Inhibition by Low Concentrations of Ouabain of Prolactin-Induced Lactogenesis in Rabbit Mammary-Gland Explants

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1. Explants of mammary tissue from pseudopregnant rabbits were cultured at 37°C in air for 24–48 h in Medium 199 buffered with 20 mM-Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid]. The medium contained insulin and corticosterone, or insulin, corticosterone and sheep prolactin in the presence or absence of ouabain, an inhibitor of Na^+/K^+ -dependent adenosine triphosphatase. The responses of explants were assessed histologically, by measuring the tissue concentration of K^+ , and by rates of synthesis of RNA, protein and fatty acids. The effect of ouabain on Na^+ and K^+ concentrations in slices of lactating rabbit mammary-gland tissue incubated for 1 h at 37°C in Krebs bicarbonate buffer was also studied. 2. Prolactin increased the concentration of K^+ in mammary explants, an effect prevented by ouabain. In slices of lactating tissue, there was a linear relationship between the log dose of ouabain (from 0.1 to 10 μM) and increased Na^+ and decreased K^+ concentrations in the tissue. 3. Ouabain at concentrations up to 1 μM did not affect the rate of synthesis of RNA, protein or fatty acids by explants cultured with insulin and corticosterone. By contrast, the stimulatory effect of prolactin on protein synthesis was diminished and the induction of medium-chain fatty acid synthesis by prolactin was almost abolished. RNA synthesis was unaffected. Histological examination showed no tissue damage by 1 μM -ouabain. 4. Explants cultured in the presence of 2 μM -ouabain for 24 h retained their ability to respond to prolactin when the ouabain was removed from the culture medium. Between 24 and 48 h they showed responses to prolactin of a magnitude similar to those of explants never exposed to ouabain. 5. These results show that a fully functional Na^+/K^+ -dependent adenosine triphosphatase system is necessary for prolactin to promote secretory activity in rabbit mammary gland.

Prolactin is the key hormone in inducing milk secretion by lobulo-alveolar mammary-gland tissue of the rabbit. The administration of sheep prolactin initiates milk secretion *in vivo* even after the removal of the ovaries, adrenals and/or pituitary (Cowie *et al.*, 1969; Denamur, 1971), whereas bromocryptine, which suppresses endogenous prolactin secretion, drastically decreases milk yield (Taylor & Peaker, 1975). Prolactin in the absence of other hormones induces secretory activity in rabbit lobulo-alveolar tissue cultured *in vitro* (Forsyth *et al.*, 1972). In the latter experiment, secretion was assessed histologically and by the ability of the explants to synthesize milk fat.

Abbreviations used: Na^+/K^+ -ATPase, Na^+/K^+ -dependent adenosine triphosphatase; Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid.

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The results of these organ-culture experiments and the effectiveness of prolactin in initiating milk secretion when injected intraductually (Lyons, 1942; Fiddler *et al.*, 1971) show that prolactin acts directly on mammary-gland tissue through specific receptors on the plasma membranes of alveolar cells (Birkinshaw & Falconer, 1972; Falconer, 1976; Shiu & Friesen, 1974, 1976; Djiane *et al.*, 1977). Investigations on the possible mechanisms of action of prolactin have demonstrated activation of the Na^+/K^+ -transport mechanism leading to an increased concentration of K^+ ions and a decreased concentration of Na^+ ions in mammary alveolar tissue (Falconer & Rowe, 1975, 1977). There is evidence in all classes of vertebrates that prolactin is involved in water and mineral metabolism, and that Na^+/K^+ -ATPase in the gills, kidney and urinary bladder of teleost fish is responsive to prolactin (see Bern, 1975).

In the present experiments ouabain has been used specifically to inhibit Na^+/K^+ -ATPase (Skou, 1965).

The effect of this inhibitor on basal and prolactin-stimulated synthesis of RNA, protein and fatty acids by explants of pseudopregnant-rabbit mammary gland has been investigated.

Materials and Methods

Animals

Sexually mature virgin Dutch rabbits were provided by the animal house of the National Institute for Research in Dairying, Shinfield, Berks., U.K. Lactating female New Zealand White rabbits were obtained from Hollymoor Rabbits, Maidenhead, Berks., U.K. Mammary lobulo-alveolar tissue for culture was obtained from rabbits on day 11–12 of pseudopregnancy by the techniques described by Forsyth *et al.* (1972). Mammary tissue for slice incubation was obtained from rabbits in their second to third week of lactation.

