coated with 2% w/v BSA in PBS. The column was eluted with 0.1% w/v BSA in PBS: peak fractions of radioactivity were pooled, treated with AG1-X8 (200–400 mesh; Biorad, Hornsby, New South Wales, Australia) resin to remove free $^{125}I^-$, and applied to an Ultrogel ACA-54 column, which had been pre-equilibrated with 2% w/v BSA in PBS, and the column developed with 0.1% w/v BSA in PBS. Peak radioactivity fractions were subjected to the talc–resin–TCA test [34], and iodinated α-LA which failed these criteria was rejected for use in RIA.

Standards for the assay were from 39–5000 pg α-LA. The purified IgG against α-LA was used at a dilution of 1:14,000 of a 1 mg/ml solution in 0.1% BSA in PBS. At this dilution about 30% of the total immunoprecipitable [^{125}I]iodo-α-LA was bound. As second antibody, donkey anti-rabbit gamma globulin was diluted 1:70 in PBS; normal rabbit serum was used as carrier at a dilution of 1:600 in PBS. These dilutions gave maximal immunoprecipitation at the stated first antibody dilution. Assay buffer was 0.1% w/v BSA in PBS, and test samples were appropriately diluted in this buffer.

In a volume of 500 μl, the incubation mixture contained standards or test samples in 100 μl of

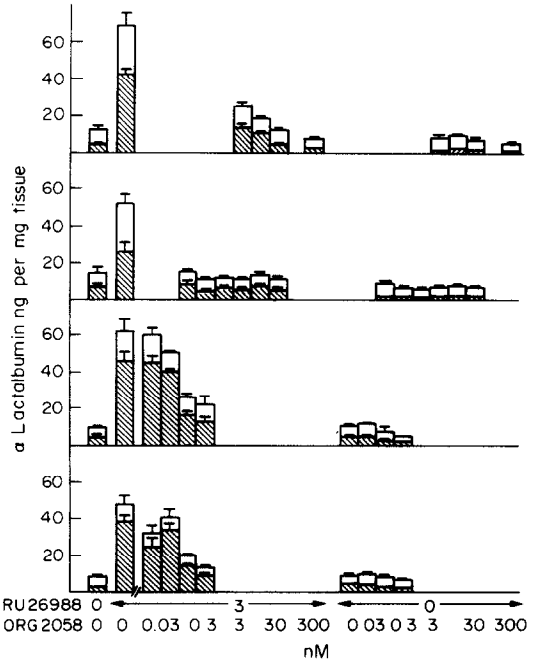

Fig. 1. Effect of RU26988 (3 nM) and ORG2058 (0.01–300 nM) on α-LA production by mammary gland explants. ORG2058 concentrations were 0.01, 0.03, 0.1 . . . 30, 300 nM; for clarity on the figure, only alternate concentrations are marked on the abscissa. The results of four separate experiments are shown; each bar represents the mean ± SEM of triplicate explant incubations in a single experiment. The height of each bar denotes α-LA production measured as the amount secreted into the medium during the last 24 h of a 48 h incubation (open portion), plus the amount found in the tissue at the end of the incubation (hatched portion). In all studies, 3 nM RU26988 elevated α-LA significantly ($P < 0.01$) above baseline (no added steroid), and this elevation was significantly ($P < 0.01$) blocked by ORG2058 concentration > 0.1 nM. In all studies ORG2058 alone progressively depressed α-LA below baseline, with a dose for threshold significance ($P < 0.05$) at 0.1–0.3 nM.

radioiodinated α-LA diluted to contain 10,000 cpm, and 100 μl of diluted rabbit anti-rat α-LA IgG. After incubation for 24 h at 4°C, 100 μl of diluted donkey anti-rabbit gamma globulin and 100 μl of diluted normal rabbit serum was added and the incubation continued at 4°C for a further 24 h. Bound radioactivity was separated from unbound radioiodinated α-LA by centrifugation at 3000 *g* for 30 min, the supernatant aspirated and discarded and the radioactivity in the pellet was measured in a gamma-counter.

Radioimmunoassays (RIA) were calculated either by use of the spline function method or by use of a computer program for RIA [35]. Sensitivity was defined as the 90% B/B_0 value and was 84 ± 6 pg/tube [mean ± SE] ($n = 14$ representing 840 ng/ml in assay samples). Non-specific binding range was 2.0–3.0%. The 50% displacement value over 14 assays was 648 pg/tube with a coefficient of variation of 13%. Over 14 assays, the coefficient of variation was 12% at 315 pg/tube and 5% at 621 pg/tube. The within assay coefficient of variation was <10% over the range 135 to 2600 pg/tube. Dilutions of both medium or tissue homogenate supernatants in assay buffer were parallel over the range of the standard curve.

When casein (0.001–10 μg) or rPRL (0.01–0.1 μg) was included in the α-LA RIA, no cross-reactivity was observed. Liver homogenate supernatant, mammary gland homogenate supernatant and serum from a 7 day lactating rat gave the following values, 0.8 μg/g tissue, 160 μg/g tissue and 1.6 μg/ml. Since the liver was not perfused, the α-LA found in the liver probably derived substantially from the blood supply. Statistical analysis was by ANOVA on log transformed data, followed by Duncan's multiple range *F*-test, on an HP 9825A calculator.

