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Prolactin Binding Analysis and Immunohistochemical Localization of Prolactin Receptor in Porcine Ovarian Cells

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Abstract. In the present study we searched for prolactin receptor (PRL-R) in porcine ovarian theca tissue (Tc) of small, medium and large follicles, as well as in early corpus luteum (ECL). The objectives of this investigation were: 1) comparison of the direct effect of PRL action on progesterone (P_4) and estradiol (E_2) secretion from Tc and ECL cells in culture with adequate effects caused by luteinizing hormone (LH). 2) detection of the presence and distribution of PRL-R in thecal tissue of porcine follicles and in ECL. Tissues were cultured as monolayers either in control M199 medium with calf serum or in medium either with PRL (100 ng/ml) or with LH (100 ng/ml). After 2 days *in vitro* cultured media were assayed for steroid concentrations by radioimmunoassays. Content and distribution of PRL-R were evaluated by Scatchard analysis and by an immunohistochemical assay. Separated theca layers as well as fragments of ECL were excised on dry ice, homogenized, and incubated with [^{125}I]-PRL. PRL stimulated P_4 secretion from Tc 10-fold versus controls. LH stimulated P_4 secretion only 2.5-fold. E_2 secretion was stimulated by PRL 2.7-fold and by LH 2.4-fold. LH enhanced P_4 secretion from ECL cells by 18% while PRL increased P_4 secretion by as much as 73%. Femtomol amounts of PRL-R protein were detected in theca tissues of medium and large follicles and also in ECL, which was in accordance with immunohistochemical results. The results showed for the first time the presence of PRL-R in porcine Tc and ECL.

Key words: Prolactin, PRL receptor, Immunohistochemistry, Ovary, Pig

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Prolactin (PRL), a multifunctional protein hormone, is synthesized and secreted by lactotrophs of the anterior hypophysis. In mammals, target cells for PRL are found in a variety of organs, of which the ovary is one. PRL is best known for its action on mammary gland [1]. However, recent data have shown its involvement in growth and maturation of ovarian follicles and maintenance of luteal function in some species [2, 3].

Rats, in which PRL is the main luteotropic hormone, have been most extensively investigated with regard to the role of this hormone in ovarian function. On the other hand, information on the direct action of PRL on ovarian cell types in porcine is limited.

Our earlier *in vitro* studies on theca cells (Tc), granulosa cells (Gc) and early corpus luteum cells (ECL) isolated from proestrus porcine ovary and cultured as monolayer showed that PRL significantly stimulated progesterone (P_4) secretion by cultures of Tc alone as well as by luteal cells of ECL. P_4 secretion was also stimulated by co-cultures of Tc and Gc but not by Gc alone in monoculture [4, 5].

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Tc in the pig, contrary to human, rat and bovine, have certain aromatizing abilities. Basal estradiol (E_2) secretion from Tc was higher than that from Gc [6, 7]. Modulation of steroidogenesis in cultured luteal cells from ECL by PRL and its distinct luteotropic action shown *in vitro* were reported elsewhere [2, 5, 8]. PRL was also detected in porcine follicular fluid [9]. This luteotropic action of PRL was later confirmed *in vivo* by Ciereszko and Dusza [10]. They showed that exogenous PRL elevated plasma P_4 concentrations during the early luteal phase of the porcine estrous cycle. In our cultures, we used medium supplemented with 5–10% calf serum, which contains low density lipoproteins (LDL). Rajkumar *et al.* [11, 12] and recently Ciereszko *et al.* [13] showed that PRL affected LDL processing in luteal tissue which resulted in enhanced P_4 secretion by ECL cells.

PRL binding by luteal cells of pig was first observed by Rolland *et al.* [14] and PRL stimulation of Gc steroidogenesis was reported by Veldhuis *et al.* [15]. PRL receptors were localized in the ovary of hamster [16], rat [17, 18], mouse [19] and human [20].

Recently, PRL-R has been cloned and identified [21]. It belongs to the growth hormone/cytokine receptor superfamily. Two different forms of the receptor, short and long have been identified [22].

PRL-Rs have not been shown as yet to be present in porcine theca tissue. Its sensitivity to PRL expressed in terms of increased steroid secretion suggested that the PRL signal could be transduced via specific receptors.

