The effect of progesterone on prolactin stimulation of fatty acid synthesis, glycerolipid synthesis and lipogenic-enzyme activities in mammary glands of pseudopregnant rabbits, after explant culture or intraductal injection

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1. The activities of lipogenic enzymes, such as acetyl-CoA carboxylase, fatty acid synthetase and glucose-6-phosphate dehydrogenase, and glycerolipid synthesis increased significantly in mammary explants of 11-day-pseudopregnant rabbits in response to prolactin, in the presence of near-physiological concentrations of insulin and corticosterone in culture. 2. Increasing the concentration of progesterone in culture resulted in suppression of glycerolipid synthesis and activities of acetyl-CoA carboxylase and fatty acid synthetase, but not the pentose phosphate dehydrogenases. However, at near-physiological concentration of progesterone, only acetyl-CoA carboxylase activity was decreased. 3. Injection of prolactin intraductally into 11-day-pseudopregnant rabbits stimulated glycerolipid synthesis, fatty acid synthesis and enzymes involved in fatty acid synthesis, after 3 days. 4. Intraductal injection of progesterone separately or together with prolactin had no significant effect on basal or stimulated lipogenesis in mammary glands. Intramuscular injection of progesterone at 10 mg/day did not suppress fatty acid synthesis stimulated when prolactin was injected intraductally, but a significant inhibition was observed at a higher dose (80 mg/day).

INTRODUCTION

The importance of progesterone and prolactin for milk synthesis in the mammary gland in most mammals is well recognized. Progesterone is concerned mainly with preparing the alveolar cells to a stage which can respond readily to prolactin and achieve the full potential of the gland. Progesterone in synergism with oestrogen is found to be mammogenic in rabbits and other mammals (Cowie *et al.*, 1980). Prolactin is also mammogenic and is the key hormone which triggers lactogenesis in most mammals, including the rabbit (Bourne *et al.*, 1974; Forsyth, 1983). Endogenous progesterone is also thought to inhibit lactation, which does not occur readily until after the end of pregnancy in the majority of mammals. Moreover, removal of the source of progesterone during pregnancy induces lactogenesis, which can be prevented by replacement therapy (Kuhn, 1983). On investigating the effect of progesterone on fatty acid synthesis in mammary-gland explants from pseudopregnant rabbits, we found that progesterone suppressed prolactin-stimulated fatty acid synthesis at hyperphysiological concentration (5 µg/ml), but a lower concentration (0.05 µg/ml) was without effect. An intermediate concentration (0.5 µg/ml) showed inhibition when either corticosterone or insulin concentration was lowered in the culture medium (Martyn & Falconer, 1984). The present paper examines the effect of the same range of progesterone concentrations in culture on the activities of the lipogenic enzymes acetyl-CoA carboxylase, fatty acid synthetase, glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase, and on glycerolipid synthesis in mammary explants from 11-day-pseudopregnant rabbits.

In addition, intraductal injection has been used to

administer prolactin into individual gland sectors, which then secrete milk without affecting uninjected glands of the same rabbit (Falconer & Fiddler, 1970). This approach has been employed to examine the effect of prolactin and progesterone on lipogenic-enzyme activities, fatty acid synthesis and glycerolipid synthesis in intact rabbits.

MATERIALS AND METHODS

Animals

Virgin rabbits about 9–10 months old and maintained under natural cycles of light and darkness were made pseudopregnant by a single intravenous injection of human chorionadotropin (100–150 i.u.) into the marginal ear vein. The day of injection was taken as day 0 of pseudopregnancy, which was confirmed by counting new corpora lutea at the time of death.

Chemicals

Medium 199 was obtained from Commonwealth Serum Laboratories, Melbourne, Australia. Corticosterone and progesterone were obtained from Sigma Chemical Co., St Louis, MO, U.S.A. Human chorionadotropin and progesterone in oil (Proluton) were obtained from Schering Pty. Ltd., Sydney, N.S.W., Australia. Sheep prolactin (NIH-P-S-14, 25 i.u./mg) was a gift from Endocrine Study Section, National Institutes of Health, Bethesda, MD, U.S.A. Sheep prolactin-16 (AFP-5915A; 30.5 i.u./mg) was a gift from National Institute of Arthritis, Diabetes, Digestive and Kidney Diseases (NIADDK), Bethesda, MD, U.S.A. Sodium [^{14}C]acetate [1.8–2.1 GBq (49–57 mCi)/mmol] and $\text{Na}_2^{14}\text{CO}_3$ [2.1 GBq (57 mCi)/mmol] were obtained from

