Role of receptors for epidermal growth factor and insulin-like growth factors I and II in the differentiation of rat mammary glands from lactogenesis I to lactogenesis II

L. E. Bussmann, I. M. Bussmann and E. H. Charreau

Instituto de Biología y Medicina Experimental-CONICET, Vuelta de Obligado 2490, Buenos Aires (1428), Argentina

In addition to ovarian steroids and lactogenic hormones from the placenta and pituitary, growth factors control the growth and differentiation of mammary glands. Lactogenesis II at the end of pregnancy is under the control of progesterone. Ovariectomy results in a significant decrease in the number of receptors for epidermal growth factor (EGF) and insulin-like growth factor I (IGF-I) and an increase in IGF-II binding sites in mammary gland acini of rats, without affecting the affinity for their respective ligand. Although concentrations of EGF, IGF-I and IGF-II receptors are regulated by oestradiol and progesterone, replacement treatment with ovarian steroids after ovariectomy showed that receptor concentrations do not mediate the restraint on lactogenesis. Progesterone treatment, which inhibits the onset of lactogenesis II, did not restore EGF receptor concentrations to control values, and the presence of oestradiol was required to reverse the effect of ovariectomy. Oestradiol, which potentiates the effect of ovariectomy on milk synthesis, increases IGF-I receptor concentrations. IGF-II receptor concentrations, after the different steroid treatments, were consistent with the steroid effect on milk synthesis. The changes observed in the concentrations of these growth factor receptors at the onset of mammary gland secretion are not considered to affect the progesterone block to lactogenesis II, but rather are a consequence of the shift of the hormonal and, hence, physiological status of the gland.

Introduction

Lactogenesis or the onset of milk synthesis is a two stage process in rats that occurs during late pregnancy (Fleet *et al.*, 1975). Lactogenesis I is characterized by the appearance of biosynthetic activity of the mammary gland in the last third of pregnancy and milk synthesis is under the control of progesterone. Lactogenesis II takes place a few hours before, or at, parturition, when progesterone serum values fall and the steroid block to milk synthesis is removed. Both stages of lactogenesis are controlled primarily in the rat by ovarian and adrenal steroids and lactogenic hormones from the pituitary and placenta (Topper and Freeman, 1980). However, the unequivocal role of a particular hormone in mammary gland differentiation is difficult to ascertain, due to the interactions between the hormones. Apart from its own particular action, each hormone modulates the secretion or the effects of the others.

A number of studies have indicated that some growth factors are involved in the differentiation of the mammary gland. Among them, insulin-like growth factors (IGFs) and epidermal growth factor (EGF) play a major role in this process (Dembinski and Shiu, 1987).

Serum concentrations of EGF increase during pregnancy in mice (Dembinski and Shiu, 1987), and studies *in vitro* have shown the mitogenic effects of EGF on the mammary epithelial cells of humans (Papadimitriou *et al.*, 1977) and mice (Turkington, 1969). The effect of EGF on milk protein synthesis *in vitro* is controversial. Initially, an inhibitory effect in a dose-dependent manner was reported (Taketani and Oka, 1983). However, when EGF was given in the presence of a physiological insulin concentration, there was no effect on casein synthesis (Sankaran and Topper, 1987).

The biological actions of EGF are mediated by a specific receptor which has protein tyrosine kinase activity and is located on the cell membrane (Carpenter and Cohen, 1979). EGF receptors are expressed in the mammary glands of mice and their concentration changes according to the different reproductive stages, reaching a maximum in mid-pregnancy and declining thereafter throughout parturition and lactation (Edery *et al.*, 1985).

The mitogenic effect of IGF-I was observed in a human breast cancer cell line (Myal *et al.*, 1984) and in mammary cells from mice (Imagawa *et al.*, 1986). IGF-I is believed to mediate the galactopoietic action of growth hormone in cows (Davis *et al.*, 1987), although in a recent report in rats with suppressed lactation, treatment with IGF-I analogues failed to mimic the action of growth hormone (Flint *et al.*, 1994).

Received 12 January 1996.

The effect of IGFs is initiated by the specific binding to their cell-surface receptors. The type I receptor has a high affinity for IGF-I and a lower affinity for IGF-II and insulin (Kasuga *et al.*, 1981). The type II receptor which has a higher affinity for IGF-II than for IGF-I and does not bind insulin was recently identified as the mannose 6-phosphate receptor (MacDonald *et al.*, 1988).

The trigger for lactogenesis at the end of pregnancy is the fall in circulating concentrations of progesterone, which allows the differentiated mammary gland to begin milk synthesis (Kuhn, 1969). The inhibitory effect of progesterone does not correlate with receptor concentrations (Shyamala and McBlain, 1979) which could indicate that the effect of progesterone on the mammary acini is indirect and acts through the modulation of the production of growth factors or their receptors. In the present study the effect of ovariectomy and steroid replacement treatment on the concentration of membrane receptors for EGF and IGFs was investigated.

Materials and Methods

All chemicals were reagent grade and obtained from Sigma Chemical Co. (St Louis, MO) unless otherwise indicated. mEGF receptor-grade and rabbit anti-mEGF antibody were obtained from Collaborative Research Inc. (Bedford, MA). Transforming growth factor- α (TGF α) AffiPure sheep antibody was from Triton Biosciences Inc. (Alameda, CA). rhIGF-I and rhIGF-II were kindly donated by J. Merryweather from Chiron Corporation (Emerville, CA). Na¹²⁵I, carrier-free was purchased from New England Nuclear Corp. (Boston, MA). Reagents for polyacrylamide gel electrophoresis and anion exchange resin AG 1X2 were purchased from Bio-Rad (Richmond, CA). Heparin-Ultrogel A4R was from IBF Biotechnics (Villeneuve-la-Garenne). Disuccinimidyl suberate (DSS) was obtained from Pierce Chemical Co. (Rockford, IL) and centricon 30 was purchased from Amicon (Beverly, MA).

