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Effect of basal lamina on progesterone production by chicken granulosa cells in vitro — influence of follicular development

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Abstract

Experiments were conducted in vitro to study the regulation of progesterone production in chicken granulosa cells by homologous basal lamina isolated from preovulatory follicles of chicken ovary. The majority of components of the basal lamina (90–95% by weight) were solubilized with guanidine-HCl (and designated fraction 1); the remaining components were solubilized with β-mercaptoethanol containing guanidine-HCl (and designated fraction 2). The ability of fraction 1 to regulate progesterone production in granulosa cells obtained from the largest (F_1, mature), third largest (F_3, growing), fifth to seventh largest (F_{5-7}, growing) follicles and a pool of small yellow follicles (SYF, immature) of chicken ovary was assessed. Granulosa cells isolated from SYF follicles were in the least differentiated (undifferentiated) and those obtained from F_1 follicles were in the most differentiated state. The ability of fraction 1 to regulate progesterone production by chicken granulosa cells was influenced both by the state of cell differentiation and the form of the matrix material (whether solid or liquid). When fraction 1 was added as liquid to the incubation mixture, it promoted progesterone production by granulosa cells at all stages of differentiation; however, it caused a greater relative increase in the amount of progesterone produced by undifferentiated (SYF) and differentiating (F_3) granulosa cells than by differentiated (F_1) ones. In the presence of the liquid-form of fraction 1, luteinizing hormone (LH) stimulated progesterone production in differentiated (F_1) and differentiating (F_{5-7}) granulosa cells. Similarly, follicle-stimulating hormone (FSH) stimulated progesterone production by differentiating (F_3) and undifferentiated (SYF) granulosa cells in the presence of the liquid-form of fraction 1 protein. In culture wells that had been pre-coated with fraction 1 (solid-form), progesterone production by less differentiated (SYF, F_{5-7}) granulosa cells was enhanced, whereas progesterone production by differentiated (F_1) cells was reduced. The solid-form of fraction 1 augmented LH-stimulated progesterone production by less differentiated (F_{5-7}) granulosa cells however, it attenuated LH-induced progesterone production in differentiated (F_1) cells. FSH-promoted progesterone production in granulosa cells from immature follicles (SYF) was augmented by solid-form of fraction 1 whereas the effect of FSH on cells obtained from older follicle (F_3) was suppressed by solid-form of fraction 1. In experiments in which gonadotropin action was attenuated by solid-form of fraction 1, the amount of progesterone produced in the presence of maximally inhibiting concentrations of fraction 1 protein was greater than control values (no fraction 1, no gonadotropin). These results show that basal lamina of the ovarian follicle can regulate progesterone production by granulosa cells. The data demonstrate that the interactions between the components of basal lamina and LH or FSH on granulosa cell function were dependent on the stage of follicular development and were influenced by the form of the matrix material. It is concluded that the basal lamina of the chicken ovarian follicle is biologically active and regulates granulosa cell function.

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1. Introduction

Basement membranes are extracellular matrix sheets that compartmentalize tissues and act as physical barriers in separating different cell types such as endothelia, epithelia and muscle fibers (Yurchenco and Schittny, 1990). Basement membranes have three layers, lamina lucida (rara) and lamina densa produced by epithelial cells and lamina fibroreticularis produced by connective tissue (Kefalides et al., 1979; Laurie and Leblond, 1985; Inoue and Leblond, 1988). Collectively, the lamina lucida (rara) and lamina densa form the basal lamina (Laurie and Leblond, 1985). Lamina fibroreticularis is usually absent. In vivo, basement membranes regulate the structure and functions of cells with which they are associated or which they surround. Several investigators have provided evidence to support the notion that basement membrane proteins modulate granulosa cell function. For example, basement membrane deposited in vitro by bovine corneal endothelial cells was shown to modulate rat and human granulosa cell function (Furman et al., 1986a,b; Aharoni et al., 1996). In addition, basement membrane extracted from Engelbreth–Holm Swarm (EHS) tumor regulated progesterone synthesis in rat granulosa cells (Aten et al., 1995). Moreover, different types of extracellular matrix proteins such as collagen matrix have been shown to alter steroid hormone synthesis in rat and human granulosa cells (Ben-Rafael et al., 1988; Carnegie et al., 1988; Aharoni et al., 1996).

Although basal lamina/basement membrane proteins appear to modulate granulosa cell function, the direct effect of homologous basal lamina of ovarian follicle on granulosa cell function has not been studied. Therefore, the goal of the present study was to isolate basal lamina from the ovarian follicle and assess its effect on progesterone production by granulosa cells. An additional goal was to investigate the effects of combinations of pituitary-derived gonadotropins and basal lamina proteins on steroidogenesis in granulosa cells. A third goal of this study was to assess the influence of the state of granulosa cell differentiation on these processes. The avian follicle was used as a model system because its anatomical structure makes possible the isolation of pure and intact basal lamina. In the mature avian ovarian follicle, the granulosal layer (*membrana granulosa*) consists of a single layer of cells located between basal lamina and perivitelline layer (Wyburn et al., 1965; Perry et al., 1978; Bakst, 1979; Callebaut et al., 1991). This unique anatomical arrangement made possible the isolation of intact basal lamina in hypotonic solution.

2. Materials and methods

2.1. Chemicals

Human recombinant follicle-stimulating hormone (hrFSH; bioactivity of 1.7 μg hFSH-13/IU) and ovine luteinizing hormone (LH; NIDDK-oLH-26; 2.3 U/mg NIH-LH-S1) were obtained from the National Hormone and Pituitary Program, Ogden BioServices Corporation (Rockville, MD). *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES), collagenase type IV, soybean trypsin inhibitor, bovine serum albumin (BSA, Fraction V), penicillin G, streptomycin, fungizone, trizma-base, triton X-100, and Tween 20 were purchased from Sigma (St Louis, MO). Medium 199 (M199) containing Hank's salts was from Gibco-BRL (Grand Island, NY).