Amersham Australia Pty. Ltd., Sydney, N.S.W., Australia. L-[U-¹⁴C]Glycerol 3-phosphate [disodium salt; 5.3 GBq (144 mCi)/mmol] was obtained from New England Nuclear, Searle Nucleonics, Sydney, N.S.W., Australia. NADPH, NADP⁺, CoA, acetyl-CoA, malonyl-CoA, bovine serum albumin (fatty acid-poor), dithiothreitol, glucose 6-phosphate, 6-phosphogluconate, *sn*-glycerol 3-phosphate (disodium salt), NaHCO₃, Hepes, 1,2-dipalmitoyl-3-*sn*-phosphatidic acid, 3-palmitoyl-*sn*-glycerol, 2,3-dipalmitoyl-*sn*-glycerol, tripalmitoylglycerol and 3-*sn*-phosphatidylcholine were purchased from Sigma. PPO (2,5-diphenyloxazole) and POPOP [1,4-bis-(5-phenyloxazol-2-yl)benzene] were obtained from Packard Instrument Co., Sydney, N.S.W., Australia. Streptopen [procaine penicillin (250 mg/ml) + dihydrostreptomycin (250 mg/ml)] was obtained from Glaxovet, Glaxo Australia Pty. Ltd., Melbourne, Vic., Australia. Nembutal (pentobarbitone sodium; 60 mg/ml) was obtained from Ceva Laboratories, Hornsby, N.S.W., Australia. Stresnil (azaperone; 40 mg/ml) was obtained from Smith, Kline and French Laboratories (Australia) Ltd., Sydney, N.S.W., Australia.

Preparation and culture of mammary explants

Explants of mammary alveoli were prepared by the method of Forsyth & Myres (1971). Groups of ten explants were cultured at 37 °C in medium 199 containing 0.6 mM-sodium acetate, 15 mM-NaHCO₃, 20 mM-Hepes, pH 7.4, and the antibiotics polymixin B sulphate (2 units/ml), neomycin sulphate (1 unit/ml) and kanamycin sulphate (10 µg/ml) in an atmosphere of air for specified periods. Viability of the mammary epithelial cells and alveolar integrity were confirmed by histological examination after staining with haematoxylin and eosin.

Rate of fatty acid synthesis

[1-¹⁴C]Acetate was used to determine the rate of fatty acid synthesis in mammary explants by the method described previously (Falconer *et al.*, 1978; Smith & Falconer, 1983).

Preparation of microsomal fraction and particle-free supernatant from mammary explants

At the end of culture, 100 explants were rinsed in 0.9% NaCl and homogenized in 0.5 ml of ice-cold 50 mM-Tris/HCl buffer containing 150 mM-KCl and 5 mM-EDTA, pH 7, with an Ultra-Turrax homogenizer with a micro-probe. The homogenization was carried out three or four times for a period of 10–15 s each. The homogenate was centrifuged at 5000 *g* for 20 min at 4 °C. The supernatant was centrifuged at 100 000 *g* for 60 min at 4 °C in a Beckman ultracentrifuge model L2-65B. The pellet (microsomal fraction) was suspended in 200 µl of 0.25 M-sucrose in 0.13 M-potassium phosphate buffer, pH 7.4, and stored in liquid N₂ (–170 °C). The supernatant (cytosol) was also stored under liquid N₂ after its volume was noted. Analysis of enzyme activities was completed within 5 days of storage.

Glycerolipid synthesis

The method used was based on that of several authors (Kuhn, 1967; Benson & Emery, 1971; Dimenna & Emery, 1971). The assay medium contained 0.25 M-sucrose in 0.13 M-potassium buffer, pH 7, 0.1 mM-CoA, 5 mg of bovine serum albumin (fatty acid-poor), 1.9 mM-dithio-

threitol, 2.6 mM-MgCl₂, 4 µCi of L-[U-¹⁴C]glycerol 3-phosphate, 18 mM-DL- α -glycerophosphate (disodium salt), 0.16 mM-potassium palmitate, 0.16 mM-potassium hexanoate, 3 mM-ATP and microsomal fraction consisting of 250–450 µg of enzyme protein in a final volume of 1.0 ml. Potassium palmitate and potassium hexanoate were prepared from the corresponding fatty acids by the method of Evans & Mueller (1963). The microsomal fraction in 0.25 M-sucrose/0.13 M-potassium phosphate buffer, pH 7.4, was homogenized with a ground-glass rod attached to an electrically driven motor, before addition to the assay medium. The reaction was started by the addition of ATP and incubated at 37 °C in a shaking water bath for 30 min. Control incubations were carried out with all the components except the microsomal fraction. The reaction was stopped by the addition of 8 ml of heptane/propan-2-ol (1:1, v/v) and 6.0 ml of 3.2 mM-NaOH. The lipid was extracted by shaking and allowed to form two clear phases. The radioactivity in samples of the heptane (top) layer was counted by scintillation spectrometry.