Animals and general methodology

White, nulliparous rats, about 3-months-old (180–200 g), were caged with a male rat during the night after pro-oestrus. The next morning was taken as day 0 of pregnancy if spermatozoa were found in the vaginal smear. In our colony, rats usually deliver on day 22. The rats were given food and water *ad libitum* and kept in a constant-temperature room ($22 \pm 2^\circ\text{C}$) with a controlled light cycle (lights on from 07:00 to 19:00 h).

Lactogenesis II was induced by bilateral ovariectomy performed between 09:00 and 11:00 h on day 18 of pregnancy through dorsolateral incisions under light ether anaesthesia; in control animals (sham-operated) only the incision was performed. Steroid replacement treatment was performed by s.c. injection. Progesterone (10 mg in 0.1 ml oil) was given after surgery and again 12 h later. Oestradiol (oestradiol benzoate 1 μg in 0.1 ml oil) was injected 12 h after ovariectomy. The rats were killed by decapitation 24 h later and the inguinal mammary glands removed immediately and placed in ice-cold PBS. The presence of milk was assessed by the oxytocin test, as described by Bussmann and Deis (1979). Lactating mammary glands were obtained from rats at day 7 of lactation.

Preparation of mammary acini and membrane isolation

The use of membranes isolated from mammary acini preparations decrease the contribution of extracellular components in the receptor determination assay due to the low proportion of acini present in the gland at day 19 of pregnancy. For this reason, acini were isolated according to Threadgold *et al.* (1982). Briefly, mammary tissue was minced and enzymatically dissociated at 37°C by treatment with 0.1% (w/v) collagenase in medium DMEM-F12 with 15 mmol Hepes l^{-1} , pH 7.4, in the presence of BSA radioimmunoassay grade (2% w/v) and Ficoll-400 (5% w/v) in a plastic tube. The tube was shaken at 200 strokes min^{-1} for 60 min. The digested tissue was filtered through Nitex 150 μm (Tobler, Ernst and Traber, Inc., New York) and the acini sedimented at 300 g for 90 s over a cushion of Ficoll-400 (35% w/v). The acini were resuspended in the same medium plus 2% (w/v) Ficoll and 1% (w/v) BSA, and sedimented as before. The entire washing procedure was repeated and finally the acini collected from the Ficoll cushion and resuspended in nine volumes (w/v) of hypotonic buffer (10 mmol Tris l^{-1} , pH 7.4 with 1 mmol EDTA l^{-1}). Similar concentrations of DNA were present in the suspensions from control and ovariectomized rats (control 2.26–2.54 mg versus ovariectomized 2.1–2.58 mg DNA per 100 mg of acini). The acini were left for 10 min on ice before homogenization by Ultra Turrax (IKA-Labortechnik, Germany) with three bursts of 15 s. The homogenate was diluted with half its volume of sucrose buffer (0.75 mol sucrose l^{-1} , 1 mmol EDTA l^{-1} in 10 mmol Tris l^{-1} , pH 7.4) and centrifuged at 10 000 g for 10 min. The supernatant was made up with 10 mmol MgCl_2 l^{-1} and centrifuged at 40 000 g for 20 min, after which the pellet was resuspended in buffer (10 mmol Tris l^{-1} , 2 mmol MgCl_2 l^{-1} , pH 7.4 in a ratio (w:v) of 1 original cell pellet after collagenization to 14 buffer) and used as the membrane preparation. Protein concentrations, between or within groups, were not different.

Homogenization and all the incubations that followed were carried out in the presence of 0.5 mmol PMSF l^{-1} (phenyl-methylsulphonyl fluoride), 0.025 mmol ZPCK l^{-1} (N-CBZ-L-phenylalanine chloromethyl ketone), 0.025 mmol TLCK l^{-1} (N'-p-tosyl-lysine chloromethyl ketone), 0.025 mmol TPCK l^{-1} (L-1-tosylamide-2-phenyl-ethylchloromethyl ketone) as proteases inhibitors.

Desaturation of the membrane receptors was performed by resuspending the pellet in 50 mmol glycine l^{-1} , 100 mmol NaCl l^{-1} , pH 3.0 and incubating for 10 min at room temperature. Then, 1/20 volume 1 mol Tris l^{-1} , pH 8.0, was added and the suspension centrifuged at 40 000 g for 20 min. The pellet was suspended in 50 mmol Tris l^{-1} , 2 mmol MgCl_2 l^{-1} , pH 7.4 and the supernatant set aside for the determination of endogenous EGF by radioimmunoassay (Molinolo *et al.*, 1987) or radioreceptor assay (Elizalde *et al.*, 1990a). The sensitivities of the assays were 10 pg and 150 pg, respectively.

Iodination

Labelling of IGF-I, IGF-II and EGF with ¹²⁵I was performed by the chloramine-T method, followed by purification by Sephadex G-50 chromatography as described by Elizalde *et al.* (1990b). Aliquots were kept frozen at -70°C and before use

they were taken up in 0.2 mol acetic acid l^{-1} and treated with AG-1-X2 anion exchange resin. The resultant tracers were characterized as having a specific activity of 100–120 $\mu Ci \mu g^{-1}$ and a maximum binding capacity of 60–70% for EGF and 30–40% for IGF-I and IGF-II, as determined by binding to excess placental membrane receptors.