Materials

Materials were obtained from the following sources: [5,6-³H]uridine (sp. radioactivity 46 Ci/mmol), L-[4,5-³H]leucine (sp. radioactivity 55 Ci/mmol), L-[U-¹⁴C]leucine (sp. radioactivity 348 Ci/mol), 3-O-methyl D-[U-¹⁴C]glucoside (sp. radioactivity 74.2 Ci/mol), [³H]inulin (sp. radioactivity 1.09 Ci/mmol) and sodium [1-¹⁴C]acetate (sp. radioactivity 61 Ci/mol) from The Radiochemical Centre, Amersham, Bucks., U.K.; ouabain octahydrate, 3-O-methyl glucoside, bovine serum albumin (fatty acid-poor) and calf thymus RNA from Sigma (London) Chemical Co., Kingston upon Thames, U.K.; Medium 199, which contained NaHCO₃ (12 mM), penicillin (200 units/ml; 0.83 mg/ml) and streptomycin (100 µg/ml), from Wellcome Reagents, Beckenham, Kent, U.K. Sheep prolactin (N.I.H. PS9; 30 i.u./mg) was a gift from the Endocrinology Study Section of the National Institutes of Health, Bethesda, MD, U.S.A.; crystalline ox insulin (approximate potency 24 units/mg) was a gift from the Wellcome Foundation, Beckenham, Kent, U.K.; corticosterone was from E. Merck, Darmstadt, Germany; Hepes was from Hopkin and Williams, Chadwell Heath, Essex, U.K.; the components of the scintillation fluid were from Sigma or from Fisons, Loughborough, Leics., U.K.

Preparation and culture of alveolar mammary explants

Explants were prepared and cultured as described by Forsyth & Myres (1971) and by Strong *et al.* (1972), except that Hepes was added to Medium 199 to a final concentration of 20 mM, and the explants were cultured in air at 37°C for 24 or 48 h. The nominal concentrations of hormones used in the culture medium were 5 µg of insulin/ml, 1 µg of corticosterone/ml and 1 µg of sheep prolactin/ml. Adsorption on Millipore filters during the sterilization of stock solutions decreases the effective final concentrations

of insulin and of prolactin. Medium 199 contains uracil (2.7 µM), L-leucine (0.46 mM) and sodium acetate (1 mM) as components of a comprehensive mixture of substrates for tissue growth.

Incorporation of radioactive precursors

To measure RNA synthesis after explants had been cultured for various times, 1 µCi of [³H]uridine (sp. radioactivity 46 Ci/mmol) was added to 2 ml of Medium 199 that contained groups of ten explants in culture. Culture was then continued for a further 3 h at 37°C and was stopped by washing the explants in ice-cold saline (0.9% NaCl) for 30 min and transferring them to 1 ml of ethanol. The explants were stored overnight at 0°C, transferred into 0.3 ml of 1 M-KOH, homogenized with a glass rod and then hydrolysed at 40°C for 1.5 h. Protein and DNA were precipitated by adding 0.15 ml of 2 M-HCl and 0.5 ml of 5% (w/v) trichloroacetic acid. The precipitate was removed by centrifugation for 20000 g_{av.}-min. A portion (0.2 ml) of the supernatant was added to 10 ml of scintillation fluid, which consisted of 4.0 g of 2,5-bis-(5-t-butylbenzoxazol-2-yl)thiophen, 80 g of naphthalene and 400 ml of 2-methoxyethanol made up to 1.4 litres in toluene. The radioactivity was measured by liquid-scintillation counting. The RNA content of the supernatant obtained after centrifugation was measured by the orcinol method of Schneider (1957).

To measure protein synthesis, either 2 µCi of [¹⁴C]-leucine (sp. radioactivity 348 Ci/mol) or 10 µCi of [³H]leucine (sp. radioactivity 55 Ci/mmol) was added to 1 ml of Medium 199, which contained groups of ten explants in culture. Culture was then continued for 1 h at 37°C and was stopped by washing the explants for 1 h in ice-cold 10% (w/v) trichloroacetic acid, which contained 5 mM-L-leucine as carrier. The explants were homogenized and the precipitate removed by centrifugation for 20000 g_{av.}-min. The precipitate was washed with 1 ml of 10% (w/v) trichloroacetic acid. Excess trichloroacetic acid was removed by washing the precipitate with diethyl ether, and the precipitate was then hydrolysed in 1 ml of 40% (w/v) KOH at 80°C for 20 min. The radioactivity in 0.1 ml portions of the hydrolysate was determined by liquid-scintillation counting. The protein content of each group of explants was determined by measuring the A₂₈₀ of the remaining hydrolysate.

