Stimulation of rat granulosa cell progesterone production but not other differentiated functions by a splenocyte-derived factor

J. M. Ness and B. G. Kasson*

Department of Pharmacology, College of Medicine, University of Iowa, Iowa City, IA 52242, USA

Concanavalin A-stimulated splenocytes secrete a factor that stimulates progesterone production in cultured rat granulosa cells. The actions of this progesterone-stimulating factor (PSF) were characterized further by purifying it by sequential chromatographies on heparin-agarose, copper chelating-Sepharose and Mono S columns. Several of its effects on granulosa cells were then compared with those of FSH. Like FSH, PSF induced accumulation of progesterone and 20 α -dihydroprogesterone in granulosa cell culture media; however, the time course of progesterone accumulation in response to PSF was much slower than that in response to FSH. In contrast to FSH, PSF induced little accumulation of oestrogen. Induction of other differentiated responses was compared by pretreating cultured granulosa cells with either FSH or PSF, and then challenging with LH, prolactin, epidermal growth factor or the β -adrenergic agonist, isoproterenol. Pretreatment with FSH induced subsequent responsiveness to all four agents, whereas pretreatment with PSF induced subsequent responsiveness only to isoproterenol. These results indicate that granulosa cell responses to PSF are much more limited than are those to FSH and that these responses are characterized by an increase in progestin production.

Introduction

Mounting evidence suggests that there are complex interactions between the immune and endocrine systems. A variety of immune cells, including neutrophils, macrophages and T lymphocytes, are present in the ovary (Brännström *et al.*, 1993). Several immune cell-derived mediators, including interleukins 1 and 6, and tumour necrosis factor α , modulate granulosa cell steroidogenesis, typically in an inhibitory fashion (Kasson and Gorospe, 1989; Veldhuis *et al.*, 1991; Gorospe *et al.*, 1992). In contrast, we have shown that rat splenocytes secrete a factor that stimulates progesterone production in freshly isolated, undifferentiated granulosa cells obtained from female weanling rats pretreated with diethylstilboestrol (Gorospe and Kasson, 1988). This progesterone-stimulating factor (PSF) is distinct from FSH, epidermal growth factor (EGF), GnRH, vasoactive intestinal peptide (VIP) and pituitary adenylate cyclase-activating peptide (PACAP), the main agents shown to induce steroidogenesis independently in undifferentiated granulosa cells (Clark, 1982; Jones *et al.*, 1982; Hsueh *et al.*, 1984; Davoren and Hsueh, 1985; Zhong and Kasson, 1994). The actions of PSF are also unlike any previously tested cytokine in that they stimulate, rather than inhibit, steroidogenesis. The actions of PSF were characterized further by purifying it and comparing its effects on granulosa cells with those of FSH.

Materials and Methods

Materials

Ovine FSH (oFSH-16; FSH activity, 20 $\times$ NIH FSH S1 units mg⁻¹), ovine LH (oLH-25; LH activity, 2.3 $\times$ NIH LH S1 units mg⁻¹), hCG (CR 125; 11 900 iu mg⁻¹) and ovine prolactin (oPRL-18; PRL activity 30 iu mg⁻¹) were obtained from the National Hormone and Pituitary Program (NIDDK, NIH, Bethesda, MD). Concanavalin A, EGF (receptor grade) and isoproterenol were purchased from Sigma Chemical Co. (St Louis, MO). Ex-Cell 320 medium was obtained from JRH Biosciences (Lenexa, KS). McCoy's 5a medium (modified, without serum), L-glutamine and gentamicin were provided by The University of Iowa Diabetes and Endocrine Research Center.