RESULTS

Figure 1 shows the effect, in four separate experiments, on α-LA production of 3 nM RU26988 alone, the antagonist effect of ORG2058 on this increase, and the effect of ORG2058 in the absence of RU26988. As reported in our previous studies [36], the synthetic glucocorticoid RU26988 (3 nM) significantly ($P < 0.01$) increased α-LA production over baseline (insulin and prolactin alone) levels. When the synthetic progestin ORG2058 (0.1–300 nM) was added together with 3 nM RU26988, a progressive decrease in α-LA production was seen, with a >50% decrease at ~0.1 nM ORG2058. In all studies, levels were significantly ($P < 0.01$) lower at >0.1 nM ORG2058, and production decreased to baseline levels by 1–30 nM ORG2058.

At levels above 0.1–0.3 nM, ORG2058 also significantly ($P < 0.01$) decreased αLA production below baseline (insulin plus prolactin, in the absence of added glucocorticoid). A feature of the effect of ORG2058 at these concentrations was the disproportionate decrease in tissue levels of α-LA (hatched bars), compared with medium levels (open bars). ORG2058 therefore appears to have a potent effect on α-LA production by rat mammary gland explants, both in the presence of glucocorticoid and incubated under baseline conditions (with insulin and prolactin alone).

DISCUSSION

There has been considerable debate over the mode of action of progesterone in several systems where glucocorticoid actions are inhibited [37, 38]. Several groups have examined casein mRNA synthesis in rat and mouse mammary gland explants, and have suggested that, since progestins can compete for glucocorticoid receptors, at least part of the inhibitory action of progesterone may be due to occupancy of glucocorticoid receptors [16, 27].

In our experiments we have found a half-maximal effect of ORG2058 on α-LA levels at doses (<0.1 nM) at least an order of magnitude lower than the dissociation contant for ORG2058 binding to progesterone receptors [29]. The first point for discussion arising from these findings concerns the question of direct effects of progesterone, versus effects due to competition for glucocorticoid receptors. At least for α-LA in the rat mammary gland, the evidence appears overwhelming for a direct action via progesterone receptors. ORG2058 is a relatively specific progestin, with an affinity for Type II dexamethasone-binding glucocorticoid receptors at least an order of magnitude lower than DM or RU26988 [29]. Given that 0.1 nM ORG2058 reduces to <50% the stimulatory effect of 3 nM RU26988 on α-LA levels, it appears impossible that such an effect is via displacement of glucocorticoid from glucocorticoid receptors, but rather than the effect of progestins on α-LA levels is mediated via occupancy of progesterone receptors.

Secondly, α-LA levels are completely suppressed at levels of ORG2058 which would minimally occupy glucocorticoid receptors, suggesting progestin occupancy of glucocorticoid receptors is inconsequential in this situation. What remains to be addressed, however, is the apparently disproportionate decrease in tissue levels of α-LA compared with medium levels, with progressively increasing doses of ORG2058; and, perhaps most importantly, the possible mechanisms whereby the system is supersensitive to progestins, in that >50% changes are seen at ligand concentrations (<0.1 nM) an order of magnitude lower than those required for half-maximal receptor occupancy.

The disproportion between the extent of the lowering of tissue and secreted levels of α-LA in response to ORG2058 is most simply explained by a differential sensitivity of synthesis and secretion to the inhibitory effects of progestins. Such a difference

in apparent sensitivity may reflect differences in steroid concentration, or time course of action, or both; the question can only be resolved by detailed time course studies, which are outside the ambit of the single, standard time-point studies described in the present paper.

A number of receptor–effector systems have been described in which the dose–response curves are shifted well to the left of the curve relating ligand concentration to binding. This effect is commonly ascribed to the presence of "spare" receptors, and has been described much more frequently for peptide than for steroid hormones [39]. It should be noted, however, that for both sex steroids [40–43] and glucocorticoids [44–47], there have been described clear examples of quite different dose–response curves for induced proteins in tissues or cell lines, with some responses closely coupled to receptor occupancy, and others shifted either to the right or to the left.

One possible explanation of the shift to the left of the progesterone dose–response curve is that of a chromatin progesterone regulatory element with very high affinity for progesterone–receptor complexes. An alternative explanation is the possible existence of multiple progesterone regulatory elements (PRE), as shown by von der Ahe *et al.*[48]. If binding to any one PRE is sufficient to abrogate transcription and/or decrease message half-life, then the dose–response curve for progestin action will be shifted to the left of that for receptor binding. The greater the number of such PRE's, the further the response curve will be shifted to the left of the receptor binding curve.

In contrast with glucocorticoid receptors [49, 50] there is currently no consensus on the nucleotide sequence of progesterone receptor binding sites on chromatin. It has recently been claimed, however, that progesterone receptors may share elements of a common consensus sequence—TGT$^{T}_{C}$CT—with glucocorticoid receptors [48]. There are 6 such sequences in the transcribed portion of the α-LA genome—one in the 5′ upstream region, 3 in intron 1 and 2 in intron 2 [51]. If such consensus sequences bind both activated glucocorticoid receptors and activated progesterone receptors, as suggested by von der Ahe *et al.*[48], there may well be a direct competition between progestins and corticosteroids—not at the receptor level, but for nuclear acceptor sites for activated steroid–receptors complexes.

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