SIGNAL TRANSDUCTION IN THE SERTOLI CELL: SERUM MODULATION OF THE RESPONSE TO FSH

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Summary—Immature Sertoli cells of the testicular seminiferous tubule maintain the expression of their differentiated phenotype when cultured in unsupplemented medium. In preliminary experiments we observed that foetal bovine serum (FBS) stimulates polyphosphoinositides (PI) hydrolysis in Sertoli cells. We then evaluated the effect of serum on the function of the immature Sertoli cell in culture, in terms of cAMP and estrogen production. Treatment of Sertoli cells for 30 min with 1–10% FBS had no effect on basal cAMP accumulation but abolished the response to FSH. The serum concentration producing half-maximal inhibition of the FSH-dependent cAMP accumulation was 0.5–1%. Comparison of the FSH-dose-response in the absence or presence of serum showed a decreased maximal response when serum was present. Sertoli cells exposed to serum were also less responsive to the β-adrenergic agonist isoproterenol, to cholera toxin, and to forskolin. The serum inhibition was rapidly reversed upon removal of serum or incubating the cells with the phosphodiesterase inhibitor MIX (methyl-isobutyl-xanthine). Similarly to what observed with cAMP, serum affected androgen aromatization stimulated by FSH, isoproterenol, cholera toxin, forskolin and dibutyryl cAMP.

These data indicate that factors present in serum can act as modulators of the Sertoli cell function in vitro by rapidly and reversibly inhibiting the cAMP and steroidogenic response of the Sertoli cell to FSH.

INTRODUCTION

The Sertoli cell of the mammalian testis serves as a nursing cell for developing germ cells, a function thought to be under the control of the gonadotropin FSH [1, 2]. The concept is emerging that gonadotropin action is modulated by a number of paracrine and autocrine factors [3, 4]. Opioids, adenosine, and growth factors have been demonstrated to integrate Sertoli cell activities [5–7]. The multiplicity of signals affecting the Sertoli cell suggests that multiple second messengers are used to modulate the function of these cells. It is well known that FSH acts via adenylate cyclase activation. The existence of another major transduction pathway (that based on PI turnover) may be inferred by recent data showing that Sertoli cells contain a functionally active protein kinase C (pkC) and that germ cell factors activate pkC-dependent phosphorylations in Sertoli cells [8–10].

Our research has focused on the interactions between the two pathways of signal transduction. In a previous paper [9] we demonstrated that tumor promoters which stimulate pkC affect Sertoli cell response to FSH: both cAMP and estrogen productions are significantly inhibited in the presence of phorbol esters. We have, therefore, attempted to identify physiological factors which can stimulate pkC in Sertoli cells. Under our experimental conditions individual growth factors known to act via PI hydrolysis [11, 12] did not exert any significant effect on the Sertoli cell response to FSH (unpublished observations). We here report that serum, which contains many of these factors [13], has profound effects on both PI turnover and the response to FSH of the Sertoli cell.

EXPERIMENTAL

Sertoli cell culture

Cell cultures were prepared from 15-day old Wistar rats following the procedure of Dorrington et al. [14] and cultured in Minimum Essential Medium (MEM) with Earle's salts at 32°C in a controlled atmosphere of 95% air–5% CO_2. The percentage of peritubular cells contaminating Sertoli cell culture, as established by fibronectin synthesis [15], was on the order of 2–5%. All treatments were started on the fourth day of culture.

Assay of cAMP production

At the end of the incubations with the substances to be tested, media were collected, boiled for 15 min,

and then centrifuged at 3000 rpm for 10 min. Intracellular cAMP was extracted with ice-cold 95% ethanol plus 0.1% trichloracetic acid (TCA). The accumulated extra- and intracellular cAMP were measured by RIA [16] after suitable dilution and acetylation of the samples [17]. The RIA employing an antibody that was a generous gift of Dr Steiner, had a sensitivity of 2–4 fmol cAMP/tube; intraassay and interassay coefficients of variation were 5 and 10% respectively. RIA performed before and after sample purification by ion exchange chromatography gave essentially the same results. The low amount of serum present in treated samples did not interfere with the assay.

Protein content was measured according to Lowry *et al.* [18] using bovine serum albumin (BSA) as standard.