Fatty acid synthesis was measured by adding 10 µCi of sodium [1-¹⁴C]acetate (sp. radioactivity 61 Ci/mol) to 1 ml of Medium 199 that contained groups of five explants in culture. The explants were then cultured at 37°C for 1 h. Culture was stopped by rinsing the explants with ice-cold Medium 199 for 10 min, followed by a further rinse in 1 ml of ice-cold saline. The explants were drained and transferred to 1 ml of 5 M-KOH in methanol/water (1:1, v/v) and

saponified at 70°C for 2 h. A standard mixture of fatty acids, consisting of 20 μg each of even-numbered acids $\text{C}_{4:0}$ to $\text{C}_{18:0}$ plus $\text{C}_{9:0}$, was then added. After 1 h at 18°C the pH was adjusted to below 3 with 11 M-HCl. The samples were extracted with 2×1 ml of n-pentane/diethyl ether (7:3, v/v) and the extracts were combined. Portions (0.2 ml) of the extract were added to 10 ml of scintillation fluid and the radioactivity was measured. The separation of ^{14}C -labelled fatty acids by radio-g.l.c. was carried out as described by Strong *et al.* (1972).

Measurement of Na^+ and K^+ concentrations

The concentrations of Na^+ and of K^+ in slices of lactating rabbit mammary gland were measured by the method of Falconer & Rowe (1977). Slices (0.7 mm thick; 100 mg wet wt.) were incubated for 1 h at 37°C in 25 ml of Krebs bicarbonate buffer, pH 7.4, which contained 15 mM-glucose (Krebs & Henseleit, 1932). The slices were continuously gassed with O_2/CO_2 (19:1). The effects of varying the concentration of ouabain on the ion concentration were then examined.

The concentration of K^+ was measured in groups of 15 explants cultured for 24 h at 37°C. The explants were rinsed with 0.25 M-sucrose/0.1 mM-EDTA at 18°C and then blotted. They were sealed in weighed vials and hydrolysed with 1 ml of 10% (w/v) acetic acid for 6 h. The concentration of K^+ was measured in quadruplicate groups of 15 explants by flame-emission spectrometry with a Unicam SP.1950 instrument.

Measurement of uptake of 3-O-methyl glucoside into cultured explants

Explants were cultured for 24 h, and 2 μmol of 3-O-methyl D-[^{14}C]glucoside (2 μCi) plus 2 nmol of [^3H]inulin (2 μCi) were then added, with mixing, to the medium (2 ml) in the culture dish. Culture was continued at 37°C for 30 min. The explants were removed, washed briefly with ice-cold 0.9% NaCl solution and blotted to remove surplus liquid. The explants were weighed and homogenized in 0.5 ml of 5% (w/v) trichloroacetic acid. The precipitate was removed by centrifugation for 4 min at 9500g_{av}. and 0.1 ml portions of the supernatant were used to measure ^{14}C and ^3H radioactivity by liquid-scintillation counting.

The extracellular-fluid space in the explants was assumed to be equivalent to the inulin space, and is expressed as a percentage of the wet weight of the explants, i.e. as

$$100 \times (\text{d.p.m. of } [^3\text{H}]\text{inulin in the explants/mg wet wt. of explants}) / (\text{d.p.m. of } [^3\text{H}]\text{inulin in the medium}/\mu\text{l of medium}).$$

The intracellular concentration of methyl glucoside was calculated as

$$(\text{d.p.m. of methyl } [^{14}\text{C}]\text{glucoside in the explants/mg wet wt. of explants} - [\text{inulin space as a percentage of wet weight of explants}/100] \times \text{d.p.m. of methyl } [^{14}\text{C}]\text{ glucoside}/\mu\text{l of medium}) \times 100 / 100 - \text{inulin space as a percentage of the wet weight of the explants}.$$

Histological examination

All culture experiments included additional explants for histological examination of viability and secretion, as described by Forsyth & Myres (1971).

Statistical analysis

Differences between treatments were tested for significance by using Student's *t* test.

Results

Effect of ouabain on the concentrations of Na^+ and K^+ in mammary tissue

Slices of lactating rabbit mammary gland were used to obtain sufficient tissue for accurate measurement of tissue Na^+ and K^+ . The slices were incubated for 1 h at 37°C in 0.1–100 μM -ouabain. Three independent experiments demonstrated a linear relationship between the log dose of ouabain (from 0.1 to 10 μM) and the concentrations of Na^+ and K^+ in the slices (Fig. 1).

