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Characterization of Somatotropin- and Prolactin-Binding Sites on Bovine Granulosa Cells Using Homologous Hormones

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Abstract—Specific binding of bovine somatotropin (BST) and bovine prolactin (BPRL) to cow granulosa cells from antrum-containing follicles of different diameter was studied. Scatchard analysis of the data revealed a single type of low affinity BST-binding sites on the granulosa cells with dissociation constants similar to those for the BPRL-binding sites. The number of BST-binding sites on the cells decreased with increasing follicle diameter from 3-5 to 6-10 mm. However, the binding capacity to BPRL decreased only in the case of cells from follicles 11-20 mm in diameter. The findings are discussed in relation to the "homologous binding" phenomenon.

Key words: somatotropin, prolactin, binding sites, granulosa cells, follicle

Follicle maturation in mammalian ovaries is a complex multistage process characterized by two main phases [1, 2]. The first phase, or basal folliculogenesis, involves the initial stages of follicle growth that is very slow and is caused by proliferation of the granulosa cells. The second phase, or tonic folliculogenesis, starts either during or after the antrum formation and is characterized by faster growth of follicles that is mainly caused by the increase in their antral cavity. After the entrance of follicles into this terminal developmental stage, the proliferative activity of the follicular cells gradually decreases and their differentiation starts, which is associated with changes not only in the cell secretory activity but also in their sensitivity to different hormones and growth factors [3, 4]. Tonic folliculogenesis is usually thought to be mainly regulated by two gonadotropic hormones, follicle-stimulating and luteinizing. However, two other pituitary hormones, prolactin (PRL) and somatotropin (ST), were recently shown also to be involved in the control of ovarian follicle maturation during the terminal phase of their development [5-8]. The immediate effect of these hormones on follicles was confirmed by findings of receptors for PRL and ST in follicular cells of various mammalian species [9-14]. Receptors for these hormones or for their mRNA were also found in cows in different ovarian structures including granulosa cells [15-18].

The binding characteristics of receptors are known to change depending on the cell differentiation stage [19]. The data available are insufficient to characterize the effect of the granulosa cell differentiation on the ability of these cells to bind ST and PRL during tonic folliculogenesis. Therefore, the purpose of the present work was to compare parameters of the interaction of bovine ST (BST) and PRL (BPRL) labeled with ^{125}I with the binding sites on granulosa cells from antrum-containing cow follicles of different diameter. Earlier we found *in vitro* the stimulating effect of physiological concentrations of BPRL and BST on DNA synthesis in these cells, and this suggests an immediate regulatory effect of these homologous hormones on cow granulosa cells [20, 21]. Moreover, we have determined characteristics of the specific binding of BPRL to granulosa cells from follicles of 1-2 to 6-10 mm in diameter [22]. However, cells from the follicles with diameter above 10 mm, which are the most differentiated and, consequently, the most interesting, were not used in those studies. Based on the classification proposed by Savio et al. [23], antrum-containing follicles were combined into groups according to their diameter: 1-5 (small), 6-10 (medium), and 11-20 mm (large). The small follicles were subdivided into two subgroups with diameters of 1-2 and of 3-5 mm because the growth of the smaller ones did not depend (at least partially) on gonadotropic hormones [1, 2].

Abbreviations: PRL) prolactin; BPRL) bovine prolactin; ST) somatotropin; BST) bovine somatotropin.

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MATERIALS AND METHODS

Granulosa cells from the antrum-containing follicles of ovaries of cows and of pubertal heifers of black particolored breed were used. Ovaries without detectable disorders obtained from a meat packing plant were used that were at the phase of follicle growth and of yellow body resorption. The granulosa cells were obtained by aspiration of contents of the follicles with diameters of 1-2, 3-5, 6-10, and 11-20 mm followed by centrifugation for 10 min at 250*g*. A fraction of the cells with picnotic nuclei was determined in the samples by the method described in [24]. For the further experiments, suspensions of granulosa cells with no more than 30% picnosis were used. After removal of the supernatant fluid, the cells were washed twice with resuspension in 10 mM Tris-HCl buffer (pH 7.1) supplemented with BSA (1 mg/ml) and Merthiolate (0.2 mg/ml), frozen and stored at –20°C. Before the freezing, the final cell concentration was calculated with a hemocytometer.