Receptor assay

EGF, IGF-I and -II receptors were assessed as described by Molinolo *et al.* (1987) and Elizalde *et al.* (1990b), with minor modifications. Duplicate 100 μl samples of membrane preparation (25–50 μg protein) were incubated with different concentrations of the tracer ranging from 1×10^{-11} to 1×10^{-9} mol l^{-1} , in a final volume of 200 μl TBSA buffer (50 mmol Tris l^{-1} , 2 mmol $MgCl_2 \cdot l^{-1}$, 1% (w/v) BSA radioimmunoassay grade) in plastic tubes overnight at 4°C. Incubation was stopped by placing the samples on ice and adding 2 ml TBSC buffer (50 mmol Tris l^{-1} , 10 mmol $MgCl_2 \cdot l^{-1}$, 0.1% (w/v) BSA, 0.1% (w/v) celite, pH 7.0).

Membrane-bound growth factor was separated by centrifugation at 6000 g for 30 min, the supernatant discarded and the pellet counted in a LKB gamma spectrometer (LKB Instruments, Wallack, Sweden). Non-specific binding was assessed in each experiment, and for each concentration of the respective tracer, by incubating samples in duplicate with an excess (3×10^{-7} mol l^{-1}) of unlabelled growth factor. The amount of growth factor bound in this condition was usually less than 2% of the total radioactivity present. The non-specific binding was subtracted from total binding to obtain specific binding. Scatchard plots (Scatchard, 1949) were used to determine equilibrium constants (K_d) and the number of receptors, using the program LIGAND (Munson and Rodbard, 1980). For comparison between groups treated with ovarian steroids and their respective controls, receptor concentrations were determined by incubating the membrane preparations with a single saturating concentration of the tracer (1×10^{-9} mol l^{-1}) in the presence or absence of an excess (3×10^{-6} mol l^{-1}) of unlabelled growth factor. Under these conditions non-specific binding was high (30–40% of the total binding) but values for receptor concentrations were in agreement with those obtained by Scatchard analysis. Binding is expressed per mg of membrane protein, instead of DNA content or number of cells, to avoid variability due to differences in the number of cells disrupted in the homogenization process.

The concentration of growth factors in the mammary glands was measured in the alcohol–acid extract obtained as described by Roberts *et al.* (1980) by radioreceptor assay using membranes from human placenta stripped of endogenous growth factors by treatment with 50 mmol glycine l^{-1} , 100 mmol NaCl l^{-1} , pH 3.0. The alcohol–acid extract was dialysed against 0.17 mol acetic acid l^{-1} and then centrifuged at 5000 g in a Centricon 30 until all the sample passed through. The retentates were rinsed once with 0.2 ml 0.17 mol acetic acid l^{-1} , and after centrifugation the residues were taken up in 1 ml 0.17 mol acetic acid l^{-1} . Aliquots of the alcohol–acid extracts, the suspended retentates (> 30 kDa) and the filtrates (< 30 kDa) were lyophilised and suspended in TBSA buffer. The incubation procedure was as for receptor determination

using 40 000 c.p.m. of tracer and various amounts of the alcohol–acid extract. The same growth factors used for iodination were used for the standard curve. The sensitivity of the assay for EGF activity was 150 pg per tube. The mean intra-assay coefficient of variation was 9%.

Affinity labelling was performed by incubating 50 μg of membrane protein with 500 000 c.p.m. of ^{125}I -labelled IGF-I or IGF-II overnight at 4°C in a final volume of 0.2 ml TBSA buffer. The reaction was stopped by adding 1 ml ice-cold PBS and further centrifugation at 10 000 g for 10 min. The pellet was resuspended in 0.195 ml PBS and cross-linking was performed by addition of 5 μl 4 mmol disuccinimidyl suberate (DSS) l^{-1} in dimethyl sulfoxide and ice-bath incubation. After 15 min, 0.2 ml 1 mol Tris l^{-1} , 1 mmol EDTA l^{-1} , pH 7.4, were added and the incubation allowed to proceed for another 10 min, followed by the addition of 1 ml cold distilled water and centrifugation at 10 000 g for 10 min. Pellets were then solubilized by boiling in sample buffer (62.5 mmol Tris l^{-1} , 15 mmol dithiothreitol l^{-1} , 1% (w/v) SDS, 15% (v/v) glycerol, pH 6.8). The proteins were separated on a 4–10% polyacrylamide gel gradient SDS-PAGE system. The gels were fixed in 40% methanol–10% acetic acid (v/v) and dried. Kodak XAR-5 film (Eastman Kodak, Rochester, USA) was exposed at $-70^\circ C$ with intensifying screen (DuPont Cronex, DuPont, Wilmington, DE) for 4–7 days. The relative intensity of each band was determined with a gel-scan densitometer (Ultrascan XL laser densitometer, LKB Broma, Sweden).

Western blot analysis of the alcohol–acid extract was performed as described by Stein *et al.* (1995) after protein separation on a 10–20% polyacrylamide gel gradient SDS-PAGE. The sensitivity of the assay for TGF α was 5 ng.

Protein determinations were performed after digestion of a sample of membrane preparation with 1 mol NaOH l^{-1} according to the method of Lowry *et al.* (1951) using BSA as standard. DNA was determined in the sonicated samples resuspended in buffer TNE (10 mmol Tris l^{-1} , 100 mmol NaCl l^{-1} , 1 mmol EDTA l^{-1} , pH 7.4). After the addition of the fluorochrome Hoechst 33258, the fluorescence was read in a TKO 100 minifluorometer (Hoefer Scientific Instruments, CA, USA). Salmon testes DNA was used as standard.