The effect of 2 μM -ouabain on the concentration of K^+ in mammary-gland explants cultured for 24 h in the presence and absence of prolactin was investigated. The results show that ouabain (2 μM) abolished

Fig. 1. Effects of ouabain on the concentrations of Na^+ and K^+ in lactating rabbit mammary gland

Slices (100 mg wet wt.; 0.7 mm thick) of lactating rabbit mammary gland were incubated for 1 h at 37°C in Krebs-Ringer bicarbonate buffer, pH 7.4, which contained 15 mM-glucose. The gas phase was O_2/CO_2 (19:1). Ouabain was added as shown. The results are means $\pm$ S.E.M. for three independent experiments. Symbols: $\bullet$, Na^+ ; $\blacksquare$, K^+ .

Table 1. *Effects of prolactin and ouabain on the concentration of K⁺ in mammary-gland explants*

Explants from pseudopregnant rabbits were cultured at 37°C in air for 24 h in Medium 199 that contained 20 mM-Hepes. Insulin (5 µg/ml), corticosterone (1 µg/ml), prolactin (1 µg/ml) and ouabain (2 µM) were added as shown. Groups of 15 explants were used, and the results are means ± S.E.M. for four groups of explants. *Significantly different from each other, and from cultures in the presence of ouabain ($P < 0.01$).

Culture conditions	Concn. of K ⁺ (µmol/g wet wt.)
Insulin, corticosterone	38.6 ± 1.6*
Insulin, corticosterone, ouabain	16.7 ± 1.2
Insulin, corticosterone, prolactin	73.6 ± 12.1*
Insulin, corticosterone, prolactin, ouabain	17.7 ± 4.5

the prolactin-induced rise in the concentration of K⁺ in the explants (Table 1).

Culture conditions

In preliminary experiments on RNA biosynthesis, culture of explants in Medium 199 in an atmosphere of O₂/CO₂ (19:1) was compared with culture in Medium 199 buffered with 20 mM-Hepes in air. RNA synthesis was measured over a 3 h period after 1, 24 or 48 h of culture with insulin and corticosterone, or with insulin, corticosterone and prolactin. In general there was a more marked response to prolactin when explants were cultured in air with Medium 199 containing Hepes. These conditions were therefore used throughout this study. Since there was little response to prolactin after 1 h in culture, all subsequent experiments used 24 or 48 h of culture.

Time course of incorporation of radioactive substrates

Triplicate groups of explants were cultured for 24 h with the following combinations of hormones and ouabain: insulin and corticosterone; insulin, corticosterone and 2 µM-ouabain; insulin, corticosterone, prolactin and 2 µM-ouabain. [³H]Uridine, [¹⁴C]leucine or sodium [¹⁴C]acetate was then added to the culture medium. Explants were removed after 1, 2 or 3 h at 37°C and were processed as described in the Materials and Methods section. The radioactivity in RNA, protein or fatty acids was then measured. With each of the culture conditions used there was a uniform rate of incorporation of radioactive substrate into RNA, protein or fatty acids during the 3 h of culture.

The incorporation of [³H]uridine into the ethanol-soluble extract of cultured explants increased uniformly for 3 h with all the culture conditions used. This was not significantly affected by ouabain

in the culture medium. The incorporation of sodium [¹⁴C]acetate into material that remained in the aqueous phase after the extraction of fatty acids increased uniformly for 2 h, and was unaffected by ouabain in the culture medium. The aqueous phase had approx. 10 times as much radioactivity as the fatty acids in the organic phase. There was no increase in the incorporation of [¹⁴C]leucine into the trichloroacetic acid-soluble fraction between 1, 2 and 3 h; the radioactivity of this fraction was unaffected by ouabain in the culture medium.

In all the experiments described subsequently, explants were cultured with [¹⁴C]leucine or [³H]leucine or with sodium [¹⁴C]acetate for 1 h, and with [³H]uridine for 3 h.

Effect of ouabain concentration on RNA, protein and fatty acid synthesis

In three independent experiments, duplicate or triplicate groups of mammary-gland explants were cultured with insulin and corticosterone, with and without prolactin for 24 h in the presence of ouabain at concentrations up to 0.1 mM. [³H]Uridine, [¹⁴C]leucine or sodium [¹⁴C]acetate was then added to the culture medium and their incorporation was measured as described in the Materials and Methods section. The biosynthetic response to prolactin and ouabain in the three experiments showed the same pattern, though the mean values for protein and RNA synthesis differed by up to 60%. To express the results graphically, all the data for protein and RNA synthesis from the first two experiments have been recalculated so that the mean response (d.p.m.) to insulin plus corticosterone was the same as that measured in the third experiment. In the absence of ouabain, prolactin stimulated the synthesis of all three products (Fig. 2) and secretion was observed by histological examination of the explants.