The main rationale of this experiment was to show the presence of PRL-R in porcine theca interna tissue and in ECL and to see whether the observed stimulation of steroidogenesis in those cells could be caused by PRL binding to its receptor.

Materials and Methods

Chemicals

Highly purified ovine prolactin (28 i.u./mg) used in cell culture was obtained from NIH, Bethesda, MD, USA, while porcine PRL (30 i.u./mg) obtained according to Kochman and Kochman [23] was used for the binding assay.

Medium M199, penicillin, trypsin and calf serum were products of the Laboratory of Sera and Vaccines, Lublin, Poland. Monoclonal (mouse) anti PRL-R antibody (IgG_1)-clone U5 was purchased from Affinity Bioreagents (Golden, CO, USA). Biotinylated anti-mouse antibody (IgG) raised in horse was obtained from Vector (Burlingame, CA, USA). Strep-ABComplex-HRP was from Dako (Glostrup, Denmark). Bio-Rad protein assay reagent was purchased from BioRad Laboratories (Richmond, CA, USA). Isotopes for RIA were obtained from Amersham (Little Chalfont, England), anti-progesterone antibody was a generous gift from Professor B. Cook, University of Glasgow, (Glasgow, Scotland) and anti-estradiol antibody was a gift from Professor R. Rembiesa (Institute of Pharmacology, Polish Academy of Sciences, Kraków, Poland). 1 mCi [^{125}I] was obtained from Amersham (Little Chalfont, England).

Tissue

Porcine ovaries were obtained from slaughterhouse animals and classified according to Akins and Morrissette [24] and Channing and Ledwitz-Rigby [25]. Large proestrous follicles 0.9–1.2 cm diam. and ECL were selected for culture. For immunohistochemical and binding studies small, medium, and large follicles (3–4 mm, 5–8mm, and 8–12 mm diam., respectively) and fragments of ECL were excised from porcine ovaries.

Cell culture

Tc were isolated using our own technique [26]. ECL, containing blood clot in the center, were removed from postovulatory ovaries (1–3 days after ovulation) and then dispersed enzymatically according to Gregoraszczuk [2]. Thecal and luteal cells were suspended in Medium M199 supplemented with 5% calf serum at densities of 3.5×10^5 and 5×10^5 cells per 1 ml medium, respectively. Cells were grown in multiwell plates (Nunc, Roskilde, Denmark) in a humidified atmosphere of 5% CO_2 in air for 48 hrs. In order to select the most effective dose of PRL, a dose response curve from 0 to 10, 100 and 500 ng of PRL was prepared in an earlier experiment [8]. The optimal dose of 100 ng PRL was used in the present experiments. Cells were cultured in the

control medium without PRL or in medium supplemented with 100 ng PRL per 1 ml. In addition, cells were also cultured in medium containing 100 ng LH per 1 ml. The latter was introduced as an additional control since it is well known that Tc contain LH receptor, the hormone which is luteotropic in pig. The dose of 100 ng LH is long known as the most effective one in cell culture experiments [2].

Steroid hormone measurement

The amount of P_4 secreted to medium was determined by radioimmunoassay according to Abraham *et al.* [27]. A highly specific antibody raised in rabbit against 11α-progesterone hemisuccinate coupled to bovine serum albumin was used. The cross-reaction with prognenolone was 2.9%. The other steroids tested showed a cross-reactivity below 1%. [1,2,6,7-^{3}H] progesterone was used as the tracer (80 Ci /mmol). The detection limit of the assay was 50 pg /ml. The coefficients of variation within and between assays were below 1.5% and 2.5% respectively.

Estradiol-17β (E_2) was analysed by the radioimmunoassay according to Hotchkis *et al.* [28]. A highly specific antibody raised in rabbit against 17β-6-oxime-BSA antigen was used. It gave negligible cross-reaction with estrone (0.8%), estriol (0.8%) and 16-keto-estradiol 17β (1%). [2,4,6,7,16,17-^{3}H]-Estradiol was used as the tracer (140 Ci/mmol). The detection limit of the assay was 5 pg. The data were expressed as nanograms of steroids secreted into 1 ml of medium per 1×10^5 cells for 48 h of culture. All data were expressed as means$\pm$SEM derived from at least three different experiments (n=3), each in quadruplicates, which means at least twelve observations. The differences between steroid concentrations in control and treated cultures were evaluated by ANOVA test.