Statistical analyses

Results are given as means $\pm$ SEM, with the number of observations in parentheses. Analysis of the data was performed by ANOVA, and differences between groups were assessed by Scheffé's multiple contrasts. The Mann–Whitney test was used for the Scatchard values owing to the number of observations. $P < 0.05$ was considered to be statistically significant.

Results

EGF receptors

Scatchard analysis of the binding of ^{125}I -labelled EGF to membrane preparations of mammary acini from intact or ovariectomized rats at day 19 of pregnancy showed a single class of high-affinity binding sites with similar K_d values

Fig. 1. Scatchard plot of the binding of ^{125}I -labelled epidermal growth factor (EGF) to mammary acini membranes from (●) control and (○) ovariectomized rats at day 19 of pregnancy. Samples of membranes (25–50 μg protein) were incubated with increasing amounts of ^{125}I -labelled EGF (4.4×10^{-11} – 1.44×10^{-9} mol l^{-1}) in the presence or the absence of 3×10^{-7} mol l^{-1} unlabelled EGF, in order to assess non-specific binding. Values are the means of four experiments. $K_d = 1.1$ nmol l^{-1} for control rats and 0.71 for ovariectomized rats.

(1.1 ± 0.05 ($n = 4$) and 0.71 ± 0.07 ($n = 5$) nmol l^{-1} , respectively) (Fig. 1). Receptor concentration was lower in the differentiated gland than in the controls (control 71.2 ± 17 ($n = 4$) fmol mg^{-1} protein; ovariectomized 24.9 ± 7.4 ($n = 5$) fmol mg^{-1} protein, $P < 0.05$) and similar to that found in those glands in which lactation was already established (25.7 ± 7 ($n = 4$) fmol mg^{-1} protein).

In spite of the presence of EGF in milk, the decrease in receptor content is not due to the occupancy of the receptor sites by endogenous ligands, since acid treatment of membranes did not change the observed results. Moreover, EGF could not be measured in the lyophilised acid supernatants either by radioimmunoassay or radioreceptor assays. These results do not exclude down regulation of receptors or ligand processing.

In the ovariectomized rats, progesterone treatment, which effectively blocks lactogenesis II, did not prevent the decrease in EGF receptors. Oestrogen, when given alone, further diminished EGF binding, but the combination of oestradiol and progesterone restored EGF receptor concentrations to values present in intact rats at day 19 of pregnancy (Fig. 2).

To find out whether the down-regulation of the EGF receptor, seen after oestrogen treatment, was caused by its own ligand, the acid-alcohol extract of the mammary glands was examined for the presence of EGF. The growth factor was not detected in any of the groups by radioimmunoassay, using a

Fig. 2. Effect of steroid replacement to ovariectomized rats on the concentration of the epidermal growth factor (EGF) receptor. Membranes from mammary gland acini of control, ovariectomized (Ov), and ovariectomized treated with progesterone (Ov + P), oestradiol (Ov + E) or progesterone plus oestradiol (Ov + PE) were incubated with ^{125}I -labelled EGF (1.44×10^{-9} mol l^{-1}) in duplicate. Specific membrane-bound labelled EGF is expressed as fmol mg^{-1} protein. Values are means $\pm$ SEM; $n = 5$ rats per group. Bars with the same letter(s) are not significantly different at $P < 0.05$.

rabbit polyclonal anti-mEGF; however when a radioreceptor assay was used, all the extracts showed EGF-like activity. Those extracts from the mammary glands of oestradiol-treated rats presented higher values of EGF-like activity and were significantly different from the other groups (oestradiol-treated 43.2 ± 7.2 ($n = 3$) ng g^{-1} of mammary gland versus intact 4.0 ± 1.7 ($n = 3$) and ovariectomized 4.55 ± 1.8 ($n = 3$) ng g^{-1}).

IGF-I binding

The binding of ^{125}I -labelled IGF-I to membranes from mammary cells from control or ovariectomized rats at day 19 of pregnancy, when analysed according to Scatchard, showed only one class of high-affinity, low-capacity binding sites with similar K_d values to those seen at the onset of lactation (0.085 ± 0.01 ($n = 4$) and 0.12 ± 0.05 ($n = 4$) nmol l^{-1} , respectively). However, a fall in receptor concentration occurred, together with the appearance of mammary gland secretions, after ovariectomy (592 ± 35 ($n = 4$) fmol mg^{-1} protein in intact rats versus 274 ± 43 ($n = 4$) fmol mg^{-1} protein in the ovariectomized rats; $P < 0.05$).

The specificity of ^{125}I -labelled IGF-I binding to membranes from intact or ovariectomized rats was determined by incubating the tracer in the presence of 0–2000 ng ml^{-1} IGF-I, IGF-II and insulin. A 50% displacement in the binding was observed with 1 ng IGF-I ml^{-1} , 200 ng IGF-II ml^{-1} and 2000 ng insulin ml^{-1} .

Covalent cross-linking of ^{125}I -labelled IGF-I to the receptor was also performed (Fig. 3). Two bands with an apparent molecular mass of 134 and 257 kDa (mean of three

Fig. 3. Representative fluorography of cross-linked ^{125}I -labelled insulin-like growth factor I (IGF-I) bound to mammary acini membranes from control (lanes 1, 3, 5 and 7) or ovariectomized rats (lanes 2, 4, 6 and 8) preincubated in the absence (lanes 1 and 2) or in the presence of $3 \times 10^{-7} \text{ mol IGF-I l}^{-1}$ (lanes 3 and 4), IGF-II (lanes 5 and 6) and insulin (lanes 7 and 8). Positions of the molecular mass markers are indicated on the right. Bars under the lanes represent the absorbance of the cross-linked receptor quantified by densitometry. (□) 257 kDa; band (▨) 134 kDa band.

experiments) were detected. Laser scanning densitometry of the autoradiography confirmed the results obtained by equilibrium-binding analysis. Ovariectomy caused a decrease in IGF-I binding of both bands and the specificity of the binding was confirmed by competition with IGF-II and insulin. The ratio between the absorbance of the lower and higher molecular mass bands (0.26) remained constant in all the cases and may indicate that the high molecular mass moiety is a dimer of the α -subunit of the IGF-I receptor.

