Rat Sertoli cells acquire a β -adrenergic response during primary culture*

(seminiferous tubules/vimentin phosphorylation/isoproterenol/(-)-[¹²⁵I]iodopindolol binding)

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ABSTRACT Two-dimensional polyacrylamide gel electrophoresis and the radioligand (-)-[¹²⁵I]iodopindolol (¹²⁵I-Pin) have been used to study isoproterenol-dependent protein phosphorylation and β -adrenergic receptor availability, respectively, in cultured Sertoli cells and freshly isolated seminiferous tubular segments of sexually immature and mature rats. Sertoli cells prepared from sexually immature rats show progressive ¹²⁵I-Pin binding in primary cultures that correlates with isoproterenol-induced cell shape changes, redistribution of immunoreactive vimentin, and phosphorylation of this intermediate filament protein. The development of ¹²⁵I-Pin binding to Sertoli cell lysates is blocked by cycloheximide. Seminiferous tubules do not show significant isoproterenol-dependent vimentin phosphorylation nor ¹²⁵I-Pin binding. However, vimentin phosphorylation can be induced by follicle-stimulating hormone or a cyclic nucleotide analog. This study stresses the need for correlating pharmacological-induced responses observed in Sertoli cell primary cultures with those in the intact seminiferous tubule.

The Sertoli cell, a somatic cell component of the seminiferous tubule, is the primary target for follicle-stimulating hormone (FSH). FSH action on rat Sertoli cells in primary culture is mediated in part by the activation of cAMP-dependent protein kinases (1, 2). Activation of cAMP-dependent protein kinases results in the phosphorylation of specific protein substrates (3), which in turn modulate many of the subsequent physiological cell responses (4). We reported that FSH stimulates the phosphorylation of various Sertoli cell intracellular proteins, including the intermediate filament protein vimentin (5, 6). FSH-dependent phosphorylation of vimentin is preceded by a surge of both intracellular cAMP and protein-bound cAMP levels (6–8), which are maximized by using a phosphodiesterase inhibitor. Protein-bound cAMP levels reflect the binding of cAMP to regulatory subunits of cAMP-dependent protein kinases as determined by using a photoaffinity analog (8). Stimulation of cultured Sertoli cells with isoproterenol results in an increase of intracellular cAMP levels that also is enhanced by the presence of a phosphodiesterase inhibitor (9–12). Isoproterenol mimics several effects induced on cultured Sertoli cells by FSH and/or cyclic nucleotide analogs (12). Although the presence of β -adrenergic receptors in cultured Sertoli cells has been reported (13), the physiological significance of β -adrenergic agonist action on components of the seminiferous tubule is not known. The question then arises: to what extent are isoproterenol responses induced in cultured Sertoli cells reproduced by this cell within the intact seminiferous tubule? This is a relevant question in light of recent evidence obtained from the study of rat hepatocytes showing that a β -

adrenergic response is acquired by these cells during primary culture as the result of newly synthesized β -adrenergic receptors (14, 15). This possibility was examined in both primary cultures of Sertoli cells and freshly isolated seminiferous tubules by comparing FSH and isoproterenol-induced protein phosphorylation. These findings were correlated with the binding of the radioligand (-)-[¹²⁵I]iodopindolol (¹²⁵I-Pin) to β -adrenergic receptors. It was found that, although in cultured Sertoli cells both FSH and isoproterenol induced the phosphorylation of vimentin and other intracellular proteins, only FSH, not isoproterenol, stimulated protein phosphorylation in isolated seminiferous tubules. These findings correlated with the time-dependent acquisition of ¹²⁵I-Pin binding to β -adrenergic receptors by cultured Sertoli cells. Sertoli cells or any other cell of the intact seminiferous tubule could be shown to contain β -adrenergic receptors.

MATERIALS AND METHODS

Materials. Ovine FSH (NIH-oFSH-S15) was from National Hormone and Pituitary Program of National Institute of Arthritis, Metabolism, and Digestive Diseases. *N*⁶,*O*²-Dibutyryl adenosine 3',5'-phosphate (Bt₂cAMP), L-isoproterenol, (-)-isoproterenol bitartrate, and cycloheximide were from Sigma. 3-Isobutyl-1-methylxanthine (iBuMeXan) was from Aldrich. Carrier-free [³²P]orthophosphate was from New England Nuclear. Gel electrophoresis reagents were from Bio-Rad and Pharmacia. Vimentin rabbit antiserum was provided by K. Burridge. Fluorescein isothiocyanate-conjugated goat anti-rabbit IgG was from Cappel Laboratories (Cochranville, PA). (-)-Pindolol was a generous gift from Sandoz Pharmaceutical. ¹²⁵I-Pin was prepared by the method of Barovsky and Brooker (16).

Sertoli Cell Culture. Sertoli cell cultures were prepared as described (17) from sexually immature rats (20–22 days old). Contamination with peritubular cells was less than 5%. Contamination with spermatogenic cells (¹²⁵I-Pin binding studies) was about 25%. Sertoli cell plating concentration was 1×10^6 cells/ml.

Seminiferous Tubules. Seminiferous tubules from sexually mature rats (75–80 days old) were gently teased apart in Hanks' balanced salt solution under sterile conditions and examined immediately by transillumination (18) with a stereomicroscope. Pools of interstitial tissue-free seminiferous tubular segments (6–8 segments, 2–3 mm in length) corresponding to specific spermatogenic stages or to a mixture of several spermatogenic stages were collected in HBSS and

Abbreviations: ¹²⁵I-Pin, (-)-[¹²⁵I]iodopindolol; FSH, follicle-stimulating hormone; Bt₂cAMP, *N*⁶,*O*²-dibutyryl adenosine 3',5'-phosphate; iBuMeXan, 3-isobutyl-1-methylxanthine.

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then transferred to wells of a multiple-well plate containing free Eagle's minimum essential medium (EME medium) (for [^{32}P]orthophosphate-labeling) or to culture tubes (for β -adrenergic receptor determination). Pools of seminiferous tubules of sexually immature rats also were examined. Specific spermatogenic stages were verified by light microscopic examination of sections (18, 19) or "squash" preparations stained with aceto-orcein.

Time-Lapse Cinematography. Sertoli cells were grown on round coverslips for 4 days and placed in a Dvorak-Stotler controlled-environment culture chamber (Zeiss). Cells were examined with a Wild M40A inverted phase-contrast microscope, and cell-shape changes were recorded on Kodak SO-115 negative black-and-white film by using an Opti-Quip 16-mm system as described (7). Film was exposed at a framing rate of two frames per min.

Indirect Immunofluorescence. Immunocytochemical experiments were carried out as described (6, 7). Controls for specificity included (i) omission of primary antiserum, (ii) absorption of antiserum with an extract of intermediate filament proteins (6), and (iii) immunoprecipitation of [^{35}S]-methionine-labeled vimentin (6).

[^{32}P]Orthophosphate Labeling. Sertoli cells plated in culture dishes for 6–7 days were maintained in serum-free EME medium for 18–24 hr prior to incubation with the chemical agents FSH, isoproterenol, and/or cyclic nucleotide analogs. Cells were preincubated for 30 min with serum-free, phosphate-free EME medium before labeling to deplete the endogenous ATP pool. [^{32}P]Orthophosphate (250 $\mu\text{Ci}/\text{ml}$; 1 Ci = 37 GBq) was added to fresh serum-free, phosphate-free EME medium prior to or with chemical agents. The endogenous ATP pool was equilibrated within 90 min after exposure to [^{32}P]orthophosphate (not shown). Cells were incubated at 32°C for 4 hr in a humidified atmosphere. Dishes were rinsed with cold phosphate-buffered saline (pH 7.2), and then 200 μl of lysis buffer (20) was added. Cells were scraped from the dishes and dispersed by sonication. Seminiferous tubular segments were incubated in serum-free EME medium for 1 hr at 32°C. The medium was replaced with serum-free, phosphate-free EME medium, and the segments were incubated at 32°C for an additional hour. The medium was aspirated, and seminiferous tubular segments were labeled with [^{32}P]orthophosphate by addition prior to or with chemical agents as described above for Sertoli cells. Segments were incubated for 4 hr at 32°C. Incubation was terminated by rinsing the wells with ice-cold phosphate-buffered saline and then adding 150 μl of lysis buffer to each well. The segments were collected on ice and dispersed by sonication.

Two-Dimensional Polyacrylamide Gel Electrophoresis and Autoradiography. Aliquots of the sonicated cell and tissue samples were counted by spotting on filter paper squares as described (6, 21). Samples containing the same number of counts were then analyzed by two-dimensional polyacrylamide gel electrophoresis and autoradiography as described (5, 6, 21).

Binding of ^{125}I -Pin. Sertoli cells were washed with EME medium and incubated for 20 min with an ice-cold hypotonic buffer containing 1 mM Tris (pH 7.8) and 0.5 mM MgCl_2 , after which cells were visibly swollen. The buffer was removed, and cells were scraped with a rubber policeman. Seminiferous tubules were homogenized in 1.5 ml of hypotonic buffer with 15 strokes of a teflon/glass homogenizer. Lysates were collected and diluted to 3 ml with 20 mM Tris, pH 7.4/140 mM NaCl/2 mM MgCl_2 . Dilutions of 1:3, 1:9, 1:27, and 1:54 were made from this concentrated solution. All dilutions were assayed by incubation for 60 min at 25°C with 130 pM ^{125}I -Pin in either 100 μM HCl (to determine total binding) or 100 μM (–)-isoproterenol bitartrate in 100 μM HCl (to determine nonspecific binding). Cell membranes were collected onto glass fiber filters, washed to lower non-

specific binding, and assayed for radioactivity at about 82% efficiency. Proteins were determined by the method of Lowry. Dilution curves (not shown) demonstrate a linear relationship between the amount of ^{125}I -Pin bound versus protein concentration.

RESULTS

Sertoli Cell-Shape Changes Induced by Isoproterenol. We have reported that FSH and cyclic nucleotide analogs induce morphologically flat Sertoli cells in culture to adopt a stellate morphology (7). Whereas cyclic nucleotide analogs induced a persistent morphological transition, FSH-triggered cell-shape changes were transient, as they reversed progressively to control conditions within 24 hr (7). Therefore, it was of interest to document the effect of isoproterenol (10 μM) on Sertoli cell morphological response. Fig. 1A illustrates a time-lapse sequence showing that after 15 min of exposure to this chemical agent (iBuMeXan not present), most Sertoli cells adopted a stellate morphology. This morphological response was transient because 150 min later the same Sertoli cells regressed to a flattened state while isoproterenol was present in the culture medium. Other Sertoli cells present in the same preparation remained contracted and initiated recovery 4 hr after exposure to isoproterenol. Fig. 1B and C show the immunofluorescent distribution of vimentin in control cells and isoproterenol-treated Sertoli cells after 60 min. As reported for cultured Sertoli cells exposed to FSH or

FIG. 1. Time-lapse cinematographic sequence of cultured Sertoli cells prepared from a 22-day-old rat and exposed to isoproterenol (10 μM) 4 days after plating. Cells were placed in serum-free EME medium for 12 hr and then were exposed continuously to isoproterenol. Numbers show time in min after initial exposure to the agent. ($\times 150$.) (B and C) Immunocytochemical localization of vimentin in control Sertoli cells (B) and after 60 min of treatment with 10 μM isoproterenol (C) ($\times 240$.) The working dilution of primary antibody was 1:50.

Bt₂cAMP (6), the diffuse and perinuclear filamentous network of immunoreactive vimentin observed in control Sertoli cells (Fig. 1B) appeared to be restricted to the long cytoplasmic processes and the perinuclear region of morphologically responsive Sertoli cells (Fig. 1C). Although treatment with FSH and isoproterenol resulted in similar vimentin immunofluorescent patterns, the morphological response of Sertoli cells to isoproterenol was more rapid and extensive.

FSH- and Isoproterenol-Dependent Phosphorylation in Cultured Sertoli Cells. Because time-lapse cinematographic and immunocytochemical results indicated that both FSH and isoproterenol induced comparable responses, we examined the effect of these two agents on protein phosphorylation in cultured Sertoli cells in the presence or absence of the phosphodiesterase inhibitor iBuMeXan (0.2 mM). Treatment of Sertoli cells with FSH (10 μ g/ml) in the presence of iBuMeXan resulted in marked [³²P]orthophosphate uptake by four polypeptides of M_r/pI 58,000/5.1, 46,000/4.95, 48,000/5.0, and 26,000/5.6 (Fig. 2). Phosphoprotein of M_r/pI 58,000/5.1 has been identified as vimentin (6, 21), and two of the additional phosphoproteins (M_r/pI 46,000/4.95 and 48,000/5.0) probably represent degradation products of vimentin (5, 6, 22). The observed changes were consistent with an earlier study showing potentiation of cAMP action in the presence of iBuMeXan (6) and correlated with the observation that only a slight increase in the phosphorylation of these four polypeptides was induced by FSH in the absence of iBuMeXan (not shown). Treatment of Sertoli cells with isoproterenol (20 μ M) in the presence of iBuMeXan induced phosphorylation of the same four polypeptides comparable to that observed with FSH and iBuMeXan (Fig. 2). Although treatment with isoproterenol or iBuMeXan alone caused no change in the protein phosphorylation patterns (not shown), the cAMP analog Bt₂cAMP (0.5 mM) mimicked the phosphorylation changes induced by FSH/iBuMeXan and isoproterenol/iBuMeXan (Fig. 2). This observation indicated

that the effects of FSH and isoproterenol on protein phosphorylation were mediated by a cAMP-dependent mechanism after binding of these chemical agents to specific cell-surface receptors.

FSH- and Isoproterenol-Dependent Protein Phosphorylation in Isolated Seminiferous Tubular Segments. Our next approach was to correlate the protein phosphorylation patterns induced by FSH and isoproterenol in cultured Sertoli cells with those observed in intact seminiferous tubular segments, free of interstitial tissue, isolated by mechanical means from sexually immature and mature rats. Fig. 3 illustrates protein phosphorylation patterns of seminiferous tubular segments of spermatogenic stages VIII–IX identified by transillumination and verified by histological methods (not shown). FSH (10 μ g/ml) treatment resulted in only slight changes in the phosphorylation pattern of the indicated proteins when compared with the control (Fig. 3). However, incubation of seminiferous tubular segments with FSH in the presence of iBuMeXan (0.2 mM) caused a significant increase in the phosphorylation of five polypeptides of M_r/pI 58,000/5.1, 46,000/4.95, 48,000/5.0, 31,000/6.25, and 26,000/5.6 (Fig. 3). Four of these phosphoproteins appear to be the same FSH-dependent phosphoproteins as those observed in cultured Sertoli cells (compare with Fig. 2). The M_r/pI 31,000/6.25 polypeptide seemed to be unique to the seminiferous tubular segments. Interestingly, isoproterenol (20 μ M) in the presence (Fig. 3) or absence (not shown) of iBuMeXan had only a slight effect on the phosphorylation of these five polypeptides. Whereas iBuMeXan alone had no effect on the protein phosphorylation pattern (not shown), Bt₂cAMP (0.5 mM) effects were comparable to these observed after FSH and iBuMeXan treatment (Fig. 3). These results suggested a discrepancy in isoproterenol effect on protein phosphorylation in cultured Sertoli cells and incubated seminiferous tubular segments. Seminiferous tubular segments corresponding to sexually immature rats and to other

FIG. 2. Two-dimensional polyacrylamide gel electrophoretic analysis of protein phosphorylation patterns of cultured Sertoli cells prepared from sexually immature rats. Cells were incubated for 4 hr in the presence of [³²P]orthophosphate (250 μ Ci/ml) in phosphate-free, serum-free EME medium with (i) FSH (10 μ g/ml) and 0.2 mM iBuMeXan (MIX), (ii) isoproterenol (ISO, 20 μ M) and 0.2 mM iBuMeXan, or (iii) Bt₂cAMP (0.5 mM). The rectangle and square indicate relevant protein phosphorylation changes. Vimentin and its degradation products are indicated by the rectangle. Numbers indicate $M_r \times 10^{-3}$ markers. The pH range of the gel was 4.5–6.8 as determined by pH measurement of gel slices. Autoradiographs were obtained after exposure of the gels for 3 days.

FIG. 3. Two-dimensional polyacrylamide gel electrophoretic analysis of protein phosphorylation patterns of freshly isolated seminiferous tubular segments corresponding to the transition between spermatogenic stages VIII and IX. Segments were incubated in the presence of [32 P]orthophosphate (250 μ Ci/ml) for 4 hr with (i) FSH (10 μ g/ml) with or without iBuMeXan (MIX) (0.2 mM), (ii) isoproterenol (ISO, 20 μ M) with iBuMeXan (0.2 mM) and (iii) Bt₂cAMP (0.5 mM). The rectangle and squares indicate relevant protein phosphorylation changes. Vimentin and its degradation products are indicated by the rectangle. Numbers indicate $M_r \times 10^{-3}$ markers. The pH range of the gel was 4.5–6.8. Autoradiographs were obtained after exposing the gels for 12–15 days.

spermatogenic stages of sexually mature rats were examined with similar results.

125 I-Pin Binding to β -Adrenergic Receptors. A radioligand with high-affinity binding to β -adrenergic receptors (23, 24) was used to determine whether the differential isoproterenol-induced protein phosphorylation in cultured Sertoli cells and incubated seminiferous tubular segments was due to the availability of receptor sites. A time-course study was carried out to determine the temporal appearance of β -adrenergic receptors in cultured Sertoli cells prepared from sexually

FIG. 4. β -Adrenergic receptor content determined by 125 I-Pin binding to lysates of cultured Sertoli cells prepared from sexually immature rats. The abscissa is culture time in hours after plating at time 0. The points are averages of different experiments (indicated by numbers on the left of the points), each using triplicate samples. Bars represent SEMs.

immature rats. Fig. 4 shows at time 0 almost undetectable 125 I-Pin binding to cell membrane preparations of trypsin/collagenase-dissociated seminiferous epithelial samples (containing mainly Sertoli cells and premeiotic and early meiotic prophase spermatocytes). Radioligand binding to Sertoli cells was detected at 4 hr after plating in serum-supplemented EME medium (calculated β -adrenergic receptor content, 4.72 ± 1.41 fmol). β -Adrenergic receptor content values were 10.07 ± 0.17 and 9.73 ± 0.11 fmol at 12 and 48 hr, respectively.

We next examined the possibility that β -adrenergic receptors were synthesized *de novo* by Sertoli cells when cultured as monolayers for 24 hr. Table 1 shows 125 I-Pin specific binding activity to β -adrenergic receptors (expressed as average cpm and determined fmol per assay) of cultured Sertoli cells for 6 and 24 hr in the presence or absence of cycloheximide (10 μ M). Results indicate that cycloheximide blocks

Table 1. Specific 125 I-Pin binding activity to lysates of cultured rat Sertoli cells and freshly-isolated and incubated seminiferous tubules in the presence or absence of cycloheximide

Sample	Time, hr	n	Mean cpm	fmol/assay
Cultured Sertoli cells (sexually immature)				
Control	0*	3	528	0.133
	6	3	515	0.130
	24	3	1548	0.399
Cycloheximide	0*	2	264	0.066
	6	2	331	0.084
	24	3	403	0.102
Seminiferous tubules (sexually immature)				
Control	0†	2	72	0.018
	4	2	58	0.015
	24	2	83	0.021
Cycloheximide	0†	2	83	0.021
	4	2	65	0.016
Seminiferous tubules (sexually mature)				
Control	0‡	1	85	0.017
Cycloheximide	—	—	ND	ND

Values are averages of experiments (n) done with duplicate and triplicate samples. ND, not determined.

*Sertoli/spermatogenic cell ratio = 3.0.

†Sertoli/spermatogenic cell ratio = 1.86.

‡Sertoli/spermatogenic cell ratio = 0.25.

the development of ^{125}I -Pin specific binding. The insignificant binding of ^{125}I -Pin observed at time 0 in trypsin (0.25 mg/ml)/collagenase-treated samples was not due to trypsin impairment of radioligand binding to specific receptor sites. β -Adrenergic receptors are resistant to the used concentration of trypsin (23, 25). In fact, homogenized seminiferous tubules isolated from sexually immature and mature rats without enzymes displayed low ^{125}I -Pin binding when incubated in serum-supplemented EME medium for 0 and 4 hr in the presence or absence of cycloheximide (Table 1). The reduced ^{125}I -Pin binding to β -adrenergic receptors in seminiferous tubular segments of sexually immature rats was not age-dependent. Pools of seminiferous tubules of sexually mature rats also showed extremely low radioligand specific binding values (Table 1).

DISCUSSION

We have used the radioligand ^{125}I -Pin, a potent β -adrenergic antagonist with high affinity for β -adrenergic receptors and a low level of nonspecific binding (23, 24), to provide experimental evidence that rat Sertoli cells in primary culture acquire β -adrenergic receptors. The ability of cultured Sertoli cells to respond to isoproterenol in a cAMP-dependent manner has been determined by protein phosphorylation studies under conditions that enhance cAMP-dependent protein kinase activity. Previous studies have shown that isoproterenol first activates and then down-regulates the cAMP-generating pathway (9–12). However, a direct measurement of β -adrenergic receptor content in cultured Sertoli cells has not been attempted.

Sertoli cells exposed continuously to isoproterenol show a shape change that is transient and correlates with both a redistribution and phosphorylation of vimentin-type intermediate filaments. Similar findings were reported for cultured Sertoli cells stimulated with FSH (6). After isoproterenol or FSH treatment, cultured Sertoli cells become refractory to a subsequent stimulation with these agents, in part due to an increase in phosphodiesterase activity that accelerates cAMP catabolism (10, 11). It is possible that this desensitization process accounts for the transient nature of Sertoli cell shape changes documented by time-lapse cinematography (7). Vimentin phosphorylation induced by FSH, isoproterenol, and Bt_2cAMP represents a measure of both Sertoli cell responsiveness and cAMP-dependent protein kinase activation (6, 8). In fact, immunocytochemical studies have shown that, in the seminiferous tubule, vimentin is mainly associated with Sertoli and peritubular cells (26). Furthermore, FSH-dependent phosphorylation of vimentin in cultured peritubular cells was not detected (unpublished data). The functional role of this phosphoprotein remains to be determined. However, because incubated seminiferous tubular segments show neither vimentin phosphorylation nor ^{125}I -Pin binding, it is possible to conclude that isoproterenol responsiveness of Sