Prolactin-induced α -lactalbumin activity in mammary explants from pregnant rabbits

A role for epidermal growth factor and glucocorticoids

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Exogenous prolactin alone can induce α -lactalbumin activity in rabbit mammary explants. Under these conditions, exogenous corticosterone has no effect. However, low levels of epidermal growth factor (EGF) can markedly inhibit the induction by prolactin, and this inhibitory effect, in turn, can be prevented by cortisol. The steroid can, in fact, convert EGF from a potent inhibitor into an agent which enhances the induction. None of the other growth factors tested inhibits induction of α -lactalbumin activity by prolactin, and no other type of steroid tested overcomes the effect of EGF. The results suggest that, under physiological conditions, glucocorticoid may be essential for induction of α -lactalbumin activity in the rabbit in order to override the effect of serum EGF.

The induction of casein synthesis in explanted mammary tissue from mice and rats requires more types of exogenous hormones than that in rabbit mammary tissue. Whereas the latter is dependent only on exogenous prolactin (Delouis & Combaud, 1977; Devinoy *et al.*, 1978), mouse (Nagaiah *et al.*, 1981; Ganguly *et al.*, 1980; Bolander *et al.*, 1981) and rat (Kulski *et al.*, 1983), mammary explants require exogenous insulin and glucocorticoid in addition to prolactin. The minimal hormone requirements for the induction of α -lactalbumin activity in the rabbit tissue *in vitro* have not been reported previously. Our intention in the present study was to determine whether the phenotypic expression of the α -lactalbumin gene in rabbit mammary explants, like that of the casein genes, can be switched on by prolactin alone. Also, we decided to determine whether the minimal hormone requirements observed in culture are in accord with those required under more physiological conditions.

In previous studies (Delouis & Combaud, 1977; Devinoy *et al.*, 1978) on isolated rabbit tissue, the only macromolecule added to the chemically defined medium was prolactin. Although there are obvious advantages in working with such a simplified system, there are also inherent diffi-

culties. Since this approach precludes the presence of potential inhibitors which may be present in the circulation of the intact animal, the minimal hormone requirements observed in this explant system may be spuriously low. The present study illustrates such a possibility. Although prolactin can induce α -lactalbumin activity in rabbit mammary explants when it is the only hormone added to the culture medium, the results suggest that at least two additional hormones are implicated physiologically in this response.

Experimental

Animals

New Zealand White rabbits, 16–17 days in term, were from Dutchland Farms, Denver, PA, U.S.A.

Chemicals

Ovine prolactin (NIH-PRL-14, PRL-15) was a gift from the Hormone Distribution Program of this Institute. Crystalline porcine insulin was a gift from Eli Lilly Co., Indianapolis, IN, U.S.A. Epidermal growth factor (EGF; receptor grade), multiplication stimulating activity, fibroblast growth factor, platelet-derived growth factor and 2.5S nerve growth factor were obtained from Collaborative Research, Waltham, MA, U.S.A.

Abbreviation used: EGF, epidermal growth factor.

Bovine α -lactalbumin, bovine milk galactosyl-transferase [4–6 units ($\mu\text{mol}/\text{min}/\text{mg}$), UTP, UDP-galactose, penicillin G, Hepes [4-(2-hydroxy-ethyl)-1-piperazine-ethanesulphonic acid], oestradiol-17 β , progesterone, corticosterone, aldosterone and testosterone were from Sigma Chemical Co., St. Louis, MO, U.S.A. Cortisol was from Calbiochem, La Jolla, CA, U.S.A. Powdered Medium 199 with Hanks' salts but without NaHCO_3 was from Grand Island Biological Co., Grand Island, NY, U.S.A. Gentamicin was from Microbiological Associates, Walkersville, MD, U.S.A. Triton X-100 was obtained from New England Nuclear Corp., Boston, MA, U.S.A. UDP-D-[6- ^3H]galactose (7 Ci/mmol) was purchased from Amersham/Searle, Arlington Heights, IL, U.S.A.

Media

Medium 199 containing Hanks' salts and 20 mM-Hepes, pH 7.5, penicillin G (35 $\mu\text{g}/\text{ml}$) and gentamicin (20 $\mu\text{g}/\text{ml}$) was used in these studies. Media containing insulin, cortisol and prolactin were sterilized with Nalgene 0.45 μm -pore-size filters, whereas those containing the growth factors and steroids were prepared by adding sterile solutions of these substances to sterilized media.

Mammary-gland organ culture

Explants, 1–2 mg each, were prepared from thoracic and abdominal mammary glands of 16–17-day-pregnant rabbits and cultured according to previously published procedures (Topper *et al.*, 1975; Dils & Forsyth, 1981), except that the gas phase was air. The media were changed daily.

Assay for α -lactalbumin activity

(a) *In explants.* Mammary-gland explants, 20–30 mg, at zero time and after culture, usually for 4 days or as otherwise indicated, were analysed for α -lactalbumin activity by measuring the amount of [^3H]lactose formed in the presence of 20 m-units of bovine milk galactosyltransferase and UDP-[6- ^3H]galactose (Nicholas & Topper, 1980). Bovine α -lactalbumin was used as standard, and the α -lactalbumin content was expressed as ng of bovine α -lactalbumin equivalents per mg of tissue.