Assay of androgen aromatization

To evaluate the conversion of androgen to estrogen, Sertoli cells were incubated with $0.5 \mu M$ androstendione and the test compounds as specified in the Results. After 24 hr, media were collected and centrifuged at 3000 rpm for 10 min. RIA of estrogen was performed according to the method of Van Damme [19] employing an antiserum that was a generous gift of Radim S.R.L. with the following cross-reactivities: 17β-estradiol, 100%; estrone, 1.2%; estriol, 0.5%; and progesterone, <0.1%. The estrogen RIA had a sensitivity of 6 pg/tube, and intraassay and interassay coefficients of variation were 9 and 14%, respectively. To equalize the serum effect on the assay, tubes containing the standard curve and untreated samples received the same amount of serum that was present in treated samples. Basal production of estrogen by the Sertoli cell was usually below the sensitivity of the assay. RIA performed before and after extraction with ether gave the same results.

Protein synthesis

Sertoli cells were cultured for 24 h with serum or FSH alone or FSH plus serum, then labeled for 5 h with 10 μCi/ml [^{3}H]leucine. At the end of the incubation, cells were extensively washed with PBS and isotope incorporation was determined in the acid precipitable material.

Inositolphosphates production

To measure inositolphosphates production, Sertoli cells were labeled for 24 h with 5 μCi/ml [^{3}H]myoinositol. After labeling, cells were rinsed 5 times with fresh medium and reincubated for 10 min with 20 mM LiCl and for additional 10 min with serum. The incubation was stopped removing medium and adding ice-cold 10% TCA. Extraction of the inositolphosphates, neutralization of the samples and ion-exchange chromatography were performed as described elsewhere [20, 21].

Statistics

Dose–response studies were analyzed by fitting the data with the four parameter logistic equation according to the method of De Lean *et al.* [22].

Materials

[^{3}H]estradiol, [^{3}H]leucine, [^{3}H]myoinositol and [^{125}I]-succinyl-cAMP were obtained from New England Nuclear Corporation (Boston, MA). Androstendione, globulins, cAMP, dibutyryl cyclic AMP (dbcAMP), bovine serum albumin, cholera toxin, Dowex resin, and charcoal were from Sigma Chemical Corporation (St Louis, MO). Eagle's Minimum Essential Medium and sera were obtained from Grand Island Biological Company (Grand Island, NJ). Forskolin was purchased from Calbiochem (San Diego, CA). MIX (methyl-isobutyl-xantine) was from Aldrich (Milwaukee, WI) and Ro 20–1742 (4-(3-Butoxy-4-methoxybenzyl)2-imidazolidinone) was a gift from Hoffman-LaRoche (Nutley, NJ). All other reagents were of analytical grade from BDH (London), Hoechst (Frankfurt, F.R.G.), or by Merck (Darmstadt, F.R.G.). Ovine FSH-S15 was supplied by the NIAKMDD rat Pituitary Hormone Distribution Program.

RESULTS

Effect of serum on polyphosphoinositides turnover

In the calcium-phospholipid-dependent signal transduction system agonist binding to its receptor leads to the hydrolysis of phosphatidylinositol-disphosphate (PIP2) and the release of the second messengers inositol trisphosphate (IP3) and diacylglycerol (DG) [23, 24].

To evaluate whether serum activates this pathway we have measured PI hydrolysis in control and FBS-treated Sertoli cells. The results obtained (Table 1) show that serum significantly stimulates PI hydrolysis in Sertoli cell pre-labelled with [^{3}H]-myoinositol. Serum was active in inducing PI hydrolysis after dialysis or incubation for 15 min at 100°C. The stimulatory effect of serum was rapid and dose-dependent with a ED_{50} of 1–2% (Monaco *et al.*, paper submitted).

Table 1. Effect of serum on PI hydrolysis in the Sertoli cells

Treatment	Inositolphosphates (cpm/dish × 10^{-3})		
	IP1	IP2	IP3
Control	2.8 ± 0.18	0.74 ± 0.06	0.80 ± 0.04
FBS (1%)	5.3 ± 0.26	1.74 ± 0.11	2.12 ± 0.09
Dialyzed FBS	5.8 ± 0.50	1.79 ± 0.23	2.23 ± 0.10
Heat-inactivated FBS	5.6 ± 0.67	1.43 ± 0.16	2.18 ± 0.26