Acetyl-CoA carboxylase (EC 6.4.1.2)

The enzyme was assayed by measuring [¹⁴C]bicarbonate fixation into malonyl-CoA by a procedure based on previous methods (Jones, 1967; Gregolin *et al.*, 1968; Mackall & Lane, 1977). The assay medium contained 60 mM-Tris/HCl buffer, pH 7.4, 8 mM-MgCl₂, 10 mM-potassium citrate, 4 mg of bovine serum albumin (fatty acid-poor), 1.9 mM-dithiothreitol, 4 mM-ATP, 3 mM-acetyl-CoA, 20 mM-NaH¹⁴CO₃ (2–4 µCi) and 180–260 µg of enzyme protein in a final volume of 0.4 ml. The reaction was started by the addition of acetyl-CoA, NaH¹⁴CO₃ and ATP after incubation of the other components for 15 min at 37 °C in a shaking water bath. Control incubations without enzyme were used as blanks. In some cases control incubations were carried out without acetyl-CoA, and the low radioactivity detected was similar to that of blank incubations without enzyme. Incubations were carried out for 5 min; then the reaction was stopped by adding 0.2 ml of 6 M-HCl and the mixture centrifuged in a bench centrifuge. Samples of supernatant were transferred to scintillation vials and heated for 1.5 h at 70 °C, and the radioactivity in the dried sample was counted by scintillation spectrometry. The scintillant consisted of 10 ml of toluene/Triton X-100 (2:1, v/v) with 0.4% PPO and 0.05% POPOP. One unit of acetyl-CoA carboxylase is defined as the amount of enzyme which catalyses the fixation of 1 µmol of H¹⁴CO₃ into malonyl-CoA/min.

Fatty acid synthetase

This enzyme was assayed essentially by the method of Speake *et al.* (1975). The assay medium contained 0.2 M-potassium phosphate buffer, pH 6.9, 0.08 mM-acetyl-CoA, 0.08 mM-malonyl-CoA, 1 mM-EDTA, 0.075 mM-NADPH and 125–175 µg of enzyme protein in a final volume of 1.0 ml. Dithiothreitol was omitted from the assay medium, as it was reported to form *O*-acyl dithiothreitol, non-enzymically (Stokes & Stumpf, 1974). The reaction was started by the addition of both acetyl-CoA and malonyl-CoA, and the rate of decrease in *A*₃₄₀ was measured with a recording spectrophotometer (DMS Varian 80). One unit of fatty acid synthetase is defined as the amount of enzyme which catalyses the oxidation of 1 µmol of NADPH/min.

Table 1. Prolactin stimulation of lipogenesis in mammary gland explants from 11-day-pseudopregnant rabbits

Explants were cultured in Medium 199 in the presence of insulin (I; 25 ng/ml), corticosterone (C; 25 ng/ml) and prolactin (P; 100 ng/ml). Glycerolipid synthesis and lipogenic-enzyme activities were determined as described in the Materials and methods section. Values represent means $\pm$ S.E.M. of data from explant cultures of tissue from three rabbits. * $P < 0.05$, † $P < 0.001$, for significance of prolactin stimulation (Student's t test).