Splenocyte cultures

Concanavalin A-stimulated culture supernatants were prepared as described by Gorospe and Kasson (1988) with some modifications. Briefly, adult male Sprague-Dawley rats (160–250 g) were killed by asphyxiation with CO₂, following procedures approved by the Institutional Animal Care and Use Committee of the University of Iowa. Their spleens were aseptically removed, washed in culture media and pressed through a sterile stainless steel screen (80 μ m mesh) to obtain individual splenocytes. The cells were washed twice by centrifugation (600 g, 5 min), resuspended in serum-free media (Ex-Cell 320) supplemented with 2.5 μ g concanavalin A ml⁻¹,

*Correspondence and reprint requests.
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50 μmol β -mercaptoethanol l^{-1} and 50 μg gentamicin ml^{-1} and incubated in T-75 flasks (7.5×10^8 cells per 50 ml) at 37°C in 95% air : 5% CO_2 for 48 h. Conditioned medium was collected, centrifuged (3000 g, 20 min, 4°C) to remove any non-adherent cells, and precipitated with ammonium sulfate. The 50–90% cut was dialysed against 50 mmol Tris-HCl l^{-1} , pH 7.4 at 4°C and used as the starting material for purification of PSF.

Granulosa cell cultures

Female Sprague-Dawley rats, 21–22 days old (Harlan, Madison, WI) were implanted s.c. with 10 mm capsules (Baxter Healthcare, McGaw Park, IL) containing diethylstilboestrol, to induce granulosa cell proliferation. Rats were maintained under a photoperiod of 12 h light:12 h dark with food and water *ad libitum* for 3–6 days and were then killed by asphyxiation with CO_2 following procedures approved by the Institutional Animal Care and Use Committee of the University of Iowa. Granulosa cells were expressed from isolated ovaries, using a 25-gauge needle. The cells were pelleted by centrifugation (100 g, 2 min), washed once and resuspended in McCoy's 5a medium. Cells were plated at 5×10^4 viable cells per well in 24-well tissue culture cluster plates (Costar, Cambridge, MA) containing 0.5 ml McCoy's 5a medium supplemented with 50 μg gentamicin ml^{-1} and 2 mmol L-glutamine ml^{-1} ; androstenedione (10^{-7} mol l^{-1}) was also included, unless indicated otherwise. Cells were incubated at 37°C in a water-saturated atmosphere with 95% air : 5% CO_2 . At the indicated times, media were removed and stored at -20°C for subsequent measurement of steroids.

Steroid assays

The progesterone and total oestrogen content of culture media were determined by enzyme-linked immunoassay using specific antisera as described in detail by Zhong and Kasson (1994). The intra-assay and interassay coefficients of variation for these assays were 7.3 and 10.2%, respectively, for progesterone, and 8.2 and 10.9%, respectively, for oestrogen. The sensitivity of both assays was 0.3 pg ml^{-1} . The 20α -dihydroprogesterone content of the culture media was determined by radioimmunoassay using a specific antiserum as described by Gorospe and Kasson (1988). The intra-assay and interassay coefficients of variation for this assay were 7.9 and 8.5%, respectively. The sensitivity was 7.8 pg ml^{-1} .

FSH radioimmunoassay and radioreceptor assay

For the FSH radioimmunoassay, reagents were obtained from the National Hormone and Pituitary Program (NIDDK, NIH) and the assay was conducted following the protocol provided. For the radioreceptor assay, FSH (receptor grade, provided by L. Reichert, Albany Medical College, NY) was iodinated using the lactoperoxidase method as described by Miyachi *et al.* (1972). The assay was started by adding freshly isolated granulosa cells (5×10^5) to 12 mm $\times$ 75 mm polypropylene tubes containing 0.2 ml Dulbecco's phosphate-buffered saline (D-PBS) with 0.2% (w/v) BSA and ^{125}I -labelled FSH

(1×10^5 c.p.m.) and increasing amounts of FSH or PSF. Cells were then incubated overnight at room temperature. The incubation was stopped by adding 2 ml ice-cold PBS, and vacuum filtration was carried out through Whatman GF/C filters pre-soaked for 5 min in D-PBS–0.2% (w/v) BSA. The filters were washed twice with 2 ml D-PBS and the amount of radioactivity was measured. Nonspecific binding was determined by including excess FSH (2 μg) in the incubation.

Statistical analyses

Radioimmunoassay and enzyme-linked immunoassay calculations and curve fitting were done using a 4-parameter logistic analysis program provided by D. Rodbard (NIH). Different treatments were assessed by ANOVA and Newman-Keuls' multiple range test; $P < 0.05$ was considered as a significant difference.