Sertoli cells were labelled for 24 h with [^{3}H]myoinositol and then stimulated with serum (1%) for 10 min. Inositolphosphates were measured as described in the Experimental. Each point is the mean ± SE of three different dishes.
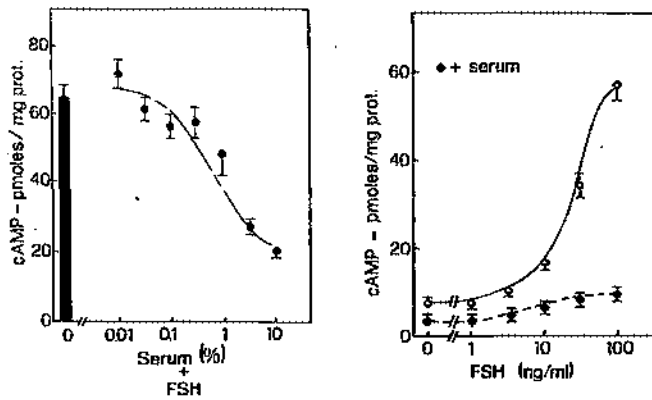
Fig. 1. Serum effect on cAMP accumulation stimulated by FSH. (a) Sertoli cells were incubated for 30 min with 100 ng/ml FSH in the absence or presence of increasing amounts of FBS. (b) Cells were incubated for 30 min with increasing concentrations of FSH in the absence or presence of FBS (3%). At the end of the incubation, medium was removed, and intracellular cAMP was measured by RIA. Each point is the mean ± SE of three different dishes, each assayed in duplicate.

Effect of serum on cAMP accumulation by the Sertoli cell

To investigate whether PI hydrolysis activation interferes with the adenylate cyclase system, we evaluated the effect of serum on the Sertoli cell response to FSH in terms of cAMP and estrogen production.

Sertoli cells were maintained in culture in MEM for 3 days and on the fourth day of culture were exposed for 30 min to foetal bovine serum (FBS). Under basal conditions cAMP levels were not affected by serum. On the contrary, serum added together with FSH had marked inhibitory effect on intracellular and extracellular cAMP accumulation. The concentration of FBS producing half-maximal inhibition was 0.5–1%, and FSH-stimulated cAMP accumulation was reduced by 70% in the presence of 10% FBS (Fig. 1a). When cells were incubated with or without 3% serum and increasing concentrations of FSH, serum inhibited the maximal response to FSH with no effect on the cell sensitivity to FSH (Fig. 1b).

Inhibition of the FSH-dependent cAMP accumulation was present whether cells were exposed to sera from different species and even after extensive dialysis of serum against MEM, or serum inactivation at 75°C for 15 min (Table 2). The serum-dependent inhibition was rapidly reversible upon serum removal (data not shown).

Table 2. Effect of serum on cAMP accumulation

Treatment	pmol cAMP/mg protein
Control	11.1 ± 3.2
FBS (3%)	7.5 ± 1.4
FSH (100 ng/ml)	68.0 ± 9.3
FSH + FBS	23.6 ± 2.7
FSH + dialysed FBS	28.1 ± 5.1
FSH + heat-inactivated FBS	23.0 ± 4.7

Sertoli cells were treated for 30 min in the presence or absence of FSH (100 ng/ml) and with or without FBS (3%). At the end of the incubation, medium was removed, and intracellular cAMP was measured by RIA. Each point is the mean ± SE of three different dishes, each assayed in duplicate.

Table 3. Serum inhibition of cAMP accumulation stimulated by FSH, cholera toxin and forskolin

	pmol cAMP/mg protein	
Treatment	−serum	+serum
Control	16.0 ± 3	15.5 ± 3
FSH (100 ng/ml)	116.5 ± 23	53.2 ± 14
Cholera toxin (1 µg/ml)	51.2 ± 7	20.4 ± 3
Forskolin (10 µM)	167.3 ± 20	94.9 ± 7

Sertoli cells were incubated for 30 min with the indicated substances in the presence or absence of 3% FBS. At the end of the incubation medium was removed,and intracellular cAMP was measured by RIA. Each point is the mean ± SE of three different dishes, each assayed in duplicate.

A possible explanation of the serum-dependent inhibition is that it is due to interference with FSH binding to its receptor. We then measured cAMP accumulation in the presence or absence of serum after stimulation with the β-adrenergic agonist isoproterenol and the non-hormonal stimulators of adenylate cyclase, cholera toxin [25] and forskolin [26]. cAMP accumulation was reduced in the presence of serum under all conditions (Table 3).

When cells were cultured in presence of the phosphodiesterase inhibitor MIX the effect of serum was abolished. On the contrary serum inhibition of the FSH response was still present when Ro 20-1742, an inhibitor of calcium-calmodulin independent phosphodiesterases, was used (Table 4).