The binding of ^{125}I-labeled BST and BPRL to the granulosa cells was studied by a conventional method based on the labeled hormone complexing with the receptor [25]. The following reagents were used: pituitary BPRL preparation (Institute of Endocrinology, Moscow, Russia); recombinant BST (Monsanto, USA); Tris (Reanal, Hungary); Na^{125}I (Izotop, Russia); BSA, Merthiolate, and $MgCl_2$ were from Sigma (USA). The hormones were radio-iodinated using a modification of the chloramine method [26]. The specific activity was 0.6-1.2 MBq/μg BST and BPRL.

After thawing at room temperature, the cells were resuspended in the initial buffer and centrifuged for 30 min at 2000*g* (4°C); then the supernatant was removed and fresh buffer of composition varied depending on the experiment conditions was added. Tris-HCl buffer (10 mM, pH 7.1) supplemented with BSA (1 mg/ml), $MgCl_2$ (5 mM), and Merthiolate (0.2 mg/ml) was used as the main buffer. The same buffer supplemented with BSA (5 mg/ml) was also used.

The reaction mixture included 100 μl of the cell suspension (0.2-3.0)·10^6 cells, 50 μl of the incubation buffer, and 50 μl of ^{125}I-labeled BST (5-7 ng, 350,000-600,000 cpm) or BPRL (5-7 ng, 500,000-700,000 cpm). The protein concentration in the cell suspension was determined by the Lowry method [27]. The nonspecific binding of the labeled hormones was determined in the presence of excess unlabeled hormones at concentration 250 μg/ml. The reaction mixture was incubated for 42 h at 37°C in an air thermostat. To determine the dependence of the labeled hormone binding on time and temperature, the incubation was performed for 2, 3, 4, 5, 6, 16, 24, and 42 h at 37°C and for 24 h at 22°C. The reaction was stopped by addition of 1 ml of cold (0°C) incubation buffer. The sample radioactivity was measured and the data were primarily processed using a Compu-Gamma counter (LKB, Sweden). The specific binding was determined as the difference between the total and nonspecific binding.

To evaluate the degradation of the labeled hormones, the radioactivity of the reaction mixture was determined after 42 h, and 1 ml of 10% TCA was added. The samples were incubated for 30 min at 0°C and centrifuged for 30 min at 2000*g* (4°C). After removal of the supernatant, the radioactivity of the precipitate was measured. The unproteolyzed fraction of the hormone was determined as the ratio of the precipitate radioactivity to the radioactivity of the samples before the TCA addition expressed in percent.

Replacement curves were obtained by incubation of the cell suspension ((1-2)·10^6 cells) with the labeled hormones (at fixed concentration) in the presence of increasing concentrations of the corresponding unlabeled hormones (2.5-50 μg/ml). The dissociation constants and concentrations of the binding sites on the cells were determined by processing the replacement data by the method of Scatchard [28]. In these experiments, 5-12% of the added ^{125}I-labeled BST and 9-20% of the added ^{125}I-labeled BPRL were specifically bound to the granulosa cells.

Every cell specimen was analyzed in three parallel samples. All experiments were repeated 3-4 times. The results were processed using single-factor analysis of variance. The reliability of the difference between the average values compared was assessed using the Sheffe test [29].