Results

Purification of progesterone-stimulating factor

Preliminary studies examining the binding of PSF to ion exchange columns indicated that most PSF activity bound well to cation exchange gels and that heparin-agarose provided the best purification. Consequently, purification was begun by loading dialysate (about 100 ml) from the ammonium sulfate-precipitated splenocyte culture supernatants onto a heparin-agarose column (15 mm $\times$ 90 mm). This column was then eluted at 0.5 ml min^{-1} with a 0–1 mol NaCl l^{-1} gradient in 50 mmol NaPO_4 l^{-1} , pH 7.4. Two peaks of progesterone-stimulating activity were obtained from this column; one peak was present in the flow-through and one peak was eluted by the NaCl gradient (Fig. 1a). Because the retained fraction contained most of the PSF, it was selected for further purification. Fractions containing active material (typically eluting between 0.3 and 0.4 mol NaCl l^{-1}) were pooled, diluted threefold with 50 mmol NaPO_4 l^{-1} , pH 7.4, and rechromatographed on the heparin-agarose column. Active fractions were again pooled, diluted and applied to a copper-chelating Sepharose column (10 mm $\times$ 60 mm) which removed, among other substances, a major haem-protein contaminant (eluting at 20–30 mmol imidazole l^{-1}). This column was eluted at 0.5 ml min^{-1} with a 0–40 mmol imidazole l^{-1} gradient in 150 mmol NaCl l^{-1} , 10 mmol Tris-HCl l^{-1} , pH 7.4 (Fig. 1b). Active fractions from this column (typically eluting between 3.5 and 5 mmol imidazole l^{-1}) were pooled, diluted threefold with 50 mmol NaPO_4 l^{-1} , pH 7.4, and applied to a Mono S FPLC cation exchange column (7 mm $\times$ 50 mm) for high resolution ion exchange chromatography. This column was eluted at 0.7 ml min^{-1} with a 0–1 mol NaCl l^{-1} gradient in 50 mmol NaPO_4 l^{-1} , pH 7.4 (Fig. 1c). The active fractions from the Mono S column (typically eluting between 0.1 and 0.2 mol NaCl l^{-1}) were then pooled.

The efficiency of this purification procedure was monitored by following the PSF activity and protein content of the combined active fractions from each preparative step. The PSF activity was assessed by first constructing dose-response curves for granulosa cell progesterone production using

Fig. 1. Chromatographic profiles from the purification of progesterone-stimulating factor (PSF). (a) Dialysate from ammonium sulfate-precipitated conditioned media from concanavalin A-stimulated splenocytes, applied to a heparin-agarose column (15 mm × 90 mm) and eluted with a 0–1 mol NaCl l⁻¹ gradient in 50 mmol NaPO₄ l⁻¹, pH 7.4. (b) Combined and diluted active fractions from the heparin-agarose column, applied to a copper-chelating Sepharose column (10 mm × 60 mm) and eluted with a 0–40 mmol imidazole l⁻¹ gradient in 150 mmol NaCl l⁻¹, 10 mmol Tris-HCl l⁻¹, pH 7.4. (c) Combined and diluted active fractions from the copper-chelating Sepharose column, applied to a Mono S FPLC cation exchange column (7 mm × 50 mm) and eluted with a 0–1 mol NaCl l⁻¹ gradient in 50 mmol NaPO₄ l⁻¹, pH 7.4. Progesterone accumulation (●) in the media of granulosa cells cultured for 2 days with 20 µl aliquots of each fraction; (---) absorbance profile at 280 nm; (—) elution gradient.

increasing amounts of the combined active fractions from each PSF purification step. These dose-response curves showed similar maximal progesterone concentrations, except for those using conditioned media and dialysate, which produced maximal concentrations that were approximately 70% lower than

material from subsequent steps. The PSF activity was then determined by defining one unit of activity as the amount required to produce half-maximal stimulation of progesterone (ED₅₀). The ED₅₀ values were then determined from the dose-response curves by fitting the curves with the four

Table 1. Purification of progesterone-stimulating factor (PSF)

Method	Activity (units) ^a	Protein (mg)	Specific activity (U mg ⁻¹)	Purification (fold)	Yield (%)
Conditioned media ^b	37 500	1255.0	30	—	—
Dialysate ^c	36 000	780.0	46	1.5	96
Heparin-Sepharose 1	30 000	263.0	114	3.8	80
Heparin-Sepharose 2	23 300	136.0	171	57.0	62
Chelating-Sepharose	14 300	5.0	2860	95.5	38
Mono S	8550	0.55	15 546	518.2	23

^aOne unit is defined as the amount of PSF required to produce half-maximal stimulation of progesterone production in cultured rat granulosa cells.