Replacement treatment of ovariectomized rats with ovarian steroids (Fig. 4) revealed that, at least in part, the decrease in ^{125}I -labelled IGF-I binding was not due to the IGF present in milk. Oestradiol, which increases milk secretion, also increased receptor concentrations to values not different from those of the control group, and progesterone which abolishes lactogenesis induced by ovariectomy, did not prevent the decrease in IGF-I binding.

IGF-II binding

IGF-II binds to the membranes from mammary cells with high affinity and with similar K_d values for both intact and ovariectomized rats at day 19 of pregnancy (0.11 ± 0.04 ($n = 3$) and 0.1 ± 0.01 ($n = 3$) nmol l^{-1} , respectively). The number of receptors slightly increased with the initiation of lactation (intact: 547 ± 29 ($n = 3$) $\text{fmol mg}^{-1} \text{ protein}$; ovariectomized:

Fig. 4. Effect of steroid replacement to ovariectomized rats on the concentration of the insulin-like growth factor I (IGF-I) receptor. Membranes from mammary gland acini of control, ovariectomized (Ov), and ovariectomized treated with progesterone (Ov + P), oestradiol (Ov + E) or progesterone plus oestradiol (Ov + PE) were incubated as described in Materials and Methods. Values are means $\pm$ SEM; $n = 5$ rats per group. Bars with the same letter(s) are not significantly different at $P < 0.05$.

666 ± 9 ($n = 3$) $\text{fmol mg}^{-1} \text{ protein}$). Affinity labelling of membranes with ^{125}I -labelled IGF-II followed by SDS-PAGE (Fig. 5) showed a band with an apparent molecular mass of 235 kDa (mean of three experiments). Laser scanning densitometry revealed an increase in binding to a receptor type II simultaneously with gland differentiation caused by ovariectomy. Binding specificity was confirmed by competition with $3 \times 10^{-7} \text{ mol l}^{-1}$ of non-radioactive IGF-I and insulin.

The identity of the receptor as a type II/mannose 6-phosphate receptor was investigated by incubation of membranes from control animals and ^{125}I -labelled IGF-II with increasing concentrations of mannose 6-phosphate ($0.5 \mu\text{mol} - 10 \text{ mmol l}^{-1}$). At the highest concentration of the sugar, binding increased by about 70%. Affinity labelling of membranes incubated with ^{125}I -labelled IGF-II in the presence of 5 $\text{mmol mannose 6-phosphate l}^{-1}$, followed by SDS-PAGE (Fig. 6) showed an enhancement of the 235 kDa band. However, when ^{125}I -labelled IGF-I was used the 257 kDa band did not increase in the presence of the sugar (data not shown).

Treatment with ovarian steroids affected IGF-II binding as well as lactogenesis. Progesterone decreased the binding in ovariectomized rats to control values, while oestradiol increased the binding to values significantly different from the control rats (Fig. 7).

Discussion

The results presented here confirm and extend previous studies on changes to EGF and IGF receptors in mammary glands at different physiological stages (Edery *et al.*, 1985; Collier *et al.*,

Fig. 5. Representative fluorography of cross-linked ^{125}I -labelled insulin-like growth factor II (IGF-II) bound to mammary acini membranes from control (lanes 1, 3, 5 and 7) or ovariectomized rats (lanes 2, 4, 6 and 8) preincubated in the absence (lanes 1 and 2) or in the presence of $3 \times 10^{-7} \text{ mol l}^{-1}$ of IGF-II (lanes 3 and 4), IGF-I (lanes 5 and 6) and insulin (lanes 7 and 8). Positions of the molecular mass markers are indicated on the right. Bars under the lanes represent the absorbance of the cross-linked receptor quantified by densitometry.

1989). The trigger for lactogenesis II at the end of pregnancy is the fall in circulating concentrations of progesterone. Ovariectomy, which induces lactogenesis (Liu and Davis, 1967), provides an appropriate model of the mammary gland with the same degree of development as that in the intact animal, but that synthesises milk. The biosynthetic activity of the mammary gland induced by ovariectomy is not of the same magnitude as that obtained by the removal of corpora lutea, but leaves the other ovarian functions intact. Replacement therapy with oestrogen is required for the stimulation of α -lactalbumin activity to be similar to that in the intact animal (Bussmann *et al.*, 1983), whereas progesterone replacement treatment effectively suppresses milk synthesis induced by ovariectomy (Kuhn, 1969).

In agreement with the findings of Edery *et al.* (1985) working on the mouse mammary gland at different reproductive stages, we found that in rats, ovariectomy and the onset of lactogenesis decreases the concentration of EGF receptors to values similar to those seen during established lactation.

Replacement treatment with ovarian steroids showed that progesterone does not prevent the fall in EGF receptor concentrations seen after ovariectomy. Treatment with oestradiol and progesterone together does not unblock the action of the latter but restores EGF receptor values to those of the intact animal. This finding confirms previous studies in non-differentiated mouse mammary glands (Vonderhaar, 1987) indicating that the stimulatory action of progesterone and oestrogen together is due to the regulation of the concentration of the EGF receptor (Haslam *et al.*, 1992).