Histological examination showed that there was no apparent damage when explants were cultured in the presence of 0.1 µM- or 1 µM-ouabain compared with explants cultured without ouabain. However, 1 µM-ouabain diminished the stimulatory effect of prolactin on protein synthesis and almost abolished the effect of prolactin on fatty acid synthesis (Fig. 2) and on secretion into the lumen of the alveoli as observed histologically. By contrast, prolactin-induced increase in RNA synthesis was observed even in the presence of 1 µM-ouabain. This indicates that RNA synthesis is less sensitive to ouabain inhibition of Na⁺ and K⁺ transport.

At 10 µM or higher, ouabain severely decreased the synthesis of all three products in both the presence and the absence of prolactin, and cell damage was observed histologically. Severe cell damage occurred after culture in the presence of 100 µM-ouabain. There was disintegration of the

Fig. 2. Effects of ouabain on synthesis of RNA, protein and fatty acid by mammary-gland explants

Explants from pseudopregnant rabbits were cultured at 37°C in air for 24 h in Medium 199 that contained 20 mM-Hepes and insulin ($5 \mu\text{g}/\text{ml}$) plus corticosterone ($1 \mu\text{g}/\text{ml}$) (Δ), or insulin ($5 \mu\text{g}/\text{ml}$), corticosterone ($1 \mu\text{g}/\text{ml}$) and prolactin ($1 \mu\text{g}/\text{ml}$) ($\blacksquare$). Ouabain was added as shown. Synthesis of (a) RNA, (b) protein and (c) fatty acids was then measured as described in the Materials and Methods section. The results are means $\pm$ S.E.M. of five determinations from three independent experiments calculated as described in the Results section.

alveolar cells, although the cells lining the mammary ducts and the connective-tissue cells were apparently not affected.

Recovery of response to prolactin by explants cultured for 24 h with ouabain

To demonstrate that a low concentration of ouabain abolished the biosynthetic responses of mammary-gland explants to culture with prolactin, without destroying the viability or altering the normal biosynthetic mechanisms of the cells, transfer experiments were carried out. From 0 to 24 h, explants were exposed to medium containing insulin and corticosterone, with or without prolactin and/or ouabain ($2 \mu\text{M}$). After 24 h in culture, the synthesis of RNA, protein and fatty acids was determined. In addition, groups of the ouabain-treated explants were washed by rinsing with Medium 199 and cultured for 1 h at 37°C with Medium 199 alone to remove residual ouabain.

These explants were then returned to culture with or without $2 \mu\text{M}$ -ouabain for a further 24 h in a medium that contained the same hormone combination as in the first period of culture. In addition, fatty acid synthesis was also investigated in explants cultured with insulin plus corticosterone, which were washed and then recultured with insulin, corticosterone and prolactin for a further 24 h. Synthesis of RNA, protein and fatty acids was measured (Table 2).

RNA synthesis showed a significant ($P < 0.05$) increase in the presence of prolactin, whether $2 \mu\text{M}$ -ouabain was present or not. This effect was seen at both 24 and 48 h of culture. The presence of ouabain had no significant effect on RNA synthesis.

Protein synthesis was increased by culturing in the presence of prolactin (Table 2, comparison of insulin and corticosterone at 24 and 48 h with insulin, corticosterone and prolactin at 24 and 48 h; $P \approx 0.05$ by t test), and this increase was abolished by $2 \mu\text{M}$ -ouabain. However, when explants that had been cultured with the three hormones plus $2 \mu\text{M}$ -ouabain for 24 h were recultured for a further 24 h with these hormones but without ouabain, rates of protein synthesis were observed comparable with those attained initially by explants cultured for 24 h with the three hormones in the absence of ouabain.

Histological examination showed no damage to the alveolar cells by $2 \mu\text{M}$ -ouabain. The only change observed was the lack of secretion by alveolar cells that had been cultured with $2 \mu\text{M}$ -ouabain for 48 h. By contrast, secretion was observed in explants that had been cultured for the first or for the second 24 h with prolactin but without ouabain.

Fatty acid synthesis showed a larger response to prolactin than did protein or RNA synthesis (Table 2 and Fig. 2). There were significant increases ($0.05 > P > 0.001$) in fatty acid synthesis when explants were cultured with insulin, corticosterone and prolactin

Table 2. *Effects of ouabain on synthesis of RNA, protein and fatty acid by mammary-gland explants*

Mammary-gland explants from pseudopregnant rabbits were cultured for 24 or 48 h with combinations of insulin (5 µg/ml), corticosterone (1 µg/ml), prolactin (1 µg/ml) and ouabain (2 µM) as shown. Synthesis of RNA, protein and fatty acids was then measured as described in the Materials and Methods section. The results are means ± S.E.M. from explants from four rabbits, each carried out in duplicate. * $P \leq 0.05$. ** $P \leq 0.01$ by *t* test comparing results in the presence and absence of ouabain obtained from analogous culture conditions with and without prolactin.