Table 4. Effect of phosphodiesterase inhibitors on the serum inhibition

	pmol cAMP/mg protein	
Treatment	−serum	+serum
FSH (30 ng/ml)	30.0 ± 1	15.6 ± 3
FSH + MIX (0.5 mM)	184.7 ± 26	178.2 ± 17
FSH + Ro 20-1742 (0.5 mM)	134.9 ± 12	74.4 ± 10

Sertoli cells were incubated for 30 min with FSH in the absence or presence of 3% FBS and with or without phosphodiesterase inhibitors. At the end of the incubation, medium was removed, and intracellular cAMP was measured by RIA. Each point is the mean ± SE of three different dishes, each assayed in duplicate.
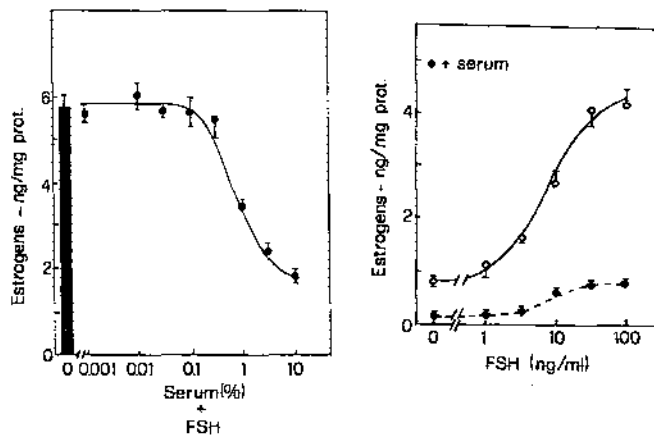
Fig. 2. Serum effect on FSH-stimulated androgen aromatization. (a) Sertoli cells were incubated for 24 h with 100 ng/ml FSH and androstenedione (0.5 μM) in the absence or presence of increasing amounts of FBS. (b) Cells were incubated for 24 h with androstenedione and increasing concentrations of FSH in the absence or presence of FBS (3%). At the end of the incubation medium was removed, and estrogen accumulated in the medium was measured by RIA. Each point is the mean ± SE of three different dishes, each assayed in duplicate.

Serum inhibition of the steroidogenic response

We also evaluated the effect of serum on androgen aromatization, a specific parameter of the Sertoli cell distal to cAMP accumulation. Similarly to cAMP, FSH-stimulated androgen aromatization was reduced in cells incubated in the presence of serum for 24 h (Fig. 2a). The dose of serum producing half maximal inhibition was again 0.5–1%. Furthermore, the sensitivity of the cell to FSH was not affected by serum, but only the maximal response in terms of estrogen produced was reduced (Fig. 2b). The FBS inhibition of androgen aromatization was not caused by a decreased substrate availability to the Sertoli cell, because inhibition was evident with androstendione concentrations in the medium up to 100 μM (data not shown). Serum also blocked steroidogenesis stimulated by forskolin, cholera toxin (Table 5), and dibutyryl cAMP (Fig. 3), thus showing that an effect distal to cAMP accumulation is responsible for the impaired response.

Effect of serum on protein synthesis

Sertoli cell viability was not affected by serum as assessed by morphological examination of the cultures. In addition, under basal and FSH-stimulated
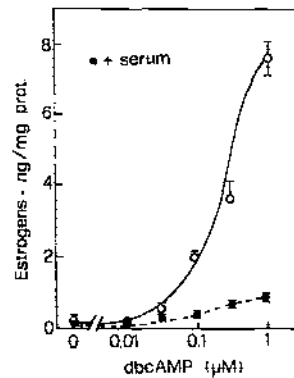

Fig. 3. Serum effect on dbcAMP-stimulated androgen aromatization. Sertoli cells were incubated for 24 h with androstenedione and increasing concentrations of dbcAMP in the absence or presence of FBS (3%). At the end of the incubation medium was removed, and estrogen accumulated in the medium was measured by RIA. Each point is the mean ± SE of three different dishes, each assayed in duplicate.

conditions, serum increased [3H]-leucine incorporation (Table 6).

DISCUSSION

The data reported in this study show that serum modulates the response of Sertoli cell to FSH. Both

Table 5. Serum inhibition of androgen aromatization stimulated by FSH, cholera toxin and forskolin

Treatment	ng Estrogen/mg protein	
	− serum	+ serum
FSH (100 ng/ml)	10.3 ± 0.63	3.01 ± 0.07
Cholera toxin (1 μg/ml)	5.5 ± 0.38	2.01 ± 0.24
Forskolin (10 μM)	7.9 ± 0.65	1.96 ± 0.18

Sertoli cells were incubated for 24 h with the specified compounds in the presence of absence of 3% FBS and in MEM containing androstendione (0.5 μM). Estrogen accumulated in the medium was measured by RIA. Each point is the mean ± SE of three different dishes, each assayed in dulplicate.