Time in culture (h)	Hormones in culture	Glycerolipid synthesis: [U- ¹⁴ C]glycerophosphate incorporation into glycerolipid (nmol/min per mg of protein)	Activity (munits/mg of protein)			
			Acetyl-CoA carboxylase	Fatty acid synthetase	Glucose-6-phosphate dehydrogenase	6-Phosphogluconate dehydrogenase
0	No hormones	0.08 $\pm$ 0.04	0.41 $\pm$ 0.07	1.5 $\pm$ 0.4	56 $\pm$ 22	84 $\pm$ 20
47	I, C	0.11 $\pm$ 0.04	0.41 $\pm$ 0.13	1.5 $\pm$ 0.3	41 $\pm$ 11	69 $\pm$ 23
47	I, C, P	0.32 $\pm$ 0.05*	1.90 $\pm$ 0.30†	8.7 $\pm$ 0.5†	92 $\pm$ 13*	88 $\pm$ 7

Glucose-6-phosphate dehydrogenase (EC 1.1.1.49)

The assay was essentially as described by Jones (1967). The assay medium contained 75 mM-Tris/HCl buffer, pH 7.3, 1 mM-glucose 6-phosphate, 5 mM-MgCl₂, 0.1 mM-NADP⁺ and 25–35 μ g of enzyme protein in a final volume of 1.0 ml. The reaction was started by the addition of glucose 6-phosphate, and the initial rate of change of A_{340} was measured in a recording spectrophotometer. Since Jones (1967) has shown that during the initial period 1 mol of NADP⁺ was reduced/mol of glucose 6-phosphate oxidized, the presence of 6-phosphogluconate dehydrogenase in the particle-free supernatant would not contribute to the production of NADPH under these conditions. Therefore one unit of glucose-6-phosphate dehydrogenase was taken as the amount of enzyme which catalyses the reduction of 1 μ mol of NADP⁺/min.

6-Phosphogluconate dehydrogenase (EC 1.1.1.44)

The medium for this assay contained 65 mM-Tris/HCl buffer, pH 9, 1 mM-6-phosphogluconate, 5 mM-MgCl₂, 0.1 mM-NADP⁺ and 25–35 μ g of enzyme protein in a final volume of 1.0 ml. The reaction was started by the addition of 6-phosphogluconate and the rate of increase in A_{340} was measured by spectrophotometer. One unit of 6-phosphogluconate dehydrogenase is the amount of enzyme which catalyses the reduction of 1 μ mol of NADP⁺/min.

Preparation of homogenate from glands injected intraductally for enzyme measurement

On days 3 or 5 after intraductal injection, the glands were dissected out individually, freed of muscle and chopped by scissors into small bits. The tissue mince was homogenized in 4 vol. of buffer (50 mM-Tris containing 150 mM-KCl and 5 mM-EDTA, pH 7.0) by using an Ultra-Turrax homogenizer, three or four times for a period of 15 s each at 4 °C, and filtered through nylon gauze. The rest of the procedure for the measurement of enzyme activities was similar to experiments with mammary explants, except for the changes noted. The microsomal pellet was stored in 1.0 ml of 0.25 M-sucrose containing 0.13 M-phosphate buffer, pH 7.0. In the assay of glycerolipid synthesis, the incubation time was 10 min.

Enzyme analysis

All the enzymes were assayed at 37 °C under conditions in which the activity was linearly related to the concentration of protein and time of incubation.

Protein analysis

Protein was determined by the method of Lowry *et al.* (1951), with bovine serum albumin as the standard.

Mammary intraductal injections

Pseudopregnant virgin rabbits (9–11 months old) were anaesthetized with 0.2 ml of Stresnil intramuscularly, followed by 1–2 ml of Nembutal intravenously. The nipples were prepared by shaving the fur around, and removing the waxy secretions that occlude the pores. Injections were carried out with 27-gauge blunt-ended needles under a dissection microscope. Each rabbit received 0.5 ml of the antibiotic Streptopen intramuscularly after intraductal injection. Prolactin was injected either as emulsion or as aqueous solution, made by dissolving prolactin in 0.1 ml of 0.1 M-NaOH and immediately diluting it with phosphate-buffered saline (137 mM-NaCl/2.7 mM-KCl/8.1 mM-Na₂HPO₄/1.5 mM-KH₂PO₄) containing 0.05–0.1% (w/v) Blue Dextran (M_r 2000000). The emulsion was prepared by sonicating briefly 2 parts of aqueous phase, consisting of phosphate-buffered saline containing 1% (w/v) bovine serum albumin and 0.05–0.1% Blue Dextran 2000000, with 1 part of safflower oil. Progesterone was dissolved in safflower oil (at 70 °C), before emulsifying.

Progesterone assay

The progesterone concentration in rabbit serum was measured by the Garvan Institute of Medical Research, St. Vincent's Hospital Sydney, N.S.W., Australia, by radioimmunoassay.