(b) *In media.* Explant culture media containing prolactin or prolactin and EGF were collected daily. Bovine serum albumin was added to a concentration of 2 mg per ml. The media were dialysed in Spectrapor dialysis tubing (M_r cut-off 3500) for 36 h against two changes of 15 litres of distilled water at 4°C, freeze-dried and reconstituted to one-tenth of the original volume in 20 mM-Tris/HCl/10 mM-MgCl $_2$ /1% Triton X-100, pH 7.4. A 40 μl sample was assayed for α -lactalbumin activity as described above.

Results

Hormonal requirements for the induction of α -lactalbumin activity in mammary explants from pregnant rabbits are shown in Fig. 1. The activity is not detectable in freshly isolated tissue. The activity is not induced in explants cultured in media containing: (1) no added hormones; (2) insulin only; (3) cortisol only; and (4) both insulin and cortisol. However, exogenous prolactin alone does induce α -lactalbumin activity. Cortisol does not affect this induction. Insulin, on the other hand, enhances the induction.

Fig. 2 shows the prolactin dose-response curves for α -lactalbumin activity in systems with prolactin alone and with prolactin plus insulin and

Fig. 1. Hormone requirements for induction of α -lactalbumin activity in rabbit mammary-gland explants

Explants from 16–17-day-pregnant New Zealand White rabbits were cultured for 4 days in Medium 199 containing Hanks' salts and 20 mM-Hepes, pH 7.5, with no hormones (NH) or insulin (I) (0.1 $\mu\text{g}/\text{ml}$), cortisol (F) (1 $\mu\text{g}/\text{ml}$) or prolactin (PRL, 1 $\mu\text{g}/\text{ml}$), singly or in combination as shown. α -Lactalbumin activity was assayed in homogenates of the explants as described in Materials and methods section. Results are means $\pm$ range or mean $\pm$ S.E.M. for the number of animals shown in parentheses, the tissue from each animal being analysed separately. For some systems, results from several experiments have been pooled.

Fig. 2. Induction of α -lactalbumin activity in rabbit mammary-gland explants as a function of prolactin concentration

Explants were cultured for 4 days in medium containing various concentrations of prolactin, alone (●) or together with insulin (0.1 μ g/ml) and cortisol (0.1 μ g/ml) (■), and were analysed for α -lactalbumin activity. Numbers in parentheses represent the number of animals for each point, tissue from each animal being analysed separately.

Fig. 3. Time course of induction of α -lactalbumin activity in rabbit mammary-gland explants

Explants from 16 day-pregnant rabbits were cultured in medium containing prolactin (1 μ g/ml) alone (●), or together with insulin (0.1 μ g/ml) and cortisol (0.1 μ g/ml) (■), and analysed for α -lactalbumin activity. Results are means $\pm$ S.E.M. for the number of rabbits shown in parenthesis, tissue from each animal analysed separately.

cortisol. In both systems the induction is linear up to 1 μ g of prolactin/ml. Over a large concentration range the induction is greater in the presence of all three hormones than in the presence of prolactin alone.

The time courses of α -lactalbumin induction in rabbit mammary explants cultured with prolactin alone, and with prolactin plus insulin and cortisol, are shown in Fig. 3. A 2-day lag period is observed in the single-hormone system; induction appears to occur more rapidly in the presence of all three hormones. Maximum activity is reached on day 6 in both systems. By day 8, α -lactalbumin activity in the prolactin system has fallen, but remains unchanged in the three-hormone system.

In order to determine whether the effect of insulin, shown above, is specific, several growth factors were tested with prolactin. Fig. 4 indicates that while platelet-derived growth factor, multiplication-stimulating activity, fibroblast growth factor and 2.5S nerve growth factor have essentially no effect, EGF markedly inhibits induction in the tissue. After 4 days of culture the cumulative amount of α -lactalbumin in the medium is only

about 20% of the total (tissue plus medium); the total induction of α -lactalbumin activity is inhibited about 75% by EGF. Homogenates from explants cultured with EGF plus prolactin did not decrease the α -lactalbumin activity in extracts of tissue cultured with prolactin alone (results not shown). This demonstrates the absence of a lactose synthetase inhibitor in extracts of tissue cultured with EGF and prolactin.

EGF titration and the effects of insulin or cortisol on the EGF-prolactin- α -lactalbumin system in rabbit mammary explants are shown in Fig. 5. EGF strongly inhibits prolactin-induced activity, even at a level of 10 ng/ml. In some experiments, EGF produced considerable inhibition at 1 ng/ml (results not shown). The presence of insulin does not diminish this inhibition. By contrast, addition of cortisol, at a concentration as low as 10 ng/ml, not only prevents the inhibition by EGF, but actually evokes enhanced induction of α -lactalbumin activity.