Culture conditions during first 24 h	$10^{-3} \times$ Incorporation of [^3H]uridine into RNA (d.p.m.)	Culture conditions during second 24 h	$10^{-3} \times$ Incorporation of [^3H]uridine into RNA (d.p.m.)
Insulin, corticosterone	44.8 ± 9.8	—	—
Insulin, corticosterone, ouabain	42.3 ± 3.8	Insulin, corticosterone	42.2 ± 7.0
Insulin, corticosterone, ouabain	42.3 ± 3.8	Insulin, corticosterone, ouabain	52.1 ± 3.4
Insulin, corticosterone, prolactin	66.0 ± 4.5*	—	—
Insulin, corticosterone, prolactin, ouabain	58.6 ± 6.7*	Insulin, corticosterone, prolactin	60.3 ± 3.0*
Insulin, corticosterone, prolactin, ouabain	58.6 ± 6.7*	Insulin, corticosterone, prolactin, ouabain	63.2 ± 4.4*
Culture conditions during first 24 h	$10^{-3} \times$ Incorporation of [^3H]leucine into protein (d.p.m.)	Culture conditions during second 24 h	$10^{-3} \times$ Incorporation of [^3H]leucine into protein (d.p.m.)
Insulin, corticosterone	42.3 ± 5.2	—	—
Insulin, corticosterone, ouabain	46.2 ± 5.3	Insulin, corticosterone	38.4 ± 4.7
Insulin, corticosterone, ouabain	46.2 ± 5.3	Insulin, corticosterone, ouabain	34.1 ± 4.1
Insulin, corticosterone, prolactin	54.2 ± 11.9	—	—
Insulin, corticosterone, prolactin, ouabain	39.4 ± 7.4	Insulin, corticosterone, prolactin	58.8 ± 15.0
Insulin, corticosterone, prolactin, ouabain	39.4 ± 7.4	Insulin, corticosterone, prolactin, ouabain	39.6 ± 4.8
Culture conditions during first 24 h	$10^{-3} \times$ Incorporation of [^{14}C]acetate into fatty acids (d.p.m.)	Culture conditions during second 24 h	$10^{-3} \times$ Incorporation of [^{14}C]acetate into fatty acids (d.p.m.)
Insulin, corticosterone	2.6 ± 0.14	Insulin, corticosterone, prolactin	14.6 ± 5.7*
Insulin, corticosterone, ouabain	2.0 ± 0.38	Insulin, corticosterone	2.5 ± 0.32
Insulin, corticosterone, ouabain	2.0 ± 0.38	Insulin, corticosterone, ouabain	1.7 ± 0.42
Insulin, corticosterone, prolactin	21.0 ± 6.20**	—	—
Insulin, corticosterone, prolactin, ouabain	2.9 ± 1.0	Insulin, corticosterone, prolactin	34.9 ± 14.0*
Insulin, corticosterone, prolactin, ouabain	2.9 ± 1.0	Insulin, corticosterone, prolactin, ouabain	8.8 ± 4.8

for the first 24 h, or for the second 24 h compared with the synthesis observed with the other culture conditions. The increase during the second 24 h in culture was not prevented by prior culture with 2 µM-ouabain from 0–24 h. Separation of the fatty acids by radio-g.l.c. showed that medium-chain fatty acids accounted for 71 mol% of the radioactivity in the fatty acids synthesized during the second 24 h in culture with insulin, corticosterone and prolactin. By contrast, explants cultured for 24 h with insulin and corticosterone, or with insulin, corticosterone, prolactin and 2 µM-ouabain, only synthesized 15 and 19 mol% respectively of these medium-chain acids.

Uptake of 3-O-methyl D-glucoside by mammary-gland explants

A non-metabolizable analogue was used to measure any effects of ouabain on the uptake of glucose by the explants. Triplicate groups of ten explants were cultured for 24 h with insulin and corticosterone in the presence and absence of 2 µM-ouabain, and with insulin, corticosterone and prolactin in the presence and absence of 2 µM-ouabain. There was no significant

change caused by prolactin or ouabain in the wet weight of the ten explants after culture (2.21 ± 0.07 mg; mean ± S.E.M. of 24 groups of explants).