RESULTS

To characterize the conditions for analysis of the labeled hormone binding to granulosa cells, some experiments were previously performed on cells from antrum-containing follicles 1-10 mm in diameter. The specific binding of ^{125}I-labeled BST and BPRL depended on the time and temperature of the hormone incubation with the cells and was close to saturation after 42 h at 37°C. Decreasing the temperature to 22°C resulted in very low levels of specific binding that made it impossible to use the Scatchard method. A linear dependence was observed between the specific binding of the ^{125}I-labeled BST and the number of granulosa cells in the range of all concentrations studied (from 0.2·10^6 to 3·10^6 cells per sample). For ^{125}I-labeled BPRL this linearity was observed on addition of 0.5·10^6 to 3·10^6 cells per sample. No significant difference was found between the specific binding of the hormones to the frozen (–20°C) and to the unfrozen granulosa cells (11.9 ± 1.8 compared to 12.1 ± 1.9% for ^{125}I-labeled BST and 9.1 ± 0.8 compared to 10.1 ± 0.9% for ^{125}I-labeled BPRL), this being consistent with the literature [30, 31]. Increasing the BSA concentration in the incubation buffer to 5 mg/ml had no effect on the binding of the labeled hormones to granulosa cells.

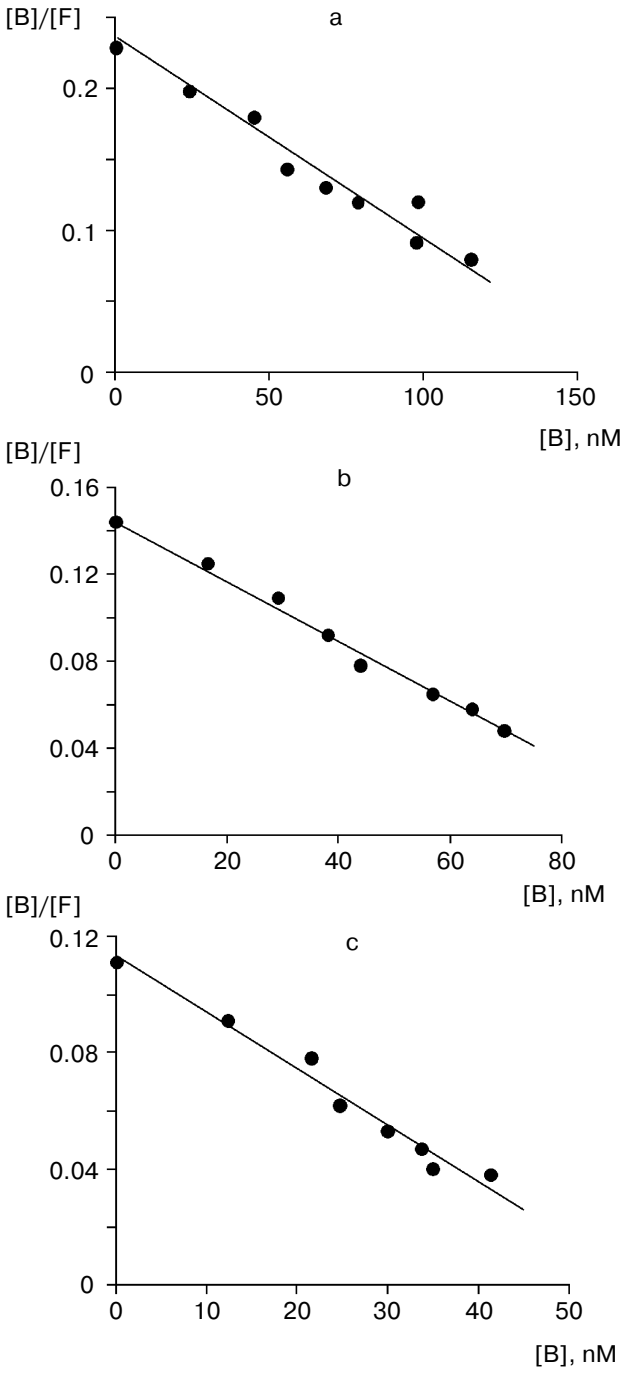

Fig. 1. Scatchard plots for BST specific binding to cow granulosa cells from antrum-containing follicles of 3-5 (a), 6-10 (b), and 11-20 mm (c) diameter. Abscissa, concentration of the hormone specifically bound to the cells ([B]); ordinate, ratio of the concentration of the specifically bound hormone to the concentration of the free hormone ([B]/[F]). The granulosa cells ($2 \cdot 10^6$) were incubated for 42 h at 37°C in 200 µl of 10 mM Tris-HCl buffer (pH 7.1) containing BSA (1 mg/ml), $MgCl_2$ (5 mM), and Merthiolate (0.2 mg/ml) in the presence of ^{125}I-labeled BST (7 ng) and of increasing concentrations of the unlabeled BST (1-10 µg). The points are mean values for three parallel samples of the cells in the same experiment. In other experiments, similar results were obtained (Table 1).

Degradation of the labeled hormones was studied, and after 42 h of the incubation with the granulosa cells proteolysis was found in 17-22% of the ^{125}I-labeled BST and 10-11% of the ^{125}I-labeled BPRL, this being acceptable for analysis of the binding.