Steroid specificity for reversal of EGF inhibition of prolactin-induced α -lactalbumin activity

Fig. 4. Effect of insulin and several growth factors on prolactin-induced accumulation of α -lactalbumin activity in rabbit mammary gland explants

Explants were cultured in media containing prolactin ($1 \mu\text{g/ml}$) alone, or together with insulin (I), epidermal growth factor (EGF), platelet-derived growth factor (PDGF), multiplication-stimulating activity (MSA), fibroblast growth factor (FGF) or 2.5S nerve growth factor (NGF), each at $0.1 \mu\text{g/ml}$, for 4 days and analysed for α -lactalbumin activity. The numbers of animals used are shown in parentheses, tissue from each animal being analysed separately.

is shown in Fig. 6. Both corticosterone and aldosterone reverse the inhibition, but do not, like cortisol, enhance the activity. Oestradiol- 17β , progesterone and testosterone do not influence the inhibition by EGF.

Discussion

The minimal hormone requirements for the induction of α -lactalbumin activity in rabbit mammary explants cultured in chemically defined medium appear to be the same as those reported previously (Delouis & Combaud, 1977; Devinoy *et al.*, 1978) for casein. Induction occurs when prolactin is the only macromolecule added. Although the addition of insulin, or insulin and cortisol enhances this induction, neither appears essential. However, the time course of the emergence of α -lactalbumin activity differs from that for the synthesis of casein. It was reported (Al-Sarraj *et al.*, 1979) that the induction of casein synthesis by mammary explants from pregnant rab-

Fig. 5. Inhibition of prolactin-induced accumulation of α -lactalbumin activity as a function of EGF concentration: ability of cortisol and inability of insulin to reverse the inhibition.

Mammary-gland explants were cultured for 4 days in medium containing prolactin ($1 \mu\text{g/ml}$) and various concentrations of EGF ($\circ$). Other explants were cultured for 4 days in medium containing prolactin ($1 \mu\text{g/ml}$) and a highly inhibitory concentration of EGF ($0.1 \mu\text{g/ml}$) to which various concentrations of insulin (I (∇)) or cortisol (F ($\blacksquare$)) were added. Values for the EGF titration curve are the means $\pm$ S.E.M. for eight rabbits, each analysed separately. Values for the insulin and cortisol titration curves are the mean $\pm$ range for two rabbits, each analysed individually.

bbits is linear during the first several days of culture. This observation has been confirmed in the present study (results not shown). By contrast, the induction of α -lactalbumin activity is linear only after a 1–2-day lag period. The reason for the difference between the two time courses is not known.

Prolactin-induced α -lactalbumin activity is profoundly inhibited by EGF. Other growth factors, such as platelet-derived growth factor, multiplication-stimulating activity, fibroblast growth factor and 2.5S nerve growth factor, have essentially no inhibitory influence. Since EGF is a normal component of serum, and since its level in serum is elevated considerably during pregnancy (Ances, 1973), it seemed reasonable that some other normal serum factor might be able to counteract the inhibitory action of EGF. It is shown that this growth factor can virtually abolish the induction of

Fig. 6. Effect of various steroids on EGF inhibition of prolactin-induced α -lactalbumin activity in rabbit mammary explants

Explants were cultured for 4 days in medium containing prolactin (1 μ g/ml), alone, or together with EGF (0.1 μ g/ml), or together with EGF and cortisol (F), corticosterone (C), aldosterone (A), oestradiol-17 β (E₂), progesterone (Prog) or testosterone (T). All steroids were added at a concentration of 0.1 μ g/ml. Results are means $\pm$ S.E.M. or means $\pm$ range for the number of rabbits shown in parentheses, each being analysed individually.

α -lactalbumin activity both in the presence and in the absence of insulin, but that this effect can be eliminated by cortisol. Moreover, this steroid, at low levels, actually converts EGF from an inhibitory into a stimulatory factor, even though cortisol does not affect induction in the presence of prolactin alone. Both corticosterone, the other major glucocorticoid present in the rabbit (Malinowska *et al.*, 1972), and aldosterone can largely overcome the inhibitory influence of EGF, but do not confer stimulatory activity on it. The other steroid hormones tested are ineffective. The level of gene

expression at which EGF exerts its inhibitory effect in the absence of glucocorticoid or aldosterone, and the level at which it enhances the induction of α -lactalbumin activity in the presence of cortisol, are not known.

Although prolactin alone can induce α -lactalbumin activity in rabbit mammary tissue *in vitro* in a chemically defined medium, it appears that under physiological conditions the minimal hormone requirements for this induction may be more complex. Assuming that an elevated level of EGF during pregnancy can simulate the inhibition reported here, cortisol may be essential for the development of α -lactalbumin activity in the intact rabbit. This function of cortisol in relation to rabbit α -lactalbumin may be different mechanistically from its essential role in mouse casein (Ganguly *et al.*, 1980; Nagaiah *et al.*, 1981) and rat casein-gene expression (Kulski *et al.*, 1983).

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