Table 6. Effect of serum on [3H]leucine incorporation by Sertoli cell cultures

Treatment	[3H]leucine (cpm/μg protein)	
	− serum	+ serum
Basal	102.0 ± 13.9	153.3 ± 16.9
FSH (100 ng/ml)	219.6 ± 20.0	381.6 ± 35.8

Sertoli cell cultures were incubated for 24 h in the presence or absence of FBS (3%) and or FSH. and then labeled for 5 h with [3H]leucine. Isotope incorporation was determined by precipitation of the proteins by TCA. Each point is the mean ± SE of three different dishes, each assayed in duplicate.

FSH-dependent stimulation of cAMP accumulation and androgen aromatization were inhibited in a dose-dependent fashion by serum derived from different species. This is consistent with those reports in which it was proposed to use the Sertoli cell steroidogenic response to gonadotropin as a parameter for an *in vitro* FSH bioassay. There it was consistently noticed that serum had inhibitory effects on the FSH-dependent androgen aromatization [19].

The effect of serum on FSH-dependent cAMP accumulation is maximal after 30 min and is rapidly reversible upon serum removal. The serum inhibition observed is not the result of generalized toxic effects. In fact FBS enhanced rather than inhibiting leucine incorporation into protein in Sertoli cells, thus indicating that the basic machinery of protein synthesis is not adversely affected under these incubation conditions. In this regard, it has been reported that the incubation of Sertoli cells with serum produces a stimulation of transferrin secretion [27].

We hypothesize that serum contains heat-stable, non dialyzable factors that affect the response of the Sertoli cell to the gonadotropin FSH. It has been reported that factors present in the serum competitively inhibit FSH binding to its receptor [28, 29]. It is possible that some of the effects we have observed are due to these naturally occurring receptor antagonists. However, it should be mentioned that cAMP and estrogen production were inhibited after FBS treatment, whether the cells were stimulated with FSH, cholera toxin or forskolin. The latter two compounds act on the hormone-dependent signal transduction system with a mechanism independent of receptor binding [25, 26]. Thus, the inhibition observed cannot be explained by the presence of inhibitors of the FSH binding. Sertoli cell aromatase activity induced by dbcAMP is also suppressed by serum. Such a finding is similar to what occurs in human adipose stromal cells. In these cells it has been reported that serum and growth factors like EGF, FGF and PDGF block the dbcAMP-dependent stimulation of aromatase [30, 31]. It is then possible that growth factors present in serum are responsible for the effects we have described. Growth factors have been shown to inhibit FSH-dependent estrogen production also in rat granulosa cells [32].

The data that we have reported are consistent with the idea that serum derived factors exerts their inhibitory effects by regulating multiple steps of the gonadotropin-dependent cascade.

We previously demonstrated that phorbol esters and other pkC activators [9] block Sertoli cell response to FSH at two sites: they inhibit FSH-dependent cAMP accumulation and androgen aromatization. Here we report that in Sertoli cells a similar effect is induced by serum which, in these cells, stimulates PI turnover. It is possible, therefore, that the observed serum inhibition of FSH response is mediated, at least in part, by activation of PI turnover and subsequent pkC activation. Also the other

second messenger IP3 produced by PI hydrolysis might have a role in the inhibition of Sertoli cell function. The observation that serum effect is reversed by MIX but not by the calcium-independent phosphodiesterase inhibitor Ro 20-1742 suggests that serum stimulates cAMP degradation by activating a calcium-calmodulin dependent phosphodiesterase. On the other hand experiments with the ionophore A 23187 showed that an increase in intracellular calcium causes an inhibition of the FSH-stimulated cAMP accumulation (unpublished data).

Further studies of serum characterization will allow to identify the factor(s) which activate PI turnover and subsequently affect the Sertoli cell response to gonadotropin. It has been reported that seminiferous tubules at stages VII–VIII of the spermatogenic cycle produce a factor that inhibits androgen aromatization [33]. It remains to be determined if homology exist between serum-derived factor(s) that inhibit aromatase and the factor secreted locally within the testis.

In conclusion our data demonstrate that a phospholipid-dependent pathway is present in the Sertoli cell and possibly interacts with the adenylate cyclase system.

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