Immunohistochemical procedures

Isolated porcine ovarian follicles of different sizes (small, medium, large) as well as early corpora lutea, were fixed in buffered 4% formalin for 24 h, dehydrated in a series of increasing gradient of ethanol and embedded in paraffin. Sections were cut at 6 μm, mounted on silanised glass slides, dewaxed in xylene, hydrated in ethanol (decreasing gradient) and washed in PBS. To retrieve antigenicity, slides were placed in a plastic box with 0.01 M citrate buffer, pH 6.0 and heated in a 750 W microwave 3 times for 5 min. Between each cycle, the evaporated buffer was supplemented with hot distilled water. After cooling, the slides were incubated with 0.3% H_2O_2 in PBS, to quench endogenous peroxidase activity and then with 3% normal horse serum to block non-specific binding. Finally, slides were incubated with monoclonal anti-PRL-R antibody (at dilution 1 : 40) overnight at 4°C. Sections were washed in 3 changes of Tris-buffered saline with 0.1% Tween 20, pH 7.6 (TBST), then incubated with biotinylated horse anti-mouse IgG for 1.5 h at room temperature, washed again with TBST and incubated with Strep-ABComplex-HRP. Colour reaction was developed in Tris-buffered saline pH 7.6 (TBS) containing diaminobenzidine (50 mg/100 mlz), 0.01 M imidazole and 0.3% H_2O_2. For negative control, the primary antibody was omitted.

PRL binding assay

Porcine PRL (2.5 μg) was labelled with 1 mCi [^{125}I] by a modified method of Greenwood *et al.* [29] using 2 μg chloramine T (1 μg added at the beginning of the reaction, and 1 μg added after 2 min.). Reaction was stopped after 4 min. Unreacted [^{125}I] was separated by gel filtration on a column of Sephadex G-50. The labelled porcine PRL was further purified on a Sephadex G-100 column equilibrated with 0.01 M PBS buffer, pH 7.5, containing 0.1% BSA. Labelled PRL was used at 50,000–200,000 cpm per tube.

Theca interna tissue from small, medium and large follicles was carefully separated from Gc and fragments of ECL were excised on ice and then frozen in -70°C until assay.

Homogenization of tissue was performed in incubation buffer 25 mM Tris-HCl, pH 7.4, 10 mM $MgCl_2$, 0.1% BSA. Homogenates obtained from 2 mg of tissue (which is equivalent to 100 μg protein) were incubated with [^{125}I] PRL (porcine) in a total volume of 0.4 ml. Protein concentration of the homogenate was measured by Bradford method using Bio-Rad protein assay reagent [30].

Incubation was carried out at 23°C for 12 hours. Incubation suspension was then applied to glass microfiber filters GF/A (Whatman, Clifton, NJ, USA) presoaked in 2% bovine serum albumin, retained in a multiplace holder, and was filtered to

separate bound [^{125}I] PRL from free ligand. Filters were washed 5 times with 2 ml of cold incubation buffer. Radioactivity of the dried pellet was counted in a γ-counter [31]. Specific binding was defined as the difference between total and nonspecific (in the presence of excess of unlabeled porcine PRL, 2 μg) binding. Scatchard analysis was performed as it described elsewhere [32].

Results

Steroid secretion

Theca cells in control cultures secreted small amounts of P_4 (0.47 ± 0.02 ng/10^5 cells); LH stimulated P_4 production up to 1.2 ± 0.05 ng/10^5 cells ($p. < 0.01$; 2.5-fold). The most significant stimulation, almost 10-fold, was observed under the influence of PRL (4.25 ± 0.5 ng/10^5 cells, $p < 0.001$) (Fig. 1).

ECL cells secreted a much higher amount of P_4, 1171 ± 12 ng/10^5 cells in control cultures, while LH stimulated secretion of this steroid up to 1390 ± 20 ng/10^5 cells ($p < 0.01$; 1.1-fold). In the presence of PRL, the increase of P_4 secretion was twofold vs control (2028 ± 40 ng/10^5 cells, $p < 0.001$) (Fig. 1).

The E_2 secretion from Tc control cultures was 0.58 ± 0.02 ng/10^5. LH stimulated markedly E_2 secretion from Tc cultures up to 1.58 ± 0.04 ng ($p < 0.001$). PRL stimulated E_2 secretion almost as strongly as LH only by cultures of theca (1.42 ± 0.06 ng/10^5 cells, $p < 0.001$) (Fig. 2).