2.2. Animals

Single Comb White Leghorn hens obtained from Purdue University Poultry Research Farms (West Lafayette, IN) in their 1st year of reproductive activity, were caged individually in a windowless, air-conditioned room with a 14 h light: 10 h darkness cycle. They had free access to a layer ration and tap water. The bird colony was patrolled routinely and the time of egg lay of each bird was noted to the nearest 30 min. The animals were killed by cervical dislocation 10–12 h before the expected time of egg lay and subsequent ovulation of the largest preovulatory follicle (which occurs 15–45 min after egg lay). The first (30–35

mm in diameter), second (25–30 mm in diameter), third (17–20 mm in diameter), fifth to seventh (12–15 mm in diameter) largest (F_1, F_2, F_3 and F_{5-7}) preovulatory follicles and a pool of small yellow follicles (SYF; 6–8 mm in diameter) were removed and placed in ice-cold modified Hank's balanced salt solution containing NaCl, 140 mM; KCl, 5 mM; $MgCl_2$, 1.1 mM; $CaCl_2$, 1 mM; HEPES, 10 mM; Glucose, 5.6 mM, pH 7.4. The follicles were classified according to the criteria of Robinson and Etches (1986). The thecal and granulosal layers (*membrana granulosa*) were separated by the method described by Gilbert et al. (1977). The granulosa cells were dissociated in M199 containing $NaHCO_3$ (350 mg/l), HEPES (10 mM), pH 7.4, penicillin G (100 000 U/l), streptomycin (100 mg/l), fungizone (250 μg/l), collagenase (500 000 U/l) and trypsin inhibitor (200 mg/l) (Novero and Asem, 1993). Cell viability, determined by the trypan blue exclusion method, was routinely >95%.

2.3. Isolation of basal lamina

The granulosal layer (*membrana granulosa*) obtained from the first (F_1) or second (F_2) largest preovulatory follicle was placed in a hypotonic solution containing Tris–HCl, 10 mM (pH 7.4); leupeptin, 0.5 mg/l; EDTA-Na_2, 1 mM; pepstatin, 0.7 mg/l; and phenylmethylsulfonyl fluoride (PMSF), 0.2 mM in a petri dish. The granulosa cells, sandwiched between the basal lamina and perivitelline layer were lysed and the basal lamina and perivitelline layer were separated. The duration of time required for complete separation of the two layers (basal lamina and perivitelline layer) was dependent on the hypotonicity of the solution. The separation was much faster (1–3 min) in the absence of Tris–HCl than in Tris–HCl-containing solution (4–8 min). This basal lamina of avian ovarian follicle (BLAOF) preparation is intact, pure, and complete. Examination of the isolated basal lamina with scanning and transmission electron microscopy revealed that it is devoid of fragments of granulosa cell membranes and its ultrastructure is similar to that of basal lamina in the intact ovarian follicle (Asem et al., 2000).

2.4. Solubilization of basal lamina

2.4.1. Fraction 1

Basal laminae were placed in a microfuge tube, and solubilization buffer, containing 6 M guanidine-HCl, 50 mM Tris–HCl pH 7.4, was added (100 μl buffer per basal lamina per follicle). After shaking at 4°C overnight some membrane fragments remained. The mixture was centrifuged at 1000–2500 × *g* for 10 min. The supernatant designated fraction 1 was placed in a 3 kD cutoff dialysis membrane and dialyzed against 150 mM NaCl, 50 mM Tris–HCl pH 7.4 overnight at 4°C. After dialysis, fraction 1 turned cloudy, presumably due to the precipitation of some proteins. The dialyzed fraction 1 was aliquoted and stored at −70°C in the same buffer. Protein content of solubilized basal lamina was determined by the method of Bradford (1976) using BSA as standard. Fraction 1 contained 90–95% of total protein in the basal lamina.

2.4.2. Fraction 2

The basal lamina fragments collected by centrifugation were solubilized with 6 M guanidine-HCl, 50 mM Tris–HCl pH 7.4 containing β-mercaptoethanol (5 mM) with shaking for 60 min at 4°C and designated fraction 2. Similar results were obtained when 8 M urea was substituted for guanidine-HCl. The fraction 2 solution was placed in a 3 kD cutoff dialysis tube and dialyzed against 150 mM NaCl, 50 mM Tris–HCl pH 7.4 overnight at 4°C. The dialysate was aliquoted and stored at −70°C in the same buffer. The dialysate of fraction 2 did not turn cloudy. The exclusion of β-mercaptoethanol from the solubilization buffer led to incomplete solubilization of the basal lamina (fragments remained). Fraction 2 contained 5–10% of total protein in the basal lamina. The effect of fraction 2 on progesterone production in granulosa cells was not examined in the present study.

2.5. Preparation of solubilized basal lamina-containing dishes for cell culture

Fraction 1 of the solubilized basal lamina was diluted with deionized water, modified Hank's balanced salt solution, or M199. Aliquots of 100–200 μl containing 10–160 μg of proteins were transferred into 96-well Falcon culture dishes (Fisher Scientific, Springfield, NJ) and allowed to dry in a tissue culture hood. These are designated pre-coated wells (solid-form of solubilized basal lamina). Some wells received vehicle only and served as controls (see below). Culture wells that

received Hank's balanced salt solution or M199 were rinsed twice with deionized water prior to the incubation of cells. Tissue isolation, solubilization, dialysis and preparation of culture dishes were carried out under sterile conditions.

2.6. Incubation of granulosa cells

2.6.1. Incubation of cells in solubilized basal lamina-containing dishes

Collagenase dispersed chicken granulosa cells were plated at a density of $0.5–2 \times 10^5$ live cells/ml in 96-well Falcon tissue culture plates (Fisher Scientific) in which different amounts of fraction 1 of solubilized basal lamina had been dried (solid-form of solubilized basal lamina, 10–160 μg/cm^2). The cells were incubated at 37°C in serum free M199 containing 0.1% (wt/vol) BSA as described by Novero and Asem (1993). In other experiments, granulosa cells, $0.25–1 \times 10^5$ live cells/ml were placed in 96-well and different amounts of solubilized basal lamina were added (liquid-form of solubilized basal lamina, 50–500 μg/ml). The mixture was incubated at 37°C in serum free M199 containing 0.1% (wt/vol) BSA as described by Novero and Asem (1993). The final volume of the incubation mixture was 0.2 ml in all experiments. Control wells (no addition) were devoid of fraction 1 and gonadotropic hormones, i.e. luteinizing hormone (LH) or follicle-stimulating hormone (FSH).