^bConditioned media was the media obtained after a treatment of cultured splenocytes for 2 days with 2.5 µg concanavalin A ml⁻¹.

^cDialysate was the material obtained after dialysing the 50–90% ammonium sulfate fraction of conditioned splenocyte media against 50 mmol Tris-HCl l⁻¹.

parameter logistic equation. Total activity was calculated by determining the number of units obtained at each preparative step. Specific activity was then determined by correcting for total protein, measured by the method of Bradford (1976). All values for each preparative step are given in Table 1. Using this method, the specific activity of the material from the Mono S column was increased over 500 times from the starting material, with yields of 23% for total activity and 0.044% for total protein.

A silver stained SDS-PAGE, performed by the method of Laemmli (1970), of the combined active fractions from each preparative step (Fig. 2) revealed the presence of three major bands (M_r = 66, 60 and 37) and three minor bands (M_r = > 200, 76 and 18) in the final Mono S preparation (lane 5), a very limited number when compared with the starting material (lane 1). Further purification was attempted using various hydrophobic interaction columns, sizing columns, and several specific columns. These subsequent attempts to separate the remaining proteins were unsuccessful, resulting either in complete loss of activity or no further purification. Consequently, material from the Mono S column was used for subsequent culture studies.

Comparison of FSH- and PSF-stimulated steroidogenesis in cultured rat granulosa cells

The steroidogenic activities of PSF and FSH were compared by adding half-maximal doses of PSF (400 ng ml⁻¹) or FSH (10 ng ml⁻¹) to freshly isolated granulosa cells in the presence of androstenedione (10⁻⁷ mol l⁻¹). Cultures were then stopped at various times over 7 days. FSH-stimulated progesterone accumulation (Fig. 3a) increased during the first 2 days of culture, reached peak values between 2 and 3 days, and then declined over the next 4 days. In contrast, PSF-stimulated progesterone accumulation gradually increased to a maximum at 5 days of culture. Although PSF-stimulated progesterone accumulation was lower than that of FSH during the first 3 days of culture, by day 5 it increased to an amount approximately equal to that seen with FSH at 3 days. The time course of 20α-dihydroprogesterone accumulation was similar for both FSH- and PSF-treated cells, reaching peak values at 5 days of

Fig. 2. SDS-PAGE of purification steps for progesterone-stimulating factor, with silver staining. Lane 1: dialysate used as starting material for the progesterone-stimulating factor (PSF) preparation (20 µg protein); lane 2: active fractions from the first heparin-agarose chromatography (15 µg protein); lane 3: active fractions from the second heparin-agarose chromatography (11 µg protein); lane 4: active fractions from the copper-chelating-Sepharose chromatography (2.3 µg protein) and; lane 5, active fractions from the Mono S chromatography (0.84 µg protein). Migration of the molecular mass standards is indicated at the right. Similar results were obtained in four separate purifications.

culture (Fig. 3b). However, PSF consistently produced higher concentrations of 20α-dihydroprogesterone than did FSH,

Fig. 3. Comparison of FSH- and progesterone-stimulating factor (PSF) stimulated steroidogenesis in rat granulosa cells. Granulosa cells (5×10^4 cells per well) were treated with FSH (10 ng ml^{-1} ; ■), PSF (400 ng ml^{-1} ; ●) or media alone (○). Culture media was removed at the indicated times and stored at -20°C until assayed for (a) progesterone, (b) 20 α -dihydroprogesterone or (c) oestrogen. Data points are means $\pm$ SEM of triplicate cultures from one of three independent experiments, all of which produced similar results. Error bars that are not shown are contained within the data point.