Figure 1 (a-c) presents findings on the competitive binding of the labeled and unlabeled BST (the replacement curve) in Scatchard's coordinates for granulosa cells from follicles of different diameter. The specific binding of the hormone to the cells in Scatchard's coordinates in all cases was described by a straight line that suggested the existence of only one type of BST-binding sites on the granulosa cells with various differentiation degree.

Parameters of specific binding of BST to granulosa cells were obtained by analysis of the replacement curves by the Scatchard method (Table 1). The dissociation constants (K_d) of BST from the complex with the binding sites were not significantly different, although the binding affinity for the cells from follicles of 11-20 mm in diameter was insignificantly increased. However, the number of BST-binding sites was significantly less on the cells from follicles of 6-10 and 11-20 mm diameter than from follicles of 1-2 and 3-5 mm diameter. Moreover, high values of the dissociation constants suggest the low affinity of the binding sites for BST during all maturation stages of the antrum-containing follicles of cows.

We found earlier that parameters of the ^{125}I-labeled BPRL specific binding to cow granulosa cells from follicles of 1-2, 3-5, and 6-10 mm diameter were not different [22]. Therefore, for characterization of BPRL binding to granulosa cells only follicles of 3-5 and 11-20 mm in diameter were used. The Scatchard analysis of the labeled hormone replacement by the unlabeled hormone revealed rectilinear dependence of the BPRL specific binding to

Table 1. Parameters of specific binding of ^{125}I-labeled BST to granulosa cells depending on the diameter of the antrum-containing follicles of cows

Follicle diameter, mm	Number of experiments	Dissociation constant (K_d) × 10^7, M	Number of binding sites per cell × 10^{-6}
1-2	3	9.18 ± 1.24	12.44 ± 0.79[a]
3-5	3	8.65 ± 0.87	11.16 ± 1.19[a]
6-10	3	8.47 ± 0.98	6.60 ± 0.71[b]
11-20	3	6.82 ± 1.23	4.32 ± 0.19[c]

Note: Mean values ± standard errors are given, the conditions of binding analysis are the same as in the caption for Fig. 1. The significance of the mean values under comparison: [a,b] $p < 0.01$; [c] $p < 0.001$.