E_2 secretion from luteal cells of ECL was not stimulated; on the contrary, it was significantly suppressed by either hormone used (Fig. 2).

Immunohistochemical localization of PRL-R

Analysis of cross-sections of whole ovarian follicles demonstrated PRL-R immunoreactivity in Gc, Tc and in ECL cells. Follicles of different sizes showed similar pattern of immunoreactivity. Immunostaining was localized on the cell membrane and in the cytoplasm. No nuclear staining was observed. Some but not all cells, showed PRL-R immunoreactivity.

In small antral follicles PRL-R was localized mainly in cells of granulosa layer, particularly in the upper part of the mural area (Fig. 3A). Negative reaction was observed in Tc. In medium and large follicles PRL-R was seen in Tc (Fig. 3B, 3C), but it expressed a much weaker reaction than that observed in Gc. In ECL cells as well as in Tc of both medium and large follicles, immunostaining for PRL-R was clearly visible (Fig. 3D).

In the control, omission of the primary antibody resulted in a lack of staining.

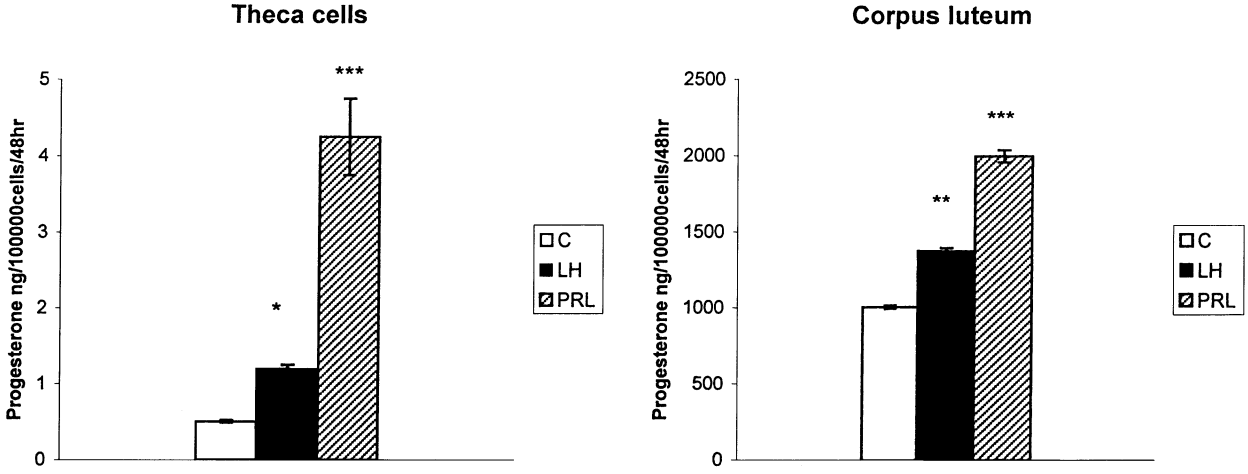

Fig. 1. Progesterone secretion (ng per 1×10^5 cells) from theca cells and early corpus luteum cells under the influence of LH (100 ng/ml) and PRL (100 ng/ml) during 48 hr culture. Each bar represents the mean $\pm$ SEM of 12 observations from 3 independent experiments (n=3). * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. The differences between progesterone concentrations in control and treated cultures were evaluated by ANOVA test.