$667 \pm 97 \text{ ng ml}^{-1}$ and $363 \pm 55 \text{ ng ml}^{-1}$, respectively. Oestrogen accumulation followed a similar pattern, with both FSH and PSF treatment; however, PSF-stimulated oestrogen accumulation was less than 5% of that seen with FSH (Fig. 3c). Measurement of protein concentrations in these cultures revealed that, even though protein content of PSF- and FSH-treated cells was 25–40% higher than that of cells treated with media at 2, 3, 5 and 7 days, these increases were not statistically significant (data not shown).

Comparison of PSF with FSH and EGF

Because both PSF and FSH induced steroidogenic responses independently in granulosa cells, we next sought to determine whether the steroidogenic activity in PSF might be FSH. Initially, PSF was assayed for FSH immunoreactivity by radioimmunoassay. Increasing amounts of the FSH standard produced increasing competition in the radioimmunoassay (Fig. 4a); however, increasing amounts of PSF did not compete in the

Fig. 4. FSH radioimmunoassay and FSH radioreceptor assay of progesterone-stimulating factor (PSF). Specific ^{125}I -labelled FSH binding to (a) FSH antiserum or (b) granulosa cells, is indicated as B:Bo, with increasing amounts of either FSH (○) or PSF (●) added to the incubations. (c) Progesterone accumulation in granulosa cells after treatment with increasing amounts of FSH (○, 2 days) or PSF (●, 5 days). Data points in (a) and (b) are means $\pm$ SEM from four independent experiments. Data points in (c) are means of triplicate samples from a single experiment. Error bars that are not shown are contained within the data point.

FSH radioimmunoassay, even when a dose eight times higher than that required to produce maximal steroidogenic activity (Fig. 4c) was used. Thus, doses of PSF that induce steroidogenic responses contain no measurable FSH immunoreactivity.

Although no FSH immunoreactivity was detected in PSF, it was possible that a bioactive, non-immunoactive form of FSH was present. Thus, PSF was tested in an FSH radioreceptor assay. Follicle-stimulating hormone competed for receptor binding in this assay (Fig. 4b), but PSF, even at a dose eight times higher than that producing maximal steroidogenic activity (Fig. 4c), again produced no competition. Thus, doses of PSF that induce steroidogenic responses contain no measurable FSH receptor binding activity. Similar studies also showed a lack of EGF activity in the PSF preparation (data not shown).

Comparison of FSH and PSF induction of granulosa cell responsiveness to other agents

In the next set of studies FSH and PSF induction of other differentiated responses in cultured rat granulosa cells were

Fig. 5. Induction of hCG, prolactin and epidermal growth factor responsiveness in rat granulosa cells. Freshly isolated granulosa cells were pretreated with media alone, FSH (30 ng ml⁻¹) or progesterone-stimulating factor (PSF; 1000 ng ml⁻¹). After pretreatment for 5 days, the cells were washed, and then challenged with media alone (□), (a) hCG (30 ng ml⁻¹ ■), (b) prolactin (1 µg ml⁻¹ ▨) or (c) epidermal growth factor (10 ng ml⁻¹ ▩). The cells were incubated for three additional days and progesterone was measured by enzyme-linked immunoassay. Data points are means ± SEM from either four (a and b) or six (c) independent experiments. **P* < 0.05 versus media treatment; ***P* < 0.02 versus media treatment; ****P* < 0.01 versus media treatment.

compared. Cells were pretreated for either 2 or 5 days with FSH (30 ng ml⁻¹) or PSF (1000 ng ml⁻¹); these doses produce maximal steroidogenic responses. After three washes with fresh media, cells were challenged for 3 days with media alone (control), hCG (30 ng ml⁻¹), prolactin (1 µg ml⁻¹), isoproterenol (10⁻⁶ mol l⁻¹), EGF (10 ng ml⁻¹), FSH (10 ng ml⁻¹) or PSF (400 ng ml⁻¹). Pretreatment times of 2 and 5 days were chosen because they were the times of near maximal FSH- and PSF-stimulated progesterone accumulation, respectively.

Cells pretreated for 5 days with FSH, but not cells pretreated with PSF, showed a significant increase in hCG-stimulated progesterone accumulation (*P* < 0.01 versus media controls, *n* = 6) (Fig. 5a). Similar responses were also seen in cells pretreated for 2 days (data not shown). This apparent induction of LH/CG receptors was confirmed by binding experiments with ¹²⁵I-labelled hCG, which also showed that only cells pretreated with FSH possessed ¹²⁵I-labelled hCG binding sites (data not shown). Similarly, cells pretreated for 5 days with

FSH, but again not those pretreated with PSF, showed a significant increase in prolactin-stimulated progesterone accumulation (*P* < 0.02 versus media controls, *n* = 4; Fig. 5b). A similar trend was observed in cells pretreated for 2 days, although the prolactin-stimulated increase in FSH-pretreated cells was not significant (*P* < 0.18, prolactin versus media controls, *n* = 4; data not shown).

Epidermal growth factor increases progestin synthesis in freshly isolated rat granulosa cells, although to much less an extent than does FSH (Jones *et al.*, 1982; St-Arnaud *et al.*, 1983). Therefore, the regulation of EGF responsiveness after pretreatment with either FSH or PSF was examined. Pretreatment of cells with FSH for 5 days increased subsequent responsiveness to EGF (*P* < 0.05 versus media controls, *n* = 6; Fig. 5c); however, a significant increase in EGF responsiveness was not observed after only 2 days of FSH treatment (*P* < 0.25; *n* = 6; data not shown). In contrast, pretreatment of cells with PSF for either 2 or 5 days did not increase subsequent responsiveness to EGF.

Binding studies have demonstrated the presence of β-adrenergic receptors in non-gonadotrophin-primed granulosa cells (Aguado and Ojeda, 1984). However, β-agonists do not stimulate steroidogenesis unless the cells are first treated with FSH (Adashi and Hsueh, 1981). Thus, as expected, granulosa cells pretreated with media alone did not produce progesterone in response to subsequent isoproterenol treatment (Fig. 6), whereas FSH-pretreated cells did acquire β-adrenergic responsiveness (*P* < 0.01 versus media controls after 2 days, Fig. 6a, and *P* < 0.02 versus media controls after 5 days, Fig. 6b, *n* = 6). Pretreatment of cells with PSF also induced subsequent β-adrenergic responsiveness (*P* < 0.07 versus media controls after 2 days, Fig. 6a and *P* < 0.05 versus media controls after 5 days, Fig. 6b, *n* = 6).

Comparison of maintenance of granulosa cell steroidogenesis by FSH and PSF

We determined whether pretreatment of granulosa cells with either PSF or FSH maintained subsequent responsiveness to these two substances. Granulosa cells were pretreated for 2 days with either media, FSH (30 ng ml⁻¹) or PSF (1000 ng ml⁻¹), and then challenged for 3 days with one of these substances (Fig. 7). Compared with media-pretreated cells, FSH-pretreated cells were significantly more responsive to subsequent challenge with FSH (*P* < 0.02, *n* = 6) or PSF (*P* < 0.05, *n* = 6). Pretreatment with FSH also induced a higher rate of basal progesterone production, which was apparent even after treatment with media for 3 days (*P* < 0.01, *n* = 6). Pretreatment with FSH for 5 days also maintained subsequent responsiveness to FSH or PSF (data not shown). We also examined the ability of pretreatment with PSF to maintain subsequent responsiveness to either PSF or FSH; however, unlike that with FSH, pretreatment with PSF did not maintain subsequent responsiveness to challenge with either FSH or PSF (*P* > 0.05, *n* = 6). Nonetheless, pretreatment with PSF did induce higher concentrations of basal progesterone production than did pretreatment with FSH (*P* < 0.01, *n* = 6).