Statistics

Significance of treatments were analysed by analysis of variance, regression analysis and Student's t test. The pooled means of enzyme activities or rates of fatty acid synthesis in untreated and hormone-free emulsion-treated glands were compared with mean activities of enzymes in treated tissue for estimation of significance, since the

Fig. 1. Differential effect of progesterone on the rate of glycerolipid synthesis and activities of some lipogenic enzymes in mammary explants from 11-day-pseudopregnant rabbits, after culture for 47 h

(a) Rate of glycerolipid synthesis; (b) acetyl-CoA carboxylase activity; (c) fatty acid synthetase activity; (d) glucose 6-phosphate dehydrogenase activity; (e) 6-phosphogluconate dehydrogenase activity. Mammary explants were cultured in Medium 199 in the presence of insulin (25 ng/ml), corticosterone (25 ng/ml), prolactin-14 (100 ng/ml) and progesterone. Glycerolipid synthesis and lipogenic enzymic activities were determined as described in the Materials and methods section. Values represent means $\pm$ S.E.M. of data from explants cultured from three rabbits. Slopes were analysed by regression analysis.

values for any of the parameters tested in hormone-free emulsion-treated glands and untreated glands did not differ significantly.

RESULTS

Effect of hormones on glycerolipid synthesis ($[U-^{14}C]$ glycerophosphate incorporation into lipid) in cultured mammary explants

Table 1 shows that the low rate of glycerolipid synthesis in the mammary gland of 11-day-pseudo-pregnant rabbits was significantly enhanced after culture for 47 h in the presence of insulin, corticosterone and prolactin ($P < 0.05$). Insulin and corticosterone without

prolactin did not have any significant stimulatory effect. Culture of the explants in the presence of progesterone at 0.05, 0.5 or 5.0 µg/ml, along with insulin, corticosterone and prolactin, resulted in a significant ($P < 0.05$) progressive inhibition of glycerolipid synthesis (Fig. 1).

Effect of hormones on lipogenic-enzyme activities in cultured mammary explants

Acetyl-CoA carboxylase. The low acetyl-CoA carboxylase activity in the mammary tissue of 11-day-pseudo-pregnant rabbits was not affected by insulin and corticosterone in the culture medium for 47 h (Table 1). Addition of prolactin stimulated acetyl-CoA carboxylase activity markedly ($P < 0.02$). This increase was suppressed

Table 2. Fatty acid synthesis in mammary explants from pseudopregnant rabbits, 3 days after intraductal injection of prolactin or progesterone or both

Prolactin-16 (50 µg/50 µl per duct) or progesterone (200 µg/50 µl per duct) or a mixture of prolactin and progesterone (50 µg and 200 µg/50 µl per duct) respectively were injected in the form of emulsion prepared as described in the Materials and methods section into the teat ducts of 11-day-pseudopregnant rabbits (four ducts/gland). Control glands received emulsion intraductally or were left untreated. The rate of fatty acid synthesis in mammary glands 3 days after intraductal injection of emulsion was similar to that in untreated glands [fatty acid synthesis in emulsion-treated or untreated glands respectively was 0.07 ± 0.02 and 0.10 ± 0.02 nmol/h per mg (means $\pm$ S.E.M. for six observations)]. Hence the mean of the pooled values is represented as 'Control' below. Each treatment was applied to two glands of three rabbits assayed in triplicate; results are means $\pm$ S.E.M. *Significantly different ($P < 0.01$) from control and progesterone-treated groups, by analysis of variance. † Not significantly different from prolactin alone.

Intraductal treatment	[1- ¹⁴ C]Acetate incorporation into fatty acids (nmol/h per mg of explant)
Control	0.09 ± 0.01
Progesterone	0.09 ± 0.01
Prolactin	$0.79 \pm 0.09^*$
Prolactin plus progesterone	$0.60 \pm 0.14^\dagger$

Table 3. Rate of glycerolipid synthesis and activities of some lipogenic enzymes in mammary glands of pseudopregnant rabbits, 3 days after intraductal injection of prolactin or progesterone or both

Prolactin-16 (50 µg/50 µl per duct) or progesterone (200 µg/50 µl per duct) or a mixture of prolactin and progesterone (50 µg and 200 µg/50 µl per duct) were injected in the form of an emulsion into teat ducts of 11-day-pseudopregnant-rabbit mammary glands. Control glands were left untreated or injected with hormone-free emulsion. Data from control glands have been pooled, as there was no significant difference between the two types of control. Significance of treatments was determined by analysis of variance. Values represent means $\pm$ S.E.M. (*n* glands) from four or five rabbits. * $P < 0.05$, † $P < 0.01$, comparing treatment with control.

Treatment	Glycerolipid synthesis: [U- ¹⁴ C]glycerophosphate incorporation into glycerolipid (nmol/min per mg of protein)	Enzyme activity (munits/mg of protein)			
		Acetyl-CoA carboxylase	Fatty acid synthetase	Glucose-6-phosphate dehydrogenase	6-Phosphogluconate dehydrogenase
Control	0.53 ± 0.12 (8)	0.66 ± 0.19 (11)	1.19 ± 0.21 (10)	15.1 ± 1.7 (8)	42.7 ± 3.9 (10)
Progesterone	0.65 ± 0.14 (8)	0.65 ± 0.10 (10)	1.24 ± 0.21 (10)	17.5 ± 2.3 (8)	42.5 ± 4.3 (10)
Prolactin	1.30 ± 0.32 (9)†	1.96 ± 0.45 (11)†	4.28 ± 0.86 (11)†	24.8 ± 2.4 (9)*	57.2 ± 3.4 (11)†
Progesterone plus prolactin	1.56 ± 0.48 (8)†	1.90 ± 0.44 (10)†	4.13 ± 0.96 (10)†	27.7 ± 5.6 (8)*	59.7 ± 5.1 (10)†

significantly ($P < 0.05$) by all concentrations of progesterone added to the culture medium (Fig. 1).

Fatty acid synthetase. In rabbit mammary tissue on day 11 of pseudopregnancy, the activity of fatty acid synthetase was low, and it was stimulated significantly ($P < 0.01$) by prolactin together with insulin and corticosterone when present in culture for 47 h (Table 1). Addition of progesterone at 0.5 and 5 µg/ml to the medium resulted in a significant ($P < 0.02$) progressive decrease in the activity of fatty acid synthetase (Fig. 1).

Glucose-6-phosphate dehydrogenase. The basal activity of glucose-6-phosphate dehydrogenase in mammary tissue of 11-day-pseudopregnant rabbits was not affected significantly when explants were cultured for 47 h in the presence of insulin and corticosterone (Table 1). With prolactin also in the culture medium, the enzyme activity rose significantly ($P < 0.05$). Addition of progesterone at concentrations of 0.05, 0.5 or 5 µg/ml to the medium containing insulin, corticosterone and prolactin had no

significant effect on glucose-6-phosphate dehydrogenase activity (Fig. 1).

6-Phosphogluconate dehydrogenase. The activity of 6-phosphogluconate dehydrogenase in the mammary tissue of 11-day-pseudopregnant rabbits was neither stimulated by prolactin nor inhibited by progesterone in the presence of insulin and corticosterone (Table 1, Fig. 1). Insulin and corticosterone in the culture medium did not affect the activity of 6-phosphogluconate dehydrogenase compared with culture in a hormone-free medium (Table 1).

Effect of intraductal administration *in vivo* of prolactin and/or progesterone on fatty acid synthesis in mammary explants

As shown in Table 2, at 3 days after intraductal injection of prolactin, fatty acid synthesis in explants of mammary gland was stimulated dramatically (8-fold, significant at $P < 0.01$) in prolactin-injected glands, compared with control glands of the same rabbits. The

Table 4. Fatty acid synthesis in mammary explants from pseudopregnant rabbits 5 days after intraductal injection of prolactin and the intramuscular injection of progesterone

Prolactin-16 as aqueous solution (50 µg/50 µl per duct) was injected intraductally into the mammary glands (four ducts/gland) of 11-day-pseudopregnant rabbits. Progesterone in oil was injected intramuscularly twice a day for 5 days, commencing on the day of intraductal injection of prolactin. Control rabbits received vehicle only. The rabbits were killed 5 days after intraductal injection. Groups of ten explants prepared under aseptic conditions were incubated in 2 ml of medium 199 containing 0.6 mM-sodium [1-¹⁴C]acetate (370 kBq) at 37 °C for 2 h. Rate of fatty acid synthesis was determined as described in the Materials and methods section. Abbreviations: i.d., intraductal; i.m., intramuscular. Results are means ± s.e.m., for the numbers of rabbits shown in parentheses, where appropriate. *Significantly higher than for control tissue ($P < 0.01$); †significantly lower ($P < 0.01$) than the rate of fatty acid synthesis in glands of rabbits which received prolactin only; by analysis of variance.

Hormones injected	[1- ¹⁴ C]Acetate incorporation into fatty acids (nmol/h per mg of explant)	Serum progesterone (ng/ml)
Phosphate-buffered saline containing Blue Dextran (i.d.)	0.16 ± 0.03 (2)	1.1 (0.9, 1.3)
Prolactin (i.d.)	2.30 ± 0.21* (4)	1.3 ± 0.15
Prolactin (i.d.) plus progesterone (i.m.) (10 mg/day)	2.05 ± 0.12* (2)	4.7 (4.5, 5.0)
Prolactin (i.d.) plus progesterone (i.m.) (80 mg/day)	1.02 ± 0.26† (4)	76.4 ± 16.8

intraductal injection of progesterone together with prolactin did not significantly affect prolactin stimulation. Progesterone alone injected intraductally had no effect on fatty acid synthesis in rabbit mammary-gland explants (Table 2).

Effect of intraductal administration *in vivo* of prolactin and/or progesterone on lipogenic enzymes in mammary tissue

As shown in Table 3, untreated and hormone-free emulsion-treated glands had basal lipogenic-enzyme activities and synthesized glycerolipids at rates which did not differ significantly from each other. In general, mammary glands 3 days after intraductal injection of prolactin, or prolactin plus progesterone, had more secretion as assessed visually than did untreated, emulsion-treated or progesterone-treated glands within the same rabbits. [U-¹⁴C]Glycerophosphate incorporation into lipid and activities of acetyl-CoA carboxylase, fatty acid synthetase, glucose-6-phosphate dehydrogenase and phosphogluconate dehydrogenase increased significantly, 3 days after intraductal injection, in prolactin-injected glands compared with control glands ($P < 0.01$, $P < 0.01$, $P < 0.01$, $P < 0.05$, $P < 0.01$ respectively; Table 3). Intraductal injection of progesterone with prolactin did not abolish the stimulatory effect of prolactin. Intraductal injection of progesterone alone into the glands did not inhibit any of the enzyme activities tested.

Effect of intramuscular injection of progesterone on fatty acid synthesis in mammary tissue explants, 5 days after intraductal injection of prolactin

Fatty acid synthesis measured by [1-¹⁴C]acetate incorporation remained low in explants of rabbit mammary glands 5 days after intraductal injection of phosphate-buffered saline into the glands on day 11 of pseudopregnancy (Table 4). When prolactin was injected intraductally into mammary glands of 11-day-pseudopregnant rabbits, the rate of fatty acid synthesis was stimulated markedly (16-fold) 5 days later. Injection of progesterone (5 mg intramuscularly, twice a day) into rabbits for 5 days after they received intraductal prolactin injection on day 11 of pseudopregnancy had no

significant effect on the extent of prolactin stimulation. However, injection of progesterone at 80 mg/day, which increased plasma progesterone to 76 ng/ml, was significantly inhibitory ($P < 0.01$).

DISCUSSION

The stimulation of lipogenic-enzyme activity and of glycerolipid synthesis (Table 1) in mammary explants of pseudopregnant rabbits in response to prolactin *in vitro* or after intraductal injection of prolactin *in vivo* follows the trend in lipogenesis which occurs naturally soon after parturition. The activities of acetyl-CoA carboxylase (Gul & Dils, 1969; Hartmann & Jones, 1970; Mellenberger & Bauman, 1974; Short *et al.*, 1977), the rate of fatty acid synthesis (Strong & Dils, 1972) and the activities of glycerolipid-synthesizing enzymes (palmitoyl-CoA synthetase, glycerol-3-phosphate palmitoyltransferase and phosphatidate phosphohydrolase; Short *et al.*, 1977) all increase *in vivo*, in rabbit mammary glands around parturition. These increases in enzyme activities are preceded by a sharp increase in circulating prolactin (McNeilly & Friesen, 1978; Muccioli *et al.*, 1982) and a progressive decrease in progesterone in peripheral circulation (Challis *et al.*, 1973; Quirk *et al.*, 1984).