The extracellular space (measured as [^3H]inulin space) was expressed as a percentage of the wet weight of the explants; the percentage of wet weight that excluded inulin was assumed to be intracellular space. The value for intracellular space so obtained ($63.7 \pm 1.0\%$; mean ± S.E.M. of 24 groups of explants) was not significantly altered by culture in the presence of prolactin or ouabain.

After explants had been cultured for 24 h, 3-O-methyl D-[U- ^{14}C]glucoside was added to the medium, and its uptake into the explants was measured 30 min later. The radioactivity of explants cultured in the presence of ouabain ($1.27 \times 10^3 \pm 0.09 \times 10^3$ d.p.m.; mean ± S.E.M. of six groups of explants) was not significantly different from that in explants cultured in the absence of ouabain ($1.12 \times 10^3 \pm 0.05 \times 10^3$ d.p.m.; mean ± S.E.M. of six groups of explants). Explants cultured with prolactin ($1.11 \times 10^3 \pm 0.03 \times 10^3$ d.p.m.; mean ± S.E.M. of six groups of explants) were not significantly different from explants cultured without

($1.28 \times 10^3 \pm 0.09 \times 10^3$ d.p.m.; mean $\pm$ s.e.m. of six groups of explants).

Discussion

Prolactin increases the concentration of K^+ and decreases the concentration of Na^+ in mammary-gland tissue from pseudopregnant and from lactating rabbits (Falconer & Rowe, 1975, 1977). The balance of these intracellular cations is regulated by the plasma-membrane Na^+/K^+ -ATPase, which transports Na^+ out of, and K^+ into, mammalian cells. This enzyme is selectively inhibited by the steroid glycoside ouabain, which blocks the transport of both ions (Skou, 1965). When slices of mammary-gland tissue from pseudopregnant or from lactating rabbits are treated with ouabain, the changes in Na^+ and K^+ concentrations observed after adding prolactin are reversed (Falconer & Rowe, 1975, 1977). The technique of organ culture of mammary-gland explants from pseudopregnant rabbits has enabled this inhibition of ion transport by ouabain to be studied in conjunction with the effects of ouabain on biochemical parameters that indicate cellular response to prolactin.

Different cell lines in culture exhibit widely different sensitivities to ouabain. Shank & Smith (1976) used 100–600 μM -ouabain to inhibit the growth of mouse lymphoblasts and to modify the intracellular concentrations of Na^+ and K^+ . With human lymphocytes, however, 0.22 μM -ouabain inhibits the stimulation by phytohaemagglutinin of protein and RNA synthesis (Quastel & Kaplan, 1970).

The sensitivity of mammary alveolar cells to ouabain was investigated by measuring the concentration of Na^+ and K^+ ions in slices of lactating rabbit mammary-gland tissue that had been incubated with a range of concentrations of ouabain. Fig. 1 demonstrates the small effect observed when slices were incubated with 0.1 μM -ouabain and the near-maximal effect when 10 μM -ouabain was used. A graded effect on the tissue concentration of Na^+ and K^+ was obtained between these two concentrations. In the light of these results, mammary explants were cultured for 24 h in the presence of 2 μM -ouabain. This decreased the concentration of tissue K^+ to values below those seen when explants were cultured with insulin and corticosterone (Table 1).

Prolactin induced milk synthesis when mammary explants from pseudopregnant rabbits were cultured with insulin, corticosterone and prolactin (Strong *et al.*, 1972). In the present experiments this response to prolactin was assessed by measuring rates of synthesis of RNA, protein and fatty acids and by histological observation of secretion within alveoli. Until now it has been difficult to demonstrate consistent effects of prolactin on RNA synthesis in

mammary explants from pseudopregnant rabbits (I. A. Forsyth & E. Jones, unpublished work), despite extensive data for increased RNA synthesis in explants from mouse mammary gland (see Topper & Oka, 1974). The use of 20 mM-Hepes to buffer the culture medium and a gas phase of air instead of O_2/CO_2 (19:1) has enabled modest, but statistically significant, effects of prolactin on RNA synthesis to be observed.

Increasing concentrations of ouabain inhibit the prolactin-induced synthesis of protein and fatty acids by explants (Fig. 2) in a manner similar to its effects in decreasing the concentration of K^+ in slices of lactating mammary gland (Fig. 1) and in mammary-gland explants from pseudopregnant rabbits (Table 1). Ouabain at 1 μM and at 2 μM significantly depleted the tissue K^+ concentration, and also inhibited the increased synthesis of protein and fatty acids induced by prolactin. There was no evidence that ouabain at these concentrations produced histologically detectable damage to the tissue, nor did 1 μM -ouabain depress the synthesis of all three products by alveoli cultured with insulin and corticosterone. Though 10 μM - and 100 μM -ouabain markedly inhibited the biosynthesis of these products, it also had damaging effects on the tissue.