2.7. Measurement of progesterone

The progesterone content of incubation media was determined with an IMMULITE progesterone kit (Diagnostic Products Corporation, Randolph, NJ) according to manufacturer's specifications.

2.8. Statistical analyses

Each experiment contained three replicate wells per treatment and was repeated three times unless otherwise noted. The data were analyzed by analysis of variance followed by post-hoc Tukey test to determine significant differences among treatment means. Student's t-test (two-tailed) was performed where applicable. Differences at $P \leq 0.05$ were considered significant.

3. Results

3.1. Effect of solubilized basal lamina on progesterone production in granulosa cells in vitro

Because basal lamina is in direct contact with granulosa cells in vivo, experiments were conducted to determine the effect of basal lamina on the function of granulosa cells. Progesterone synthesis was monitored because it is the primary steroid hormone produced by chicken granulosa cells; moreover progesterone production varies with the state of cell differentiation (stage of follicular development). The effects of fraction 1 of solubilized basal lamina on progesterone synthesis in chicken granulosa cells were examined in (a) experiments in which different amounts of fraction 1 were added as liquid (liquid-form) to the incubation medium, or (b) in experiments in which granulosa cells were incubated in culture wells in which fraction 1 had been dried (solid-form).

3.1.1. Liquid-form

When fraction 1 of solubilized basal lamina was added as liquid to the incubation medium, it stimulated progesterone production in a concentration-dependent manner in granulosa cells obtained from mature (F_1), developing (F_3, F_{5-7}) and immature (SYF) follicles (Fig. 1). Under this condition, fraction 1 caused a greater relative increase in progesterone production by less differentiated SYF granulosa cells (5–50-fold) than observed for differentiating F_{5-7} (0.5–5-fold) and F_3 (0.25–0.8-fold) or differentiated F_1 (0.08–0.25-fold) granulosa cells. Thus, the relative stimulatory effect of fraction 1, added as liquid on progesterone production was least in wells that contained F_1 cells that are already producing large amounts of progesterone (Fig. 1).

3.1.2. Solid-form

Granulosa cells were incubated in wells in which fraction 1 of protein had been dried (solid-form; 10–160 μg/cm^2). When tested in this form, fraction 1 alone caused a 25–35% decrease in progesterone production by differentiated cells (F_1; Fig. 2A). By contrast, fraction 1 alone significantly ($P < 0.05$) increased the amount of progesterone produced by less differentiated (F_{5-7} and SYF) granulosa cells (Fig. 2C and D). The stimulatory effect of solid-form of fraction 1 was great

est in the least differentiated SYF granulosa cells. In the solid-form, fraction 1 caused a numerical decrease in the amount of progesterone produced by differentiating F_3 granulosa cells (Fig. 2B), but this effect was not significant. Thus, F_3 represents a transition stage of regulation of progesterone production by the solubilized basal lamina in solid-form. The results show clearly that homologous basal lamina (in the absence of other known physiological modifiers such as gonadotropins) can regulate granulosa cell function and that its effect is dependent on the state of cell differentiation.

3.2. *Effect of solubilized basal lamina on LH-induced progesterone production in vitro*

Experiments were conducted to determine the effects of fraction 1 protein on LH-induced steroidogenesis in granulosa cells. LH-stimulated progesterone production in a dose-dependent manner, by granulosa cells incubated in culture wells pre-coated with fraction 1 (Fig. 3). In confirmation of the results shown in Fig. 2, the solid-form of fraction 1 alone suppressed basal progesterone production in differentiated (F_1) cells but increased it in differentiating (F_{5-7}) ones (compare Fig. 3A with Fig. 3C).

Additional experiments were conducted to examine further the interactions between the solid-form of fraction 1 protein and LH in regulating progesterone production by granulosa cells. Granulosa cells were stimulated with a single concentration of the LH in culture wells pre-coated with different amounts of fraction 1. The results shown in Fig. 4 demonstrate that the solid-form of fraction 1 suppressed LH-induced progesterone production in differentiated (F_1) and differentiating