Fig. 6. Induction of β -adrenergic responsiveness in rat granulosa cells. Freshly isolated granulosa cells were pretreated with media alone, FSH (30 ng ml^{-1}) or progesterone-stimulating factor (1000 ng ml^{-1}). After pretreatment for (a) 2 days or (b) 5 days, the cells were washed, and then challenged with media alone ($\square$) or isoproterenol ($10^{-6} \text{ mol l}^{-1}$; $\blacksquare$). The cells were incubated for a further 3 days and progesterone was measured by enzyme-linked immunoassay. Data points are means $\pm$ SEM from six independent experiments. * $P < 0.05$ versus media treatment; ** $P < 0.02$ versus media treatment; *** $P < 0.01$ versus media treatment.

Discussion

In the study reported here a progesterone-stimulating factor secreted by concanavalin A-stimulated rat splenocytes was purified over 500-fold and it was determined that this factor, like FSH, stimulated both progesterone and 20α -dihydroprogesterone production but, unlike FSH, stimulated only a minimal amount of oestrogen production. When compared in the same cultures, PSF maximally stimulated approximately the same amount of progesterone as did FSH, but required a substantially longer time to do so. This is in contrast to other agents, including EGF, GnRH, VIP and PACAP, that independently stimulate only 10–30% the amount of progesterone seen in response to FSH but do so with time courses similar to that of FSH (Clark, 1982; Jones *et al.*, 1982; Davoren and Hsueh, 1985; Zhong and Kasson, 1994). PSF also stimulated nearly twice the amount of 20α -dihydroprogesterone as did FSH; however, the time course for this effect was similar to that of FSH.

Even though these initial studies revealed differences between the steroidogenic activity of PSF and the other agents

Fig. 7. Maintenance of FSH and progesterone-stimulating factor, PSF, responsiveness in rat granulosa cells. Freshly isolated granulosa cells were pretreated with media alone, FSH (30 ng ml^{-1}) or PSF (1000 ng ml^{-1}). After pretreatment for 2 days, the cells were washed, and then challenged with media alone ($\square$), FSH (10 ng ml^{-1} ; $\square$) or PSF (400 ng ml^{-1} ; $\blacksquare$). The cells were incubated for a further 3 days and progesterone concentration was measured by enzyme-linked immunoassay. Data points are means $\pm$ SEM from six independent experiments. * $P < 0.05$ versus media treatment; ** $P < 0.02$ versus media treatment; *** $P < 0.01$ versus media treatment.

described above, we determined whether the activity of PSF might still result from the presence of one of these other agents. Initial experiments demonstrated that the steroidogenic activity of PSF could not be attributed to the presence of an FSH-like molecule, because PSF did not contain FSH immunoreactivity nor did it contain FSH receptor-binding activity. Moreover, PSF activity could not be attributed to an EGF-like molecule, because PSF did not contain EGF receptor-binding activity. It was thought unlikely that the other smaller peptides (GnRH, VIP and PACAP) could be responsible for the steroidogenic activity of PSF, because they would have been removed during the dialysis and subsequent chromatographic steps used in the purification of PSF. Consequently, these data indicate that PSF is distinct from FSH, EGF, GnRH, VIP and PACAP.

The chromatographic procedure used in the purification of PSF from conditioned splenocyte media isolated two distinct progesterone-stimulating activities; one activity flowed through the heparin-agarose column and one activity bound to the column but could be eluted by NaCl. The non-retained activity was difficult to detect upon heparin-agarose chromatography (25–150 min) probably because of its low concentration in the eluant. However, it was clearly present upon DEAE chromatography of dialysate. Initial characterization of this activity showed that it was quite different from the heparin-binding material, and was much more anionic and much larger in apparent size; whether this activity is related to the heparin-binding activity is unclear. The heparin-binding activity was purified over 500 times from material present in the splenocyte culture supernatants; however, the active fractions from the Mono S column still contained several proteins as assessed by SDS-PAGE. Subsequent attempts to purify this activity further to a single protein band resulted either in complete loss of activity or no further purification. Thus, two or more of the proteins seen on SDS-PAGE may be required to

elicit the steroidogenic activity present in this preparation, or the remaining proteins may have very similar physical and chemical properties, making them difficult to separate by chromatography.