The increase in fatty acid synthesis in mammary explants *in vitro* in response to prolactin together with insulin and corticosterone is greater (2.0 nmol/min per mg of explant; Martyn & Falconer, 1984) than *in vivo* at 3 days after intraductal injection of prolactin (0.8 nmol/min per mg; Table 2). The presence of progesterone at 0.05, 0.5 or 5 µg/ml in culture appears to affect lipogenic enzymes differentially. Since progesterone did not significantly inhibit the dehydrogenases of the pentose phosphate pathway, even at high concentration (5 µg/ml; Fig. 1), it follows that progesterone exerts its effect in a specific manner rather than by general metabolic toxicity. This is suggested by the effects on protein synthesis in mammary explants under comparable conditions (Martyn & Falconer, 1984). The significant negative correlation between progesterone concentration and fatty acid synthetase activity is in agreement with the

similar trend observed in overall fatty acid synthesis in response to progesterone (Martyn & Falconer, 1984). At the near-physiological concentration of progesterone used, 0.05 µg/ml (Challis *et al.*, 1973; Quirk & Currie, 1984), an inhibitory effect was observed only on the activity of acetyl-CoA carboxylase (Fig. 1).

Prolactin, when given by mammary intraductal injection, localized in the injected gland and induced lactation, as evidenced by visible milk in injected ducts (Lyons, 1942; Bradley & Clarke, 1956; Chadwick, 1962; Fiddler & Falconer, 1968; Birkinshaw & Falconer, 1972) without effect on the adjacent glands. In the present study, on day 3 after intraductal injection of prolactin, a significant increase in enzymes necessary for the synthesis of fatty acids (Table 3) and a significant increase in overall synthesis of fatty acids from acetate was observed. This, together with increased use of fatty acids from circulating lipoproteins mobilized by lipoprotein lipase (Falconer & Fiddler, 1970), provides the source of fatty acids for esterification as triacylglycerols. A stimulation of glycerolipid synthesis (Table 3) was also observed. Histological examination of the tissue showed that most of the alveoli in prolactin-injected glands were expanded with secretion much more than those in the unstimulated control glands of the same rabbit.

The lack of a significant inhibitory effect of progesterone injected intramuscularly at 10 mg/day on prolactin stimulation was unexpected (Tables 3 and 4), since Assairi *et al.* (1974) showed suppression of lactose synthesis by this treatment. However, measurement of circulating progesterone indicated that only a small increment on the normal concentrations on day 16 of pseudopregnancy resulted from the intramuscular injection (Table 4). On day 11 of pseudopregnancy the plasma progesterone content in the same rabbits before treatment was 21.0 ± 2.9 ng/ml, appreciably higher than at day 16 in rabbits injected with 10 mg of exogenous progesterone/day.

At the higher dosage of progesterone used (80 mg/day), which increased plasma progesterone concentration to 76.5 ± 16.8 ng/ml, a significant inhibition of fatty acid synthesis was observed (Table 4). This is in general agreement with the inhibition *in vitro* seen in explant culture.

The molecular mechanism of progesterone inhibition of prolactin-stimulated lipogenesis is obscure. Progesterone may bind to glucocorticoid receptors, as shown in the mammary gland of the lactating rat (Quirk *et al.*, 1983), and prevent the synergistic stimulatory effect of corticosterone with insulin and prolactin, and may also exert its effect by binding to its specific receptors, as demonstrated in the mammary gland of pregnant rats (Quirk *et al.*, 1982). Recent data have shown an overlap in DNA binding sites for progesterone- and corticosteroid-receptor proteins (Von der Ahe *et al.*, 1985). Sapag-Hagar & Greenbaum (1974) have reported that progesterone at concentrations of 0.6 and 6 µg/ml activates adenylate cyclase activity by 20–80% in mammary-gland preparations of pregnant rats, but not of lactating rats. This is supported by the demonstration of inhibition of fatty synthetase activity by dibutyryl cyclic AMP and the inhibition of prolactin-stimulated fatty acid synthesis by methylxanthine (Cameron & Rillema, 1983) in mammary explants. Acetyl-CoA carboxylase from lactating rabbit mammary gland has been shown to be inhibited by phosphorylation, which can be carried out by a cyclic

AMP-dependent protein kinase (Hardie & Cohen, 1979).

It has been demonstrated that prolactin stimulation of mammary tissue in pseudopregnant rabbits causes an increase in acetyl-CoA carboxylase activity, fatty acid synthetase activity, glucose-6-phosphate dehydrogenase activity, 6-phosphogluconate dehydrogenase activity, glycerolipid synthesis and overall fatty acid synthesis from acetate. Progesterone *in vitro* inhibited acetyl-CoA carboxylase activity at near-physiological concentrations and fatty acid synthetase and glycerolipid synthesis at pharmacological concentration. *In vivo*, intramuscular injection of progesterone was shown to inhibit fatty acid synthesis in the prolactin-stimulated mammary gland.

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