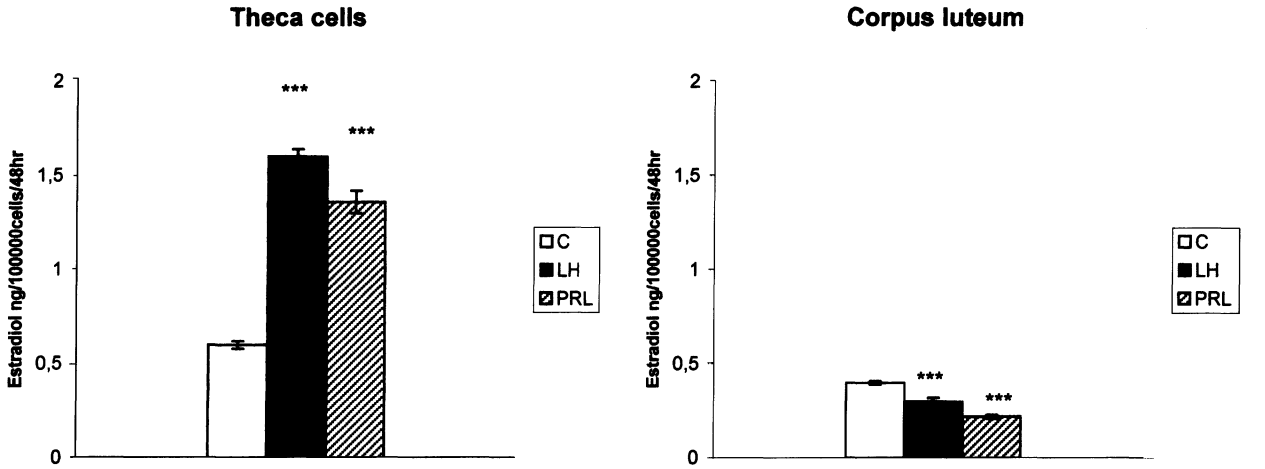

Fig. 2. The effect of LH (100 ng/ml) and PRL (100 ng/ml) on estradiol-17β secretion (ng per 1×10^5 cells) from theca cells and early corpora lutea cells during 48 hr culture. Each bar represents the mean $\pm$ SEM of 12 observations from 3 independent experiments (n=3). * $p<0.05$; ** $p<0.01$; *** $p<0.001$. The differences between estradiol concentrations in control and treated cultures were evaluated by ANOVA test.

Identification of PRL-R by binding assays

The amount of PRL-R protein (B_{max}) in Tc of small, medium and large follicles and in ECL tissue was calculated by Scatchard plot (Fig. 4A, 4B, 4C), and results are summarised in Table 1. In accordance with the result of immunohistochemical analysis (Fig. 3A), PRL binding was negative in Tc of small antral follicles. First appearance of PRL binding was seen in Tc of medium follicles (B_{max} $=0.97\pm0.052$, n=5), in Tc of large follicle (1.573 ±0.45, n=7) and in ECL cells (1.7 ± 0.14, N=8) which contained the largest number of PRL-R.

Discussion

PRL binding to ovaries was reported in rat [17, 33], hamster [16] mouse [19] and human [20]. Magoffin and Erickson [18, 34] demonstrated specific high affinity PRL-Rs in cultured interstitial cells of the rat ovary. On the other hand, data on PRL binding to porcine ovaries are scarce. Rolland and Hammond [35] first showed PRL binding to pig ovaries and Veldhuis *et al.* [15, 36] detected PRL binding to cultured Gc and indicated its dependency on cytodifferentiation.

No information is available on PRL binding with pig follicular theca interna cells as well as by cells of ECL in which, during a short postovulatory phase of its formation, the thecal component is quite substantial [37]. In the present study, cultured porcine Tc exhibited distinct sensitivity to exogenous PRL as measured by P_4 and E_2 secretion to culture medium [4, 5]. The amount of secreted steroids was comparable to that secreted in response to LH (Fig. 1). It is well known that Tc are equipped with LH receptors that are involved in stimulation of P_4 and androgen synthesis in majority of mammalian species. However, porcine Tc, unlike Tc of the other species, have certain aromatizing ability. In culture, they secreted significant amount of E_2, which was markedly stimulated by testosterone [6, 7]. Results of the present experiment showed that PRL acts directly on Tc and ECL cells. The recent publication by Ciereszko *et al.* [13] showed that PRL action on ovarian cells is mediated by LDL. These lipoproteins were apparently present in the serum supplement of the medium in which cells in this experiment were grown. However, this finding and earlier reports by Rajkumar *et al.* [11, 12] do not exclude the role of PRL-R in the mechanism underlying Tc and ECL functions in the porcine ovary. The present preliminary study using Scatchard analysis revealed for the first time in the pig, femtomol levels of PRL-R protein in Tc of medium and large follicles. PRL-R was also detected in ECL tissue (Table 1). These results were strengthened by results of im-