While the steroidogenic activities of PSF and FSH showed similarities, their actions with regard to the induction of granulosa cell responsiveness to other agents differed markedly. As demonstrated here and by Hsueh *et al.* (1984), pretreatment of granulosa cells with FSH induced subsequent responsiveness to LH/CG, prolactin and isoproterenol; however, pretreatment with PSF induced responsiveness only to isoproterenol. Previous work has shown fundamental differences in the induction of these three responses. Binding studies have demonstrated the presence of β -adrenergic receptors on the surface of freshly isolated rat granulosa cells (Aguado and Ojeda, 1984), but activation of these receptors with β -agonists does not stimulate steroid production unless the cells have been previously exposed to FSH (Adashi and Hsueh, 1981). This type of data has been interpreted as indicating that FSH is required to couple β -adrenergic receptors to adenylate cyclase. In contrast, LH/CG and prolactin receptors are not present on the surface of freshly isolated rat granulosa cells but, following exposure to FSH, these receptors are induced through gene transcription and translation (Wang *et al.*, 1979; Hu and Dufau, 1991; Piquette *et al.*, 1991). Thus, the present results suggest that PSF may be able to induce coupling of β -adrenergic receptors to adenylate cyclase, but may not be able to induce the transcriptional and translational processes necessary to produce granulosa cell responsiveness to LH/CG and prolactin.

Although the actions of a variety of cytokines on granulosa cells have been investigated (Kasson and Gorospe, 1989; Adashi *et al.*, 1989; Gorospe *et al.*, 1992; Dorrington *et al.*, 1993), PSF remains essentially unique in that it can independently stimulate steroidogenesis in non-gonadotrophin-primed cells. Only one other immune cell-derived activity, produced by peritoneal macrophages, increases progesterone production in a dose-dependent manner in human and mouse luteinized granulosa cells (Kirsch *et al.*, 1981; Halme *et al.*, 1985). This activity has characteristics similar to those of the splenocyte-derived activity investigated here, in that both are heat- and trypsin-sensitive, stimulated by concanavalin A and have two apparent sizes on gel filtration chromatography, 26 and 41 kDa for the macrophage activity (Kirsch *et al.*, 1983) and 27 and 55 kDa for the splenocyte activity (Ness and Kasson, 1993). However, unpublished, preliminary studies (J. M. Ness and B. G. Kasson) have shown that the splenocyte activity is associated with slowly adherent splenocytes (thought to be lymphocytes) rather than rapidly adherent splenocytes (thought to be macrophages). Although some of the characteristics of PSF resemble those of the macrophage factor, these two factors are apparently produced by different types of cell.

In the present study, several characteristics of PSF activity, but not their potential physiological roles, have been defined. Because PSF activity is produced by splenocytes, which are composed mainly of lymphocytes with a limited number of macrophages, it is possible that PSF activity secreted from these leukocytes, either in the spleen or in the circulation, could affect ovarian function through an endocrine mechanism.

Alternatively, because T lymphocytes and macrophages are also present within rat ovaries (Adashi, 1990; Brännström *et al.*, 1993), intraovarian leukocytes may produce this activity, which could then affect ovarian function through a paracrine mechanism. Although physiological stimulators of PSF activity have not yet been identified, concanavalin A binding sites within the ovary have been documented (Lee and Ryan, 1979; Hsueh *et al.*, 1984). These sites may represent receptors for physiological stimulators of PSF that are nonspecifically activated by concanavalin A. The nature of these sites and their potential relationship to PSF remains to be investigated.

The present study showed that PSF stimulates progesterone production but does not induce significant oestrogen production or any other differentiated functions of granulosa cells. These observations, combined with the finding of Brännström *et al.* (1993) that the number of certain ovarian leukocyte subtypes increases during the luteal phase of the cycle, suggest that PSF may play a more significant role during the luteal phase of the cycle, when there is prominent progesterone production, rather than during the follicular phase of the cycle. However, further studies on when and where PSF may be produced within the ovary are required to establish these hypotheses. Nonetheless, in the light of the very limited number of factors that independently stimulate granulosa cell function, the actions of PSF are unique. The activity of PSF may provide an important link between intraovarian leukocytes and the regulation of ovarian function as well as a mechanism for diversifying the regulation of steroidogenesis in granulosa and luteal cells.

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