Fig. 6. Representative fluorography of cross-linked ^{125}I -labelled insulin-like growth factor II (IGF-II) bound to mammary acini membranes from rats at day 19 of pregnancy preincubated in the absence (lane 1) or in the presence of $3 \times 10^{-7} \text{ mol l}^{-1}$ IGF-II (lane 2) or 5 mmol mannose 6-phosphate l^{-1} (lane 3). Positions of the molecular mass markers are indicated on the right. Bars under the lanes represent the absorbance of the cross-linked receptor quantified by densitometry.

In the uterus, oestradiol upregulates the EGF receptor in immature rats (Makku and Stancel, 1985) and in ovariectomized adult mice (Das *et al.*, 1994). Oestradiol, given in combination with progesterone, produces an increase in mRNA encoding the EGF receptor, which is not concomitant with the increase in receptor concentration in the mouse uterus (Das *et al.*, 1994). In the human cell line T-47D, both the EGF receptor and its mRNA are upregulated by progestagens (Murphy *et al.*, 1986, 1988). The differences in responses may be due to tissue- or physiological stage-specific conditions.

Treatment of ovariectomized pregnant rats with oestradiol produces a further decrease in EGF receptors together with the appearance in the acid-alcohol extract of an EGF-like molecule. Western blot analysis of the acid-alcohol extract with a rabbit polyclonal antibody against TGF α , did not reveal any specific band for TGF α . However, the EGF-like molecule, the concentration of which was increased by oestradiol treatment, was found to bind to heparin-Sepharose and eluted with 0.75 mol NaCl l^{-1} (L. Bussmann and E. Charreau, unpublished observations). These results indicate a paracrine control of the mammary growth by an EGF-like growth factor which has an

Fig. 7. Effect of steroid replacement to ovariectomized rats on the concentration of the insulin-like growth factor II (IGF-II) receptor. Membranes from mammary gland acini of control, ovariectomized (Ov), and ovariectomized treated with progesterone (Ov + P) or oestradiol (Ov + E) were incubated. Values are means $\pm$ SEM; $n = 5$ rats per group. Bars with the same letter(s) are not significantly different at $P < 0.05$.

affinity for heparin that lies between platelet-derived growth factor (PDGF) and those of heparin-binding EGF (Higashiyama *et al.*, 1992). In the mouse mammary gland, an EGF-like growth factor activity, MDGF, has been described by Vonderhaar (1984), and the presence of amphiregulin, which binds to the EGF receptor and heparin, was recently reported by Kenney *et al.* (1995).

The presence of high-affinity binding sites for IGF-I has been reported in rat mammary glands and their concentrations were higher in rats at day 7 of pregnancy compared with virgin or lactating rats (Collier *et al.*, 1989). Ovariectomy, which initiates milk secretion and is responsible for the change in the mammary gland from a growing stage to a secretory one, induces down-regulation of the receptors for IGF-I and EGF. A similar fall in concentrations of the IGF-I receptor has been described in sheep at parturition and lactation (Winder *et al.*, 1993). In contrast, lactogenesis in cows is accompanied by an increase in IGF binding and the appearance of a 127 kDa IGF-I subunit, in addition to the 134 kDa subunit (Dehoff *et al.*, 1988; Hadsell *et al.*, 1990).

Colostrum and milk are major sources of growth-promoting factors (Corps and Brown, 1987), and immunohistochemical studies have shown that in rat mammary glands IGF-I is present only in myoepithelial cells (Marcotty *et al.*, 1994). Downregulation of the IGF-I receptor during lactogenesis could indicate the uptake and transfer of the growth factor from the myoepithelial cells or serum to milk, as described in goats (Prosser *et al.*, 1991).

The IGF-I and EGF receptor content in mammary glands of ovariectomized rats showed a different pattern after replacement treatment with ovarian steroid hormones. Oestrogen was shown to regulate the IGF-I receptor, since ovariectomy produced a decrease in the binding sites and administration of oestradiol partly restored the sites, even when injected

together with progesterone. This would allow IGF-I to exert its mitogenic effect during pregnancy, when progesterone concentrations are high and preclude a lactogenic type II action by IGF-I. In humans, using an oestrogen-responsive cell line, oestradiol was found to act in a similar manner (Steward *et al.*, 1990). When incubated in the absence of oestrogen, these cells showed a six-fold decrease in IGF-I binding sites, with a similar effect observed on mRNA encoding the IGF-I receptor. This finding suggests that oestradiol exerts its action at the transcriptional level. The decrease in receptors after ovariectomy observed in the work reported here was not of the same magnitude as that observed by Steward *et al.* (1990), possibly due to the shorter time of hormone deprivation before the experiment and to the action of some residual oestradiol.

Affinity cross-linking of ¹²⁵I-labelled IGF-II showed only one band corresponding to the type II receptor. No radioactivity was observed associated with either the type I receptor or with IGF-binding proteins such as IGFBP3, as described in cultured mammary cells from mice (Hadsell *et al.*, 1994). Recently, the IGF-II receptor was confirmed as being identical to the mannose 6-phosphate receptor (MacDonald *et al.*, 1988) which targets acid hydrolases to lysosomes (Pfeffer, 1988). The results of the covalent cross-linking of ¹²⁵I-labelled IGF-II to the receptor when incubated in the presence of mannose-6-phosphate presented here support the hypothesis that both receptors are identical. In agreement with Collier *et al.* (1989) we showed that, after differentiation of the mammary gland to a secretory state, the concentration of the IGF type II receptor changed slightly. When covalent cross-linking was carried out, an increase in labelling of more than 50% was found in membranes from ovariectomized rats. Oestradiol treatment, which increases lactose synthesis (Bussmann *et al.*, 1983), significantly increased concentrations of the IGF-II/mannose-6-phosphate receptor. This may reflect the shift from growth and development of the gland to a secretory role, and hence to an increase in lysosomal movement.