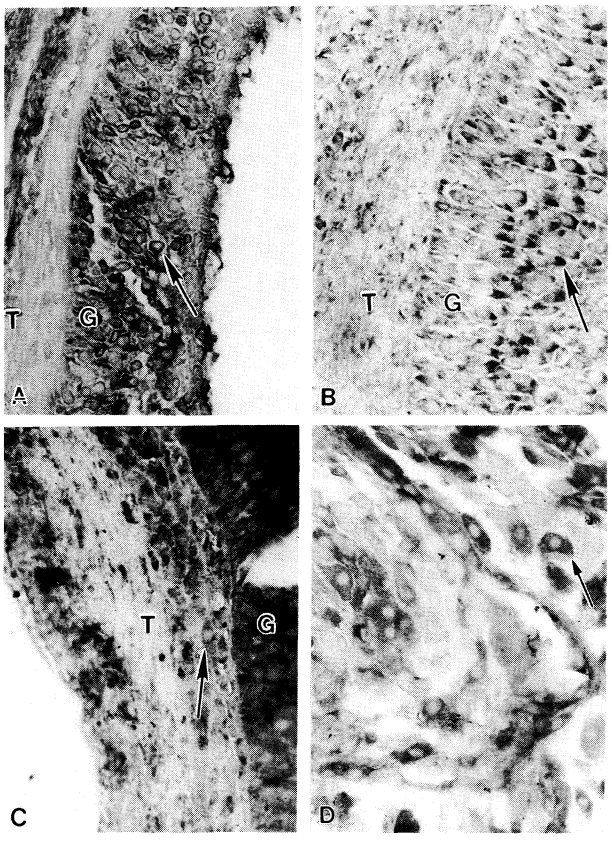

Fig. 3. Immunohistochemical localization of prolactin receptor in the porcine follicles and corpus luteum. A. small antral follicle, with PRL-R present in granulosa layer (arrow); B. medium follicle, with PRL-R localized in both granulosa cells (arrow) and theca layer; C. large follicle, with immunopositive staining in granulosa and theca (arrow); D. early corpus luteum, with prolactin receptor present in several cells (arrow). Magnification × 200.

munohistochemical detection of PRL-R in porcine ovary. Similarly as in the quantitative assay, Tc of small antral follicles was not immunopositive (Fig. 3A). As there are some immunogenic domains of the receptor conserved between species, antibody to the rat liver PRL-R, U5, could be used in the experiments carried on the porcine ovary [38]. Antibody U5 is very useful in characterization of the structure of the PRL-R in various stages of receptor processing. In partially purified receptors from pig mammary gland, in the immunoblot analysis, bands of 66,000 and 36,000 mol wt were seen [38]. Using slices of ECL tissue it was difficult to discriminate which cell type was the bearer of PRL-R. Gregoraszczuk [8, 39], and Gregoraszczuk and Krzysztofowicz [40] showed that the large luteal cells of ECL responded to PRL action by increase in P_4 secretion. These results allowed us to assume that large luteal cells are equipped with PRL-R. PRL binding experiments should be performed in the future using separated luteal cell types. Taking into consideration, significant increase in steroidogenesis in cultured Tc and luteal cells, one would predict the presence of higher receptor number. However, the possibility can not be excluded, that PRL-R, similar to receptors for the other gonadotropins and those of steroid hormones, undergo a time dependent dynamics of up- and down-regulations, which are rather difficult to target during the much longer pig estrous cycle [41–43] than rat estrous cycle. PRL-R gene expression was visualized in various tissues by *in situ* hybridization. The levels and localization of PRL-R mRNA in the rat ovary changes throughout the reproductive cycle. In the rat Tc the short form was present during early proestrus, while in diestrus the long form of PRL-R mRNA appeared to be expressed in the Tc region [44]. On the contrary, using the same technique, no differences in the abundance of PRL-R mRNA were observed between follicles of different sizes, healthy vs. atretic or stage of the estrous cycle or pregnancy, as well as between CL from pregnant and nonpregnant deer [45]. In all follicles, PRL-R mRNA was localized to the Tc layer. These results suggest a direct role of PRL in red deer ovarian physiology [45]. However this animal is not a proper sample, because it is a seasonal, short day breeder. The timing of hormonal events is incomparable with animals dependent on long day breeding.