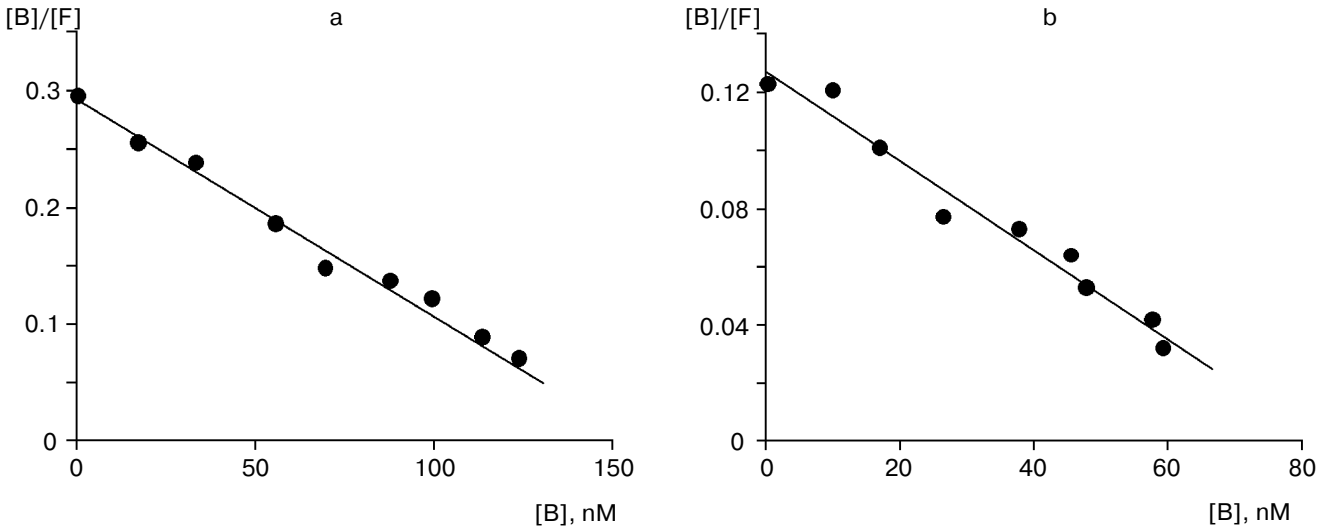

Fig. 2. Scatchard's plots for the BPRL specific binding to cow granulosa cells from antrum-containing follicles of 3-5 (a) and 11-20 mm (b) diameter. The designations are the same as in Fig. 1. The granulosa cells ($2 \cdot 10^6$ cells per sample) were incubated for 42 h at 37°C in 200 µl of 10 mM Tris-HCl buffer (pH 7.1) containing BSA (1 mg/ml), $MgCl_2$ (5 mM), and Merthiolate (0.2 mg/ml) in the presence of ^{125}I-labeled BPRL (7.1 ng) and of increasing concentrations of the unlabeled BPRL (0.5-10 µg). The points are mean values for three parallel samples of the cells in the same experiment. Similar results were obtained in other experiments (Table 2).

cells from the small and large follicles (Fig. 2, a and b), and this suggests the existence of a single class of the BPRL-binding sites during both the early and late stages of follicle development. The number of the BPRL-binding sites on the cells from the follicles of 11-20 mm in diameter was significantly lower than on the cells from the follicles of 3-5 mm in diameter (Table 2). The dissociation constants were not significantly different and indicated that the binding site affinity for BPRL was low. However, the affinity of the BPRL specific binding to granulosa cells from follicles of 3-5 mm diameter was significantly higher ($K_d = 5.13 \cdot 10^{-7}$ M) than that in the case of BST ($K_d = 8.65 \cdot 10^{-7}$ M, $p < 0.05$), although these K_d were not different in the large follicles.

Table 2. Parameters of specific binding of ^{125}I-labeled BPRL to granulosa cells depending on the diameter of the antrum-containing follicles of cows

Follicle diameter, mm	Number of experiments	Dissociation constant (K_d) × 10^7, M	Number of binding sites per cell × 10^{-6}
3-5	3	5.13 ± 0.33	9.64 ± 0.55[a]
11-20	3	5.61 ± 0.29	5.42 ± 0.73[b]

Note: Mean values ± standard errors are given, the conditions of binding analysis are the same as in the caption for Fig. 2. The significance of the mean values under comparison: [a,b] $p < 0.01$.

It should be noted that the BST- and BPRL-binding sites were characterized for cells that mainly represented two subpopulations of granulosa, namely, the cumulus cells surrounding the oocyte and the granulosa adjacent to the antral cavity of the follicle.