Our results suggest that, while the crucial role of EGF and IGF-I in the growth and differentiation of the mammary gland might be mediated by changes in receptor concentration accompanying lactogenesis II, the progesterone block of this physiological event is not mediated by these growth factors.

The authors thank J. C. Calvo and A. Colman Lerner for critical reading of the manuscript. This work was supported by grants from the CONICET and BID-CONICET II programs.

References

- Bussmann L and Deis RP (1979) Studies concerning the hormonal induction of lactogenesis by prostaglandin $F_{2\alpha}$ in pregnant rats *Journal of Steroid Biochemistry* **11** 1485–1489
- Bussmann L, Konyncx A and Deis RP (1983) Effect of estrogen and placental lactogen on lactogenesis in pregnant rats *Biology of Reproduction* **29** 535–541
- Carpenter G and Cohen S (1979) Epidermal growth factor *Annual Review of Biochemistry* **48** 193–216
- Collier RJ, Ganguli S, Menke PT, Buonomo FC, McGrath MF, Kotts CE and Krivi GG (1989) Changes in insulin and somatomedin receptors and uptake of insulin, IGF-I and IGF-II during mammary growth, lactogenesis and lactation. In *Biotechnology in Growth Regulation* pp 153–163 Eds RB Heap, CG Prosser and GE Lamming. Butterworth, London