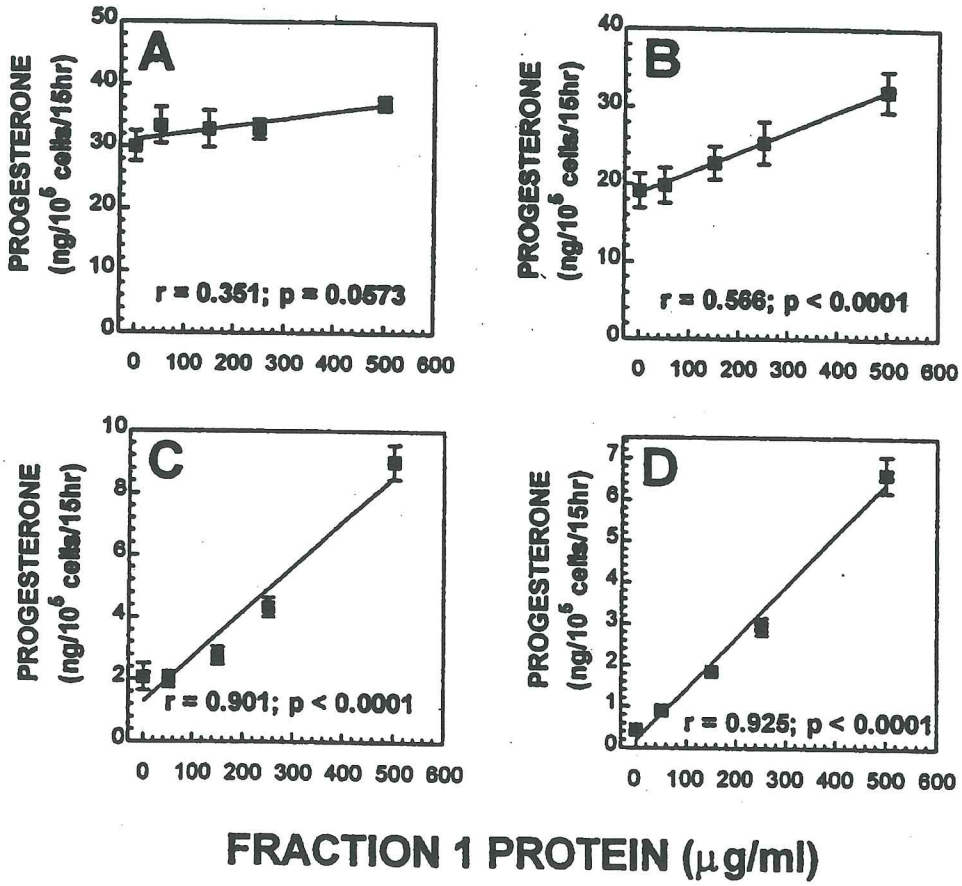

Fig. 1. Effect of fraction 1 of solubilized basal lamina protein (liquid-form) on progesterone production. Granulosa cells isolated from first (F_1, panel A), third (F_3, panel B) and developing fifth, sixth and seventh (F_{5-7}, panel C) largest preovulatory follicles and small yellow follicles (SYF, panel D) were placed in 96-well plates and different amounts of fraction 1 were added as liquid. The mixture was incubated for 15 h and the progesterone content of the incubation medium was measured. Data are mean ± S.E.M. of 6 to 15 incubations from three separate experiments. Note differences in the scale of y-axes.

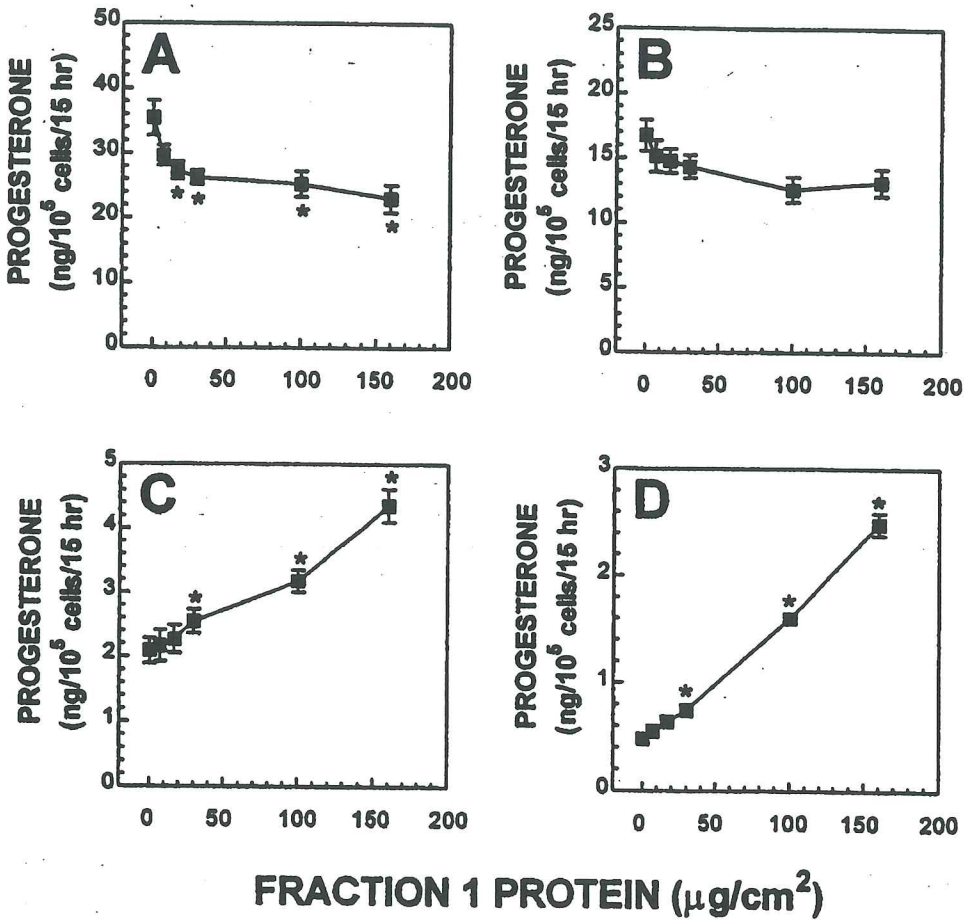

Fig. 2. Effect of pre-coated fraction 1 (solid-form) of solubilized basal lamina on progesterone production. Granulosa cells isolated from first (F_1, panel A), third (F_3, panel B) and developing fifth, sixth and seventh (F_{5-7}, panel C) largest preovulatory follicles and small yellow follicles (SYF, panel D) were incubated in 96-well plates that were pre-coated with different amounts of fraction 1 protein. The cells were incubated for 15 h and the progesterone content of the incubation medium was measured. Data are mean ± S.E.M. of 9 to 18 incubations from three to six separate experiments. Note differences in the scale of y-axes. $^*P < 0.05$ vs. control.

(F_3) cells by 13–30 and 19–36%, respectively. By comparison, LH-induced progesterone production in less differentiated (F_{5-7}) cells was not attenuated by the solid-form of fraction 1 (Fig. 4C). It is noteworthy that in Fig. 4A and B the level of progesterone in culture wells containing the maximally inhibiting concentration of solid-form of fraction 1 was greater than the amount of progesterone produced by cells in control wells. Because, in Fig. 4A and B 160 μg/cm² of solid-form of fraction 1 suppressed LH-induced progesterone production, it could be assumed that the results in Fig. 3A and B represent LH effects that were attenuated by the solid-form of fraction 1.

LH interacted with the liquid-form of fraction 1 as well to regulate progesterone production (Fig. 5). In the liquid-form, fraction 1 protein did not antagonize the stimulatory action of LH on granulosa cells from F_1 follicle as occurred with the solid-form (Fig. 4A). The stimulatory effect of LH on progesterone production in differentiating (F_{5-7}) granulosa cells was augmented by the liquid-form of fraction 1 (Fig. 5A) but this effect was not observed in differentiated (F_1) cells. Throughout these experiments basal and LH-induced progesterone production was greater in granulosa cells from mature (F_1) follicle than in granulosa cells from developing (F_3 or F_{5-7}) follicles (Figs. 3–5).