Measurements of the incorporation of precursors into specific products may be affected by changes in the intracellular pools of these precursors. In the experiments reported here there was, however, no evidence of significant changes in precursor concentrations in the explants caused by 2 μM -ouabain. Measurement of the radioactivity from [^{14}C]acetate in the explants that was not incorporated into fatty acids showed a 10-fold greater radioactivity than had been incorporated into the fatty acids in explants cultured in the presence of ouabain, indicating an abundance of [^{14}C]acetate entering the tissue. Radioactivity from [3H]leucine in the trichloroacetic acid-soluble fraction showed a constant value in explants sampled at 1, 2 or 3 h, with no effects due to ouabain. This is supported by the finding that when mammary-gland explants are cultured with insulin, corticosterone and prolactin, the protein synthesized is derived from an amino acid pool that rapidly equilibrated with amino acids in the culture medium (Speake *et al.*, 1976).

Thus the marked decrease by 1 μM - or 2 μM -ouabain of the prolactin-stimulated synthesis of protein and fatty acids (Fig. 2, Table 2) cannot be attributed to alterations in the size of the pools of their precursors, or to their intracellular concentration.

The possibility of an inhibition of glucose entry to alveolar cells by ouabain was also investigated. When explants were cultured for 24 h with 2 μM -ouabain, the inward transport of 3-*O*-methyl [^{14}C]-glucoside measured over 30 min was non-significantly increased compared with explants cultured without

ouabain. Other studies using ouabain ($1\text{ }\mu\text{M}$ – 1 mM) to inhibit Na^+/K^+ -ATPase in muscle and fat-cells have shown increases in transport of glucose or of 3-*O*-methyl glucoside that tend to decrease as the insulin concentration is raised in the incubation medium (Bihler, 1968; Clausen, 1969; Letarte *et al.*, 1969).

Ouabain decreases the gradient between intra- and extra-cellular pools of Na^+ , and this gradient is implicated in the entry of amino acids into cells. Hence high concentrations of ouabain can be expected to exert inhibitory effects both on protein synthesis and on cell growth (Shank & Smith, 1976). With $10\text{ }\mu\text{M}$ - or $100\text{ }\mu\text{M}$ -ouabain, there were marked decreases in the synthesis of the three products. This may contribute to the histological damage observed.

The prolactin-induced synthesis of RNA showed relatively lower sensitivity to ouabain than did synthesis of protein or fatty acids. This may reflect the synthesis of rRNA, which is markedly increased in the presence of prolactin (see Denamur & Gaye, 1967). The possibility of decreased synthesis of mRNA in explants cultured with $1\text{ }\mu\text{M}$ -ouabain in the presence of prolactin can be inferred from the decreases in synthesis of protein and fatty acids.

The action of ouabain is reversible and it can be readily removed by washing the cells after culture. The cells then rapidly recover their former Na^+/K^+ -ATPase activity (Shank & Smith, 1976). The reversibility of the effects of ouabain has been confirmed in the present experiments, which show the lack of permanent damage to mammary alveolar cells by culture in $2\text{ }\mu\text{M}$ -ouabain. Even after they had been cultured for 24 h in the presence of $2\text{ }\mu\text{M}$ -ouabain (Table 2), explants showed an undiminished response in fatty acid synthesis when they were recultured with prolactin. The predominant synthesis of medium-chain fatty acids shows that the explants had responded to prolactin in the characteristic manner of rabbit mammary-gland tissue undergoing biochemical differentiation during lactogenesis (Strong *et al.*, 1972). These medium-chain fatty acids were not appreciably synthesized in the absence of prolactin, nor in the presence of prolactin plus ouabain. This shows that ouabain did not merely decrease the rate of fatty acid synthesis, but prevented the differentiation of the tissue.

Ouabain abolishes changes in the concentrations of Na^+ and K^+ ions induced by prolactin and also inhibits increased biosynthesis of protein and fatty acids induced by prolactin. When ouabain is removed, the tissue recovers its ability to undergo prolactin-stimulated induction of milk biosynthesis, as seen histologically and by measuring fatty acid biosynthesis. These results clearly indicate the necessity for an active Na^+/K^+ -ATPase system for prolactin to produce its effects. They are consistent with the

possibility that an initial action of prolactin is to modulate Na^+/K^+ -ATPase activity. The resulting change in the intracellular concentrations of Na^+ and K^+ or in the ratio of their concentrations might then act as a 'second messenger' for prolactin.

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