- Corps AN and Brown KD (1987) Stimulation of intestinal epithelial cell proliferation in culture by growth factors in human and ruminant mammary secretions *Journal of Endocrinology* **113** 285–290
- Das SK, Tsukamura H, Paria BC, Andrews GK and Dey SK (1994) Differential expression of epidermal growth factor receptor (EGF-R) gene and regulation of EGF-R bioactivity by progesterone and oestradiol in the adult mouse uterus *Endocrinology* **134** 971–981
- Davis SR, Gluckman PD, Hart IC and Henderson HV (1987) Effects of injecting growth hormone or thyroxine on milk production and blood plasma concentrations of insulin-like growth factors I and II in dairy cows *Journal of Endocrinology* **114** 17–24
- Dehoff MH, Elgin RG, Collier RJ and Clemmons DR (1988) Both type I and II insulin growth factor receptor binding increase during lactogenesis in bovine mammary tissue *Endocrinology* **122** 2412–2417
- Dembinski TC and Shiu PC (1987) Growth factors in mammary gland development and function. In *The Mammary Gland Development, Regulation and Function* pp 355–381 Eds MC Neville and CW Daniel. Plenum, New York
- Ederly M, Pang K, Larson L, Colosi T and Nandi S (1985) Epidermal growth factor receptor levels in mammary glands in various physiological states *Endocrinology* **117** 405–411
- Elizalde P, Lanari C, Kordon E, Tezon J and Charreau EH (1990a) Transforming growth factor- β activities in lines of hormone-dependent and independent mammary adenocarcinomas induced by medroxyprogesterone acetate in BALB/c mice *Breast Cancer Research and Treatment* **16** 29–39
- Elizalde P, Lanari C, Bussmann L, Kordon E, Guerra F, Savin M and Charreau EH (1990b) Insulin-like growth factor-I production and receptors in mammary adenocarcinomas induced by medroxyprogesterone acetate in BALB/c mice *Journal of Experimental and Clinical Cancer Research* **9** 193–203
- Fleet IR, Goode JA, Hamon MH, Laurie MS, Linzell JL and Peaker M (1975) Secretory activity of goat mammary glands during pregnancy and the onset of lactation *Journal of Physiology* **251** 763–773
- Flint DJ, Tonner E, Beattie J and Gardner M (1994) Several insulin growth factor-I analogues and complexes of insulin-like growth factor-I and -II with insulin-like growth factor-binding protein-3 fail to mimic the effect of growth hormone upon lactation in the rat *Journal of Endocrinology* **140** 211–216
- Hadsell DL, Campbell PG and Baumrucker CR (1990) Characterization of the change in type I and II insulin-like growth factor receptors of bovine mammary tissue during the pre- and postpartum periods *Endocrinology* **126** 637–643
- Hadsell DL, Gibson CA and Baumrucker CR (1994) Insulin-like growth factor (IGF) binding to a murine mammary epithelial cell line *Journal of Cell Physiology* **161** 435–440
- Haslam SZ, Counterman LJ and Nummy KA (1992) EGF receptor regulation in normal mouse mammary gland *Journal of Cell Physiology* **152** 553–557
- Higashiyama S, Lau K, Besner GE, Abraham JA and Klagsbrun M (1992) Structure of heparin-binding EGF-like growth factor *Journal of Biological Chemistry* **267** 6205–6212
- Imagawa W, Spencer EM, Larson L and Nandi S (1986) Somatomedin-C substitutes for insulin for the growth of mammary epithelial cells from normal virgin mice in serum-free collagen gel culture *Endocrinology* **119** 2695–2699
- Kasuga M, Van Obberghen E, Nissley SP and Rechler MM (1981) Demonstration of two subtypes of insulin-growth factor receptors by affinity crosslinking *Journal of Biological Chemistry* **256** 5305–5308
- Kenney NJ, Huang RP, Johnson GR, Wu JX, Okamura D, Matheny W, Kordon E, Gullick WJ, Plowman G, Smith GH, Salomon DS and Adamson ED (1995) Detection and location of amphiregulin and cripto-1 expression in the developing postnatal mouse mammary gland *Molecular Reproduction and Development* **41** 277–286
- Kuhn NJ (1969) Progesterone withdrawal as the lactogenic trigger in the rat *Journal of Endocrinology* **44** 39–54
- Liu TM and Davis JW (1967) Induction of lactation by ovariectomy in pregnant rats *Endocrinology* **80** 1043–1050
- Lowry OH, Rosebrough NJ, Farr AL and Randall RJ (1951) Protein measurement with the Folin phenol reagent *Journal of Biological Chemistry* **193** 265–276
- MacDonald RG, Pfeffer SR, Coussens L, Tepper MA, Brocklebank CM, Mole JE, Anderson JK, Chen E, Czech MP and Ulrich A (1988) A single receptor binds both insulin-like growth factor II and mannose 6-phosphate *Science* **239** 1134–1137
- Makku VR and Stancel GL (1985) Regulation of epidermal growth factors receptors by oestrogen *Journal of Biological Chemistry* **260** 9820–9824
- Marcotty Ch, Franke F, Meuris S and Hennen G (1994) Immunolocalization and expression of insulin-like growth factor I (IGF-I) in the mammary gland during rat gestation and lactation *Molecular and Cellular Endocrinology* **99** 237–243
- Molinolo AA, Lanari C, Charreau EH, Sanjuan N and Dosne Pasqualini C (1987) Mouse mammary tumours induced by medroxyprogesterone acetate: immunohistochemistry and hormonal receptors *Journal of National Cancer Institute* **79** 1341–1350
- Munson PJ and Rodbard D (1980) Ligand: A versatile computerized approach for characterization of ligand-binding systems *Analytical Biochemistry* **107** 230–239
- Murphy LC, Murphy LJ and Shiu RPC (1988) Progesterin regulation of EGF-receptor mRNA accumulation in T-47D human breast cancer cells *Biochemical and Biophysical Research Communications* **150** 192–196
- Murphy LJ, Sutherland RL, Stead B, Murphy LC and Lazarus L (1986) Progesterin regulation of epidermal growth factor receptor in human mammary carcinoma cells *Cancer Research* **46** 728–734
- Myal Y, Shiu RPC, Bhanmick B and Bala M (1984) Receptor binding and growth-promoting activity of insulin-like growth factors in human breast cancer cells (T47D) in culture *Cancer Research* **44** 5486–5490
- Papadimitriou JT, Shearer M and Stocker MGP (1977) Growth requirements of human mammary epithelial cells in culture *International Journal of Cancer* **20** 903–910
- Pfeffer SR (1988) Mannose 6-phosphate receptors and their role in targeting proteins to lysosomes *Journal of Membrane Biology* **103** 7–16
- Prosser CG, Fleet IR, Davis AJ and Heap RB (1991) Mechanism of secretion of plasma insulin-like growth factor-I into milk of lactating goats *Journal of Endocrinology* **131** 459–466
- Roberts AB, Lamb LC, Newton DL, Sporn MB, DeLarco JE and Todaro GJ (1980) Transforming growth factors: isolation of polypeptides from virally and chemically transformed cells by acid/ethanol extraction *Proceedings of the National Academy of Sciences USA* **77** 3494–3498
- Sankaran L and Topper YJ (1987) Is EGF a physiological inhibitor of mouse mammary casein synthesis? Unphysiological responses to pharmacological levels of hormones *Biochemical and Biophysical Research Communications* **146** 121–125
- Scatchard G (1949) The attractions of proteins for small molecules and ions *New York Academy of Sciences* **51** 660–672
- Shyamala G and McBlain WA (1979) Distinction between progesterin- and glucocorticoid-binding sites in mammary glands *Biochemical Journal* **178** 345–352
- Stein P, Bussmann L and Tessone M (1995) *In vivo* regulation of the steroidogenic activity of rat luteal cells by insulin *Journal of Steroid Biochemistry and Molecular Biology* **52** 329–335
- Steward AJ, Johnson MD, May FEB and Westley BR (1990) Role of insulin-like growth factors and the type I insulin-like growth factor receptor in the oestrogen-stimulated proliferation of human breast cancer cells *Journal of Biological Chemistry* **265** 21 172–21 178
- Taketani Y and Oka T (1983) Epidermal growth factor stimulates cell proliferation and inhibits functional differentiation of mouse mammary epithelial cells in culture *Endocrinology* **113** 871–877
- Threadgold LC, Coore HG and Kuhn NJ (1982) Monosaccharide transport into lactating rat mammary acini *Biochemical Journal* **204** 491–501
- Topper YJ and Freeman CS (1980) Multiple hormone interaction in the developmental biology of the mammary gland *Physiological Review* **60** 1049–1106
- Turkington RW (1969) The role of epidermal growth factor in mammary gland development *in vitro Experimental Cell Research* **57** 79–85
- Vonderhaar BK (1984) Hormone and growth factors in mammary gland development. In *Control of Cell Growth and Proliferation* pp 11–33 Ed. CM Veneziale. Van Nostrand-Reinhold, Princeton, NJ
- Vonderhaar BK (1987) Local effects of EGF, TGF α , and EGF-like growth factors on lobuloalveolar development of the mouse mammary gland *in vivo Journal of Cell Physiology* **132** 581–584
- Winder SJ, Wheatley SD and Forsyth IA (1993) Receptor binding of insulin-like growth factor-I to mammary microsomes from non-pregnant, pregnant and lactating sheep *Journal of Endocrinology* **136** 297–304