3.3. Effect of solubilized basal lamina on FSH-induced progesterone production in vitro

The effects of solubilized basal lamina on FSH-stimulated progesterone production by granulosa cells were also assessed. Similar to observations

made for LH, FSH-stimulated progesterone production by differentiating (F_3) granulosa cells was attenuated by solid-form of fraction 1 (Fig. 6A); in contrast, the stimulatory effects of FSH in undifferentiated (SYF) cells was enhanced by thesolid-form of fraction 1 (Fig. 6C). Similar to observations made for LH effect, FSH-induced progesterone production in the presence of maximally inhibiting concentrations of solid-form of fraction 1 was greater than the amount of progesterone produced by cells in control wells (Fig. 6A).

FSH also interacted with the liquid-form of fraction 1 to regulate progesterone production by granulosa cells. The liquid-form of solubilized basal lamina protein did not antagonize the stimulatory action of FSH (Fig. 7) rather, it augmented progesterone production in both differentiating (F_3, F_{5-7}) or undifferentiated (SYF) granulosa cells (Fig. 7).

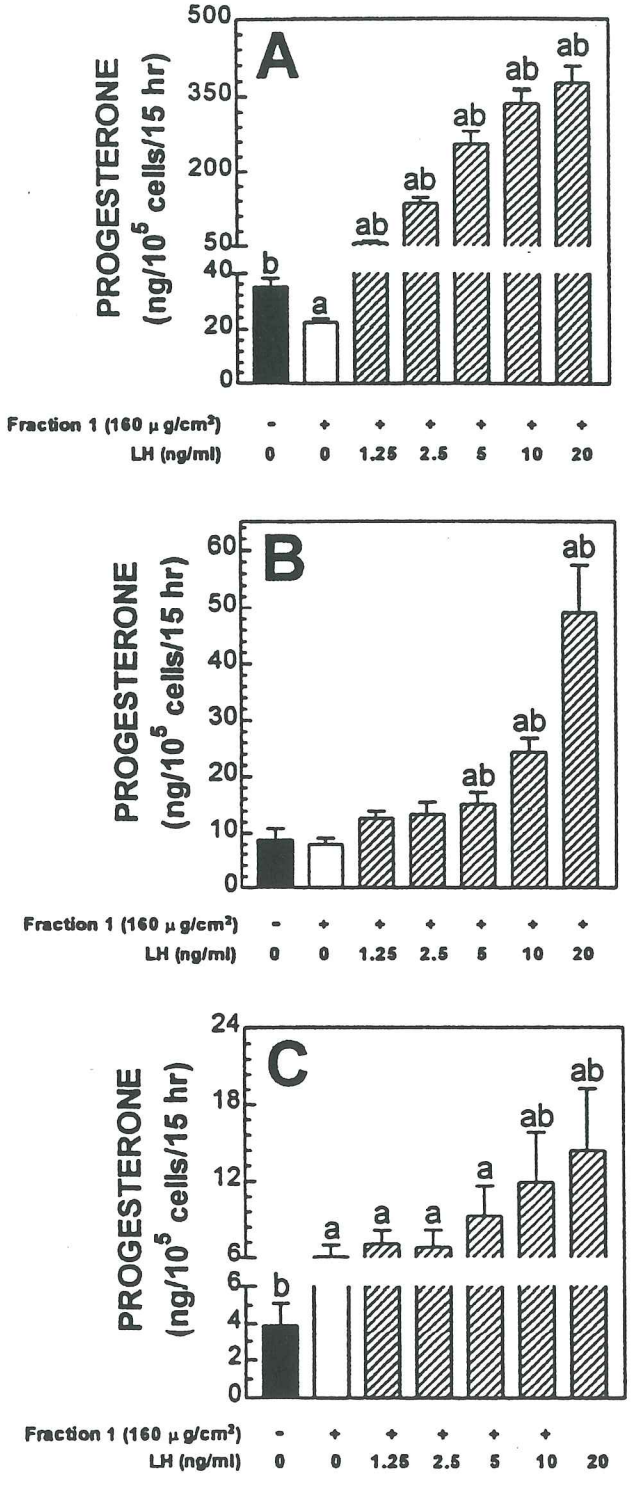

Fig. 3.

4. Discussion

The present results demonstrate that basal lamina isolated from avian ovarian follicle is capable of regulating granulosa cell function and supports the view that it is biologically active. Another major finding of the present study was that the effect of fraction 1 of solubilized basal lamina on steroidogenesis was influenced by the state of granulosa cell differentiation. Fraction 1, containing the bulk of basal lamina proteins, was more effective in stimulating steroidogenesis in undifferentiated granulosa cells isolated from immature follicles than in differentiated cells obtained from mature follicles. The exact loci of action of fraction 1 proteins are unknown. Neither are their mechanisms of action known. The present observations point to the complex nature of the relationships that exist between granulosa cells and components of basal lamina in vivo. The nature of these interactions will be elucidated in the future. Basal laminae used in the present study were isolated from the first and the second largest

Fig. 3. Dose-response effect of LH on progesterone production in the presence of solid form of fraction 1 basal lamina. Granulosa cells isolated from the first (F_1, panel A), third (F_3, panel B) and developing fifth, sixth and seventh (F_{5-7}, panel C) largest preovulatory follicles were incubated in 96-well plate that were pre-coated with fraction 1 (160 μg/cm²) of fluidized basal lamina proteins. The cells were incubated for 15 h in the presence and absence of different amounts of LH and the progesterone content of the incubation medium was measured. Data are mean ± S.E.M. of nine incubations from three separate experiments. Note differences in the scale of y-axes. $^{a}P < 0.05$ vs. control (no LH, no fraction 1). $^{b}P < 0.05$ vs. fraction 1 alone.

preovulatory follicles. Future studies will determine the effects of basal lamina from developing and immature follicles on the function of differentiating and undifferentiated granulosa cells. Ovarian follicles at all stages of development pos-