DISCUSSION

The decrease in the number of BST-binding sites found on granulosa cells from the medium size and large follicles of cows compared to the cells from the small follicles is, in general, consistent with changes in the ability of granulosa cells for binding BST found during folliculogenesis in sows [10]. Moreover, the decrease in the concentration of the BST-binding sites on cells from the follicles of 11-20 mm diameter correlates with a similar decrease in the PRL-binding sites in sheep and in the large follicles of swine [11]. Thus, notwithstanding the significant difference in the mechanisms regulating ovarian folliculogenesis in cows and sows [1], the ability of granulosa cells for binding PRL or ST changes similarly during the growth of the antrum-containing follicles.

The binding characteristics of the BST- and BPRL-binding sites on the cow granulosa cells were rather similar. And high values of the dissociation constant (about 10^{-7} M) suggested very low affinity of the binding sites for BST and BPRL. It is suggested that these results should be explained by the "homologous binding" phenomenon that was found by some researchers when hormones and

tissues of the same mammalian species were used for radioreceptor analysis. Thus, lactogen receptors of swine endometrium had $K_a = 3.26 \cdot 10^7$ M^{-1} for swine PRL [32]. The K_d of the binding of membrane preparations of cow milk gland to BPRL was $9 \cdot 10^{-8}$ M, and the binding capacity and K_d were 100 times higher than when using human growth hormone [33]. The concentration of unlabeled swine ST which corresponded to the half maximal inhibition ($[IC]_{50}$) of the ^{125}I-labeled swine ST to membranes of swine fat tissue was more than tenfold higher than $[IC]_{50}$ for the BST binding and was 10^{-8} M [34].

The cellular affinities for homologous and non-homologous PRL were compared to biological activities of these hormones determined by their effects on the same cells, and unexpected results were obtained. Although the receptor affinity of the swine milk gland for the sheep PRL was 200 times higher than to the swine PRL, the *in vitro* effect of the latter on the rate of lipogenesis and glucose oxidation in the swine milk gland explants was even higher than the effect of the sheep PRL [35]. The K_d for the rabbit PRL binding to membranes of the rabbit milk gland was $6.2 \cdot 10^{-8}$ M, that is, 300 times higher than the K_d for the binding of sheep PRL determined in the same experiments. However, both hormones nearly equally stimulated the synthesis of β-casein in the rabbit milk gland explants [36]. Based on these findings, the authors concluded that the low affinity of receptors for the homologous PRL was not responsible for the decrease in its regulatory effect on the cells compared to the non-homologous hormone. We showed earlier that BPRL and BST stimulated *in vitro* synthesis of DNA in granulosa cells from cow follicles 3-5 mm in diameter, and the maximum mitogenic effect was found at the BST concentration of 10 ng/ml ($4.4 \cdot 10^{-10}$ M) and at 50 ng/ml BPRL ($2.2 \cdot 10^{-9}$ M) [20, 37].

Thus, our findings on the BST and BPRL binding to cow granulosa cells are, in general, consistent with the "homologous binding" phenomenon. Data on the interaction parameters (especially on the binding capacity) for the homologous ST and PRL with the receptors are very scarce in the available literature because most of the researchers used non-homologous hormones in their radioreceptor studies. But Kazmer et al. [33], who used for binding analysis homologous and non-homologous lactogenic hormones, found similar changes in the concentration of lactogen receptors of the cow milk gland depending on the differentiation stage, notwithstanding the 100-fold difference in the absolute values of this parameter. Therefore, it is suggested that, independently of the species specificity of the hormone used, changes in its binding capacity to the cells during their differentiation should be the most informative. Thus, based on the findings presented, it is concluded that the granulosa cell competence in cows for ST binding is higher during the early stages of tonic folliculogenesis, while the PRL-binding ability of these cells decreases only during the terminal stage of follicle maturation.

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