Apart from receptor mediated action, the likelihood of an additional paracrine mechanism of PRL action within porcine ovary was suggested by Einspanier [46]. The author found detectable concentrations of PRL mRNA in pig Gc and luteal cells, and also described the capacity of these cells to produce PRL. Gonadotropins may act on ovarian cells before they develop appropriate receptors [47]. According to the authors [47], FSH affected ovarian androgen synthesis via a paracrine fashion involving increased expression of rat Tc P450-17α enzyme. Since Tc do not express FSH receptors, the authors suggested involvement of some paracrine proteins (possibly inhibin or IGF-I) produced by Gc in

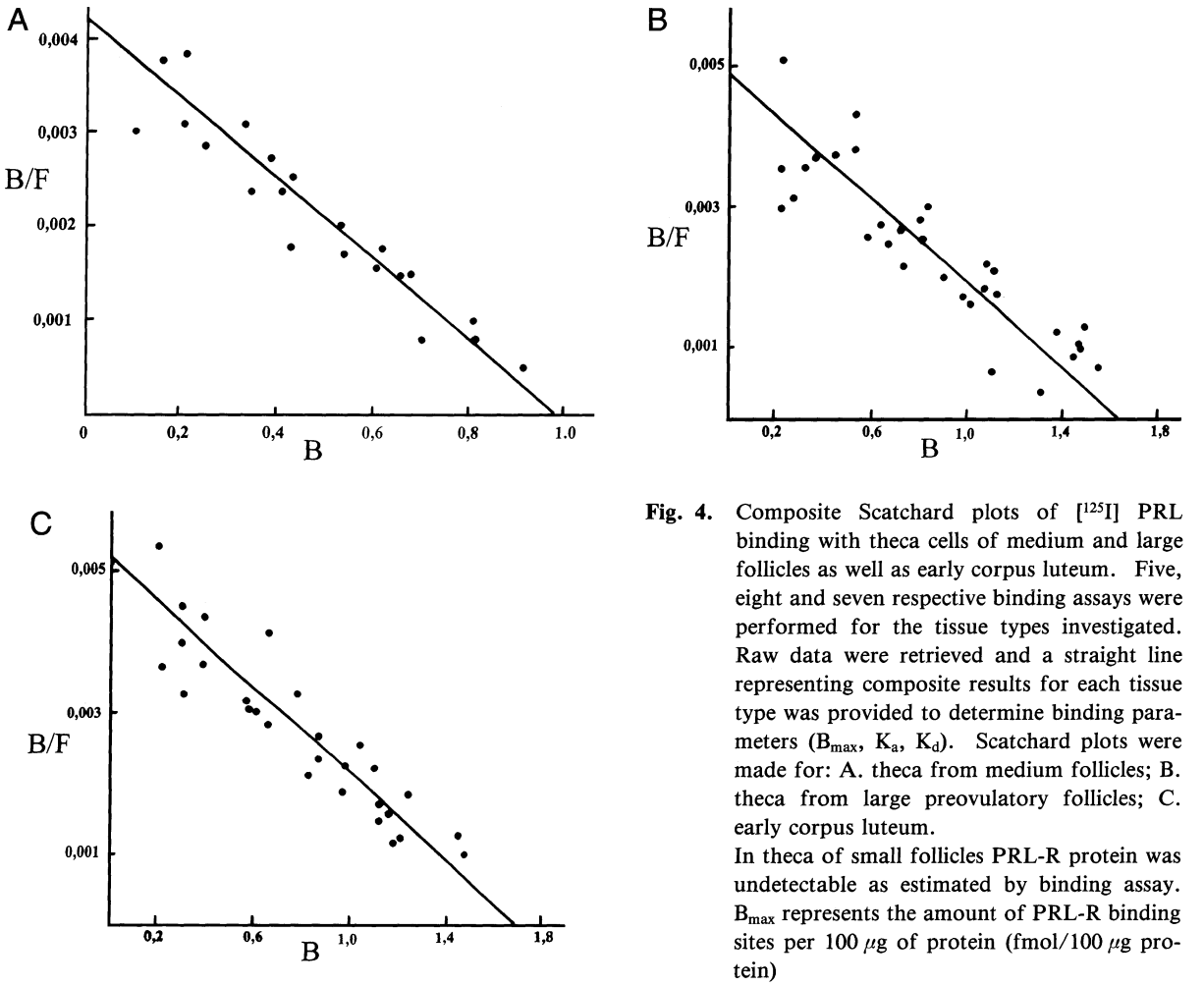

Fig. 4. Composite Scatchard plots of [^{125}I] PRL binding with theca cells of medium and large follicles as well as early corpus luteum. Five, eight and seven respective binding assays were performed for the tissue types investigated. Raw data were retrieved and a straight line representing composite results for each tissue type was provided to determine binding parameters (B_{max}, K_a, K_d). Scatchard plots were made for: A. theca from medium follicles; B. theca from large preovulatory follicles; C. early corpus luteum.
In theca of small follicles PRL-R protein was undetectable as estimated by binding assay. B_{max} represents the amount of PRL-R binding sites per 100 μg of protein (fmol/100 μg protein)

Table 1. Summary of PRL-R binding assay in theca cells (Tc) of small, medium and large follicles as well as in early corpus luteum cells (ECL).

Cell type	$B/F_{(0)}$	B_{max}	K_a	K_d
Tc-small follicle	ND	ND	ND	ND
Tc-medium follicle	0.00419 ± 0.00027	0.97 ± 0.052	$2.15 \pm 0.22 \times 10^{12}$ M.$^{-1}$	$4.46 \pm 0.56 \times 10^{-13}$ M
Tc-large follicle	0.004985 ± 0.000646	0.573 ± 0.45	$1.489 \pm 0.29 \times 10^{12}$ M.$^{-1}$	$6.97 \pm 1.32 \times 10^{-13}$ M
ECL	0.0052 ± 0.00063	1.7 ± 0.14	$1.53 \pm 0.23 \times 10^{12}$ M.$^{-1}$	$6.54 \pm 1.29 \times 10^{-13}$ M

Data are presented as mean$\pm$SD; Tc small follicle (n=5); Tc medium follicle (n=5); Tc large follicle (n=8); ECL (n=7).
B/F, ligand ratio while binding is 0; B_{max}, maximum binding (fmol/100 μg protein); K_a, affinity constance; K_d, dissociation constance, ND, undetectable