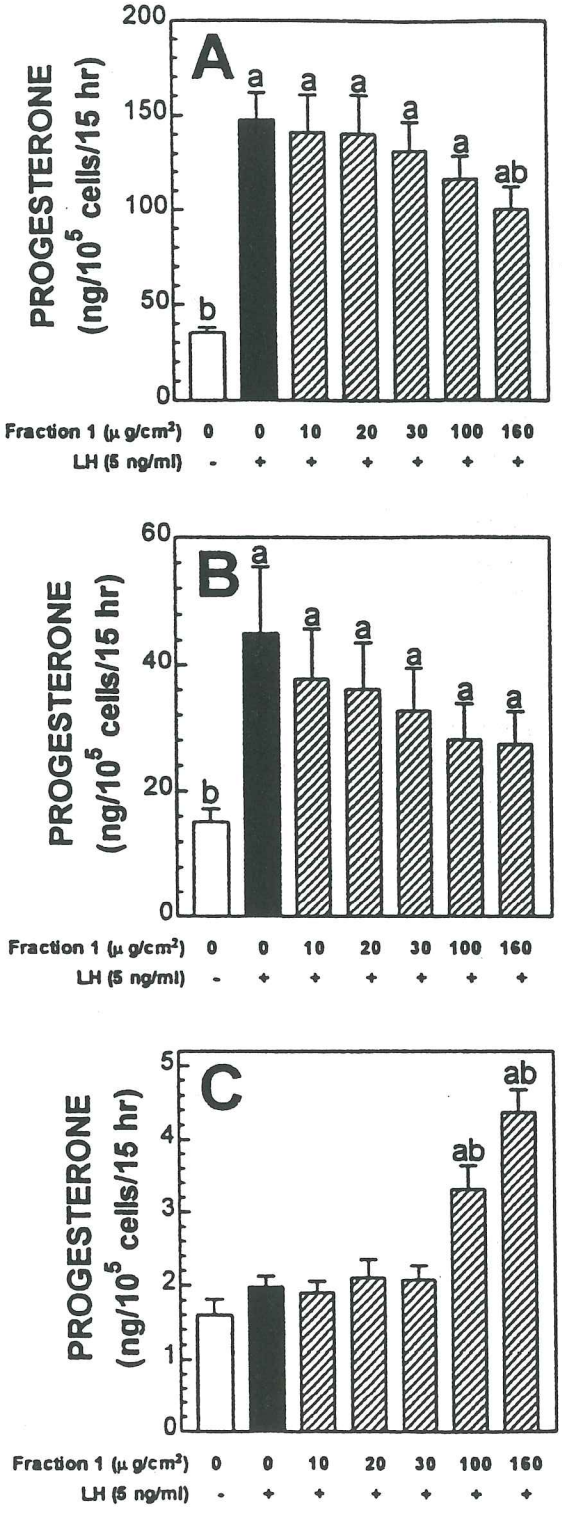

Fig. 4.

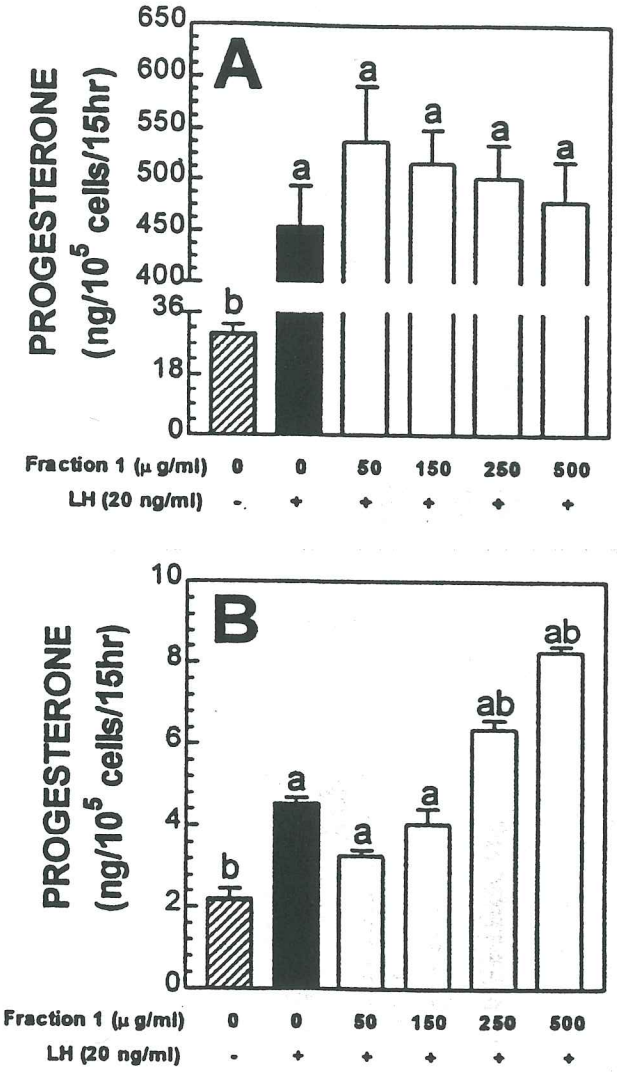

Fig. 5. Dose-response effect of liquid-form of fraction 1 of basal lamina on LH-induced progesterone production. Granulosa cells from the first (F_1, panel A), fifth, sixth and seventh (F_{5-7}, panel B) largest preovulatory follicles were placed in 96-well plates and different amounts of fraction 1 were added as liquid. The mixture was incubated for 15 h in the presence and absence of LH and the progesterone content of the incubation medium was measured. Data are mean ± S.E.M. of three to six incubations from two experiments. Note differences in the scale of y-axes. $^aP < 0.05$ vs. control (no LH, no fraction 1). $^bP < 0.05$ vs. LH alone.

Fig. 4. Dose-response effect of solid-form of fraction 1 of basal lamina on LH-induced progesterone production. Granulosa cells from the first (F_1, panel A), third (F_3, panel B) and fifth, sixth and seventh (F_{5-7}, panel C) largest preovulatory follicles were incubated in 96-well plate that were pre-coated with different amounts of fraction 1 of fluidized basal lamina. The cells were incubated for 15 h in the presence and absence of LH (5 ng/ml) and the progesterone content of the incubation medium was measured. Data are mean ± S.E.M. of nine incubations from three separate experiments. Note differences in the scale of y-axes. $^aP < 0.05$ vs. control (no LH, no fraction 1). $^bP < 0.05$ vs. LH alone.

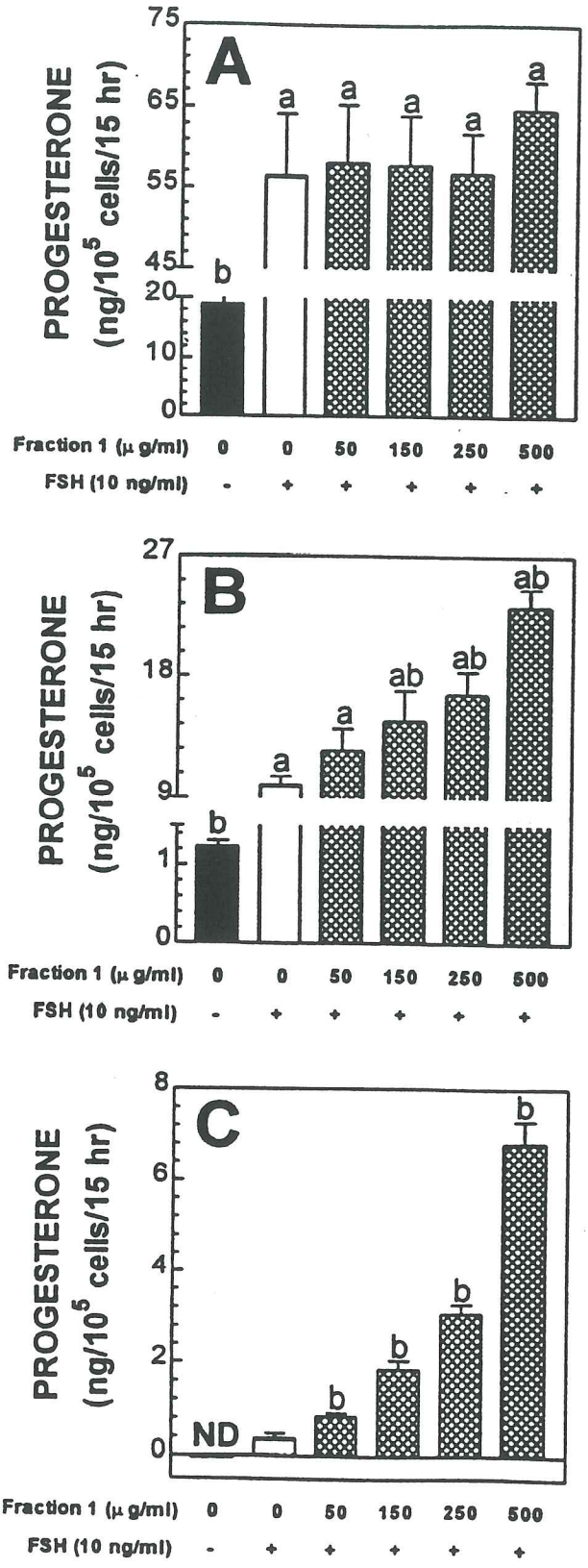

Fig. 6.

sess basal lamina, however, the similarities and/or differences in their compositions have not been studied. It is our hypothesis that there are significant differences between the types and amounts of components of basal lamina derived from follicles at different stages of development.

A very important observation of the present study was that the effect of fraction 1 of solubilized basal lamina on progesterone production by granulosa cells was influenced by the form in which the matrix material was introduced into the culture system. When added as liquid to the incubation mixture, fraction 1 of solubilized basal lamina enhanced progesterone production by chicken granulosa cells at all stages of differentiation. In contrast, when presented in solid-form, fraction 1 proteins increased progesterone production by less differentiated granulosa cells but suppressed it in differentiated ones. The explanation for this intriguing observation is unknown. It is noteworthy that other investigators have observed similar phenomenon, that is, the differential effects of different forms of extracellular proteins on granulosa cell function. For example, Aten et al. (1995) observed that rat granulosa cells incubated on Matrigel matrix, basement membrane reconstituted from extracts of EHS tumor, produced less progesterone than those incubated on plastic (similar to results obtained with the solid-form of fraction 1 in the present study). In addition, Aten et al. (1995) showed that when laminin or fibronectin, major components of basal lamina, were added in liquid-form to rat granulosa cell culture steroid hormone synthesis increased. The effect of coated laminin (solid-form) on steroid hormone synthesis in granulosa cells is controversial. Whereas Aten et al. (1995) demonstrated that progesterone production by rat granulosa cells incubated in laminin-coated wells was enhanced,

Fig. 6. Dose-response effect of liquid-form of fraction 1 of basal lamina on FSH-induced progesterone production. Granulosa cells from the third (F_3, panel A), fifth, sixth and seventh (F_{5-7}, panel B) largest preovulatory and a pool of small yellow follicles (SYF, panel C) follicles were placed in 96-well plates and different amounts of fraction 1 were added as liquid. The mixture was incubated for 15 h in the presence and absence of FSH (10 ng/ml) and the progesterone content of the incubation medium was measured. Data are mean ± S.E.M. of six to nine incubations from three separate experiments. Note differences in the scale of y-axes. $^{a}P < 0.05$ vs. control (no FSH, no fraction 1). $^{b}P < 0.05$ vs. FSH alone. ND, not detectable.

Aharoni et al. (1996) showed that progesterone production by rat granulosa cells in laminin-coated wells was decreased. In other studies, when human granulosa cells retrieved from patients undergoing in vitro fertilization were cultured in laminin-coated wells, steroidogenesis was suppressed (Fujiwara et al., 1997).Similarly, human chorionic gonadotropin(hCG)induced progesterone synthesis was attenuated in laminin-coated dishes (Fujiwaraet al., 1997). Therefore, reports in the literature and results of the present studies indicate that the incubation of granulosa cells in dishes pre-coated with reconstituted or components of basement membranes or solubilized basal lamina results in the reduction of steroid hormone synthesis. The observations made with the solid-form of fraction 1 would be expected to more closely mimic the condition in vivo than the results obtained with the liquid-form because the basal lamina exists in solid-form.