response to FSH. Magoffin and Magarelli [48] and Magarelli *et al.* [49] demonstrated that developing preantral follicles secrete paracrine substances, proteinaceous in nature, possesing androgen stimulating activity. These, termed theca differentiating factors (TDF), might be involved in initiating a genetic program resulting in an expression of LH receptor and steroidogenic enzymes specific for Tc. This interesting interpretation indicates an indirect way by which FSH may stimulate not only aromatase activity in Tc

but also androgen substrate production.

Recently, the role of testicular macrophages in stimulating steroidogenesis of Leydig cells (LC) was suggested by Hutson [50]. It appeared that macrophages residing in the testis are equipped with FSH receptors. The digitations connecting neighbouring LC and macrophages were observed. The author postulated that FSH bound with macrophages but not with LC, which is lacking in FSH receptor, stimulates synthesis and secretion of various peptides and cytokines which in a paracrine way may enhance steroidogenesis in LC. It has been shown that macrophages reside in pig ovaries [51, 52] and their number varies in different stages of estrous cycle. Gc in coculture with macrophages secreted more P_4 than cultured alone [53]. Interestingly, Standaert *et al.* [51] found that conditioned medium from splenocytes as well as monocytes and lymphocytes influenced steroid secretion by pig granulosa cells. Similarly, in co-cultures splenic macrophages enhanced stimulatory action of PRL on P_4 secretion by Gc [54]. Considering homology between testicular and ovarian cells, it is reasonable to suggest that ovarian macrophages could also bear receptors of FSH and its binding could trigger a paracrine mechanism in cells which are not the direct target for this hormone. In this experiment immunostained slices of Tc revealed that not all Tc showed positive staining for PRL-R. However, this preparation made it impossible to prove whether PRL-R antibody was bound with Tc or to macrophages or to another cell type present in this complex tissue. On the basis of the concepts presented above, one may speculate that in addition to the classical signal transduction via adequate membrane receptors, PRL could also act on Tc by a similar but as yet unknown mechanism. This interesting idea should be clarified in further investigations using separated ovarian cell types.

In conclusion Tc of porcine medium and large follicles as well as ECL respond to PRL in terms of increased steroid secretion. This action is mediated by PRL-R as detected in both tissues by binding assay and immunohistochemical technique.

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