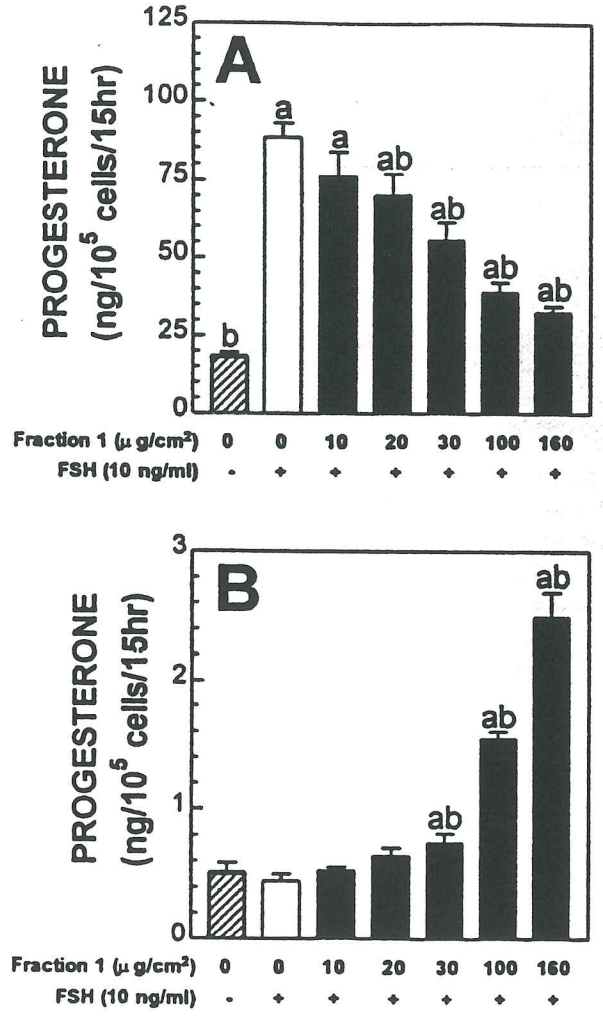

Fig. 7. Dose-response effect of solid-form of fraction 1 of basal lamina on FSH-induced progesterone production. Granulosa cells from the third largest preovulatory follicle (F_3, panel A) and small yellow follicles (SYF, panel B) were incubated in 96-well plate that were pre-coated with different amounts of fraction 1 of fluidized basal lamina. The cells were incubated for 15 h in the presence and absence of FSH (10 ng/ml) and the progesterone content of the incubation medium was measured. Data are mean ± S.E.M. of nine incubations from three separate experiments. Note differences in the scale of y-axes. $^aP < 0.05$ vs. control. $^bP < 0.05$ vs. FSH alone.

In the present study, solubilized basal lamina interacted with pituitary-derived gonadotropic hormones to regulate chicken granulosa cell function. LH- or FSH-induced progesterone production by chicken granulosa cells was modulated by both liquid- and solid-forms of fraction 1 of solubilized basal lamina. Although not proven, these data suggest that the basal lamina or its components interact with gonadotropic hormones to regulate granulosa cell function in vivo. Furthermore, the current data may be a reflection of the differential effects of basal lamina or its components on granulosa cells at different states of differentiation during follicular development in vivo.

An interesting finding of the present study was that in differentiated granulosa cells, gonadotropin-stimulated progesterone production was attenuated in the presence of solid-form of fraction 1. This finding is intriguing because the solid-form of solubilized basal lamina most closely mimics the in vivo condition. The observation is suggestive of the possibility that basal lamina or some of its components belong to an intra-ovarian system that exerts feedback regulatory effect on gonadotropin-stimulated steroidogenesis in granulosa cells in vivo. The effect of FSH on steroidogenesis in granulosa cells obtained from mature F_1 follicle was not examined in the present study because it was shown that FSH had little or no effect on these cells (Hammond et al., 1981; Tilly et al., 1991; Johnson, 1993; Novero and Asem, 1993). In contrast, it is well established that FSH regulates the functions of granulosa cells from immature and developing follicles (Hammond et al., 1981; Tilly et al., 1991; Johnson, 1993; Novero and Asem, 1993). It is well documented that granulosa cells in F_1 follicle produce large amounts of progesterone whereas those in SYF follicles produce little or no progesterone (Hammond et al., 1981; Tilly et al., 1991; Johnson, 1993).

It was reported that extracellular matrix proteins modulate steroid hormone synthesis by granulosa cells. For example, rat (Carnegie et al., 1988) or human (Ben-Rafael et al., 1988) granulosa cells grown in collagen gel matrix produced greater amounts of progesterone than their counterparts that were incubated on plastic. In addition, basement membrane deposited by bovine corneal endothelial cells enhanced steroidogenesis in rat and human granulosa cells (Furman et al., 1986a,b; Amsterdam et al., 1989). The present results demonstrate for the first time that basal lamina obtained from the ovarian follicle is capable of regulating granulosa cell function. It was shown previously that basement membrane deposited by bovine corneal endothelial cells caused rat and human granulosa cells to become more differentiated and have increased steroidogenesis (Furman et al., 1986a,b; Amsterdam et al., 1989). It is possible that the stimulatory effects of fraction 1 of basal lamina on progesterone production in undifferentiated cells (observed in the present study) was due to differentiation caused by the matrix proteins. Future studies will examine this possibility.

The relation between spherical shape and elevated progesterone production in granulosa cells was reported (Soto et al., 1986; Carnegie et al., 1987; Ben-Rafael et al., 1988; Carnegie et al., 1988). It was shown that extracellular matrix protein-induced increase in progesterone production by granulosa cells was accompanied by changes in cell shape. Rat (Carnegie et al., 1988) or human (Ben-Rafael et al., 1988) granulosa cells grown in collagen gel matrix were rounded and produced greater amounts of progesterone than their counterparts that were spread on plastic culture surface. It is noteworthy that fraction 1 of solubilized basal lamina caused F_1, F_3, F_{5-7}, and SYF granulosa cells to become more spherical (Asem et al., 2000) thus, the effect of solubilized basal lamina on progesterone production in chicken granulosa cells was accompanied with rounding of the cells.

In summary, pure, intact and complete basal lamina was isolated from the largest preovulatory follicles of the chicken ovary. The solubilized basal lamina, alone, regulated steroid hormone synthesis in granulosa cells. In addition, it modulated the stimulatory actions of LH or FSH on progesterone synthesis in chicken granulosa cells. These effects of fluidized basal lamina were influenced by the state of granulosa cell differentiation (stage of follicular development). The BLAOF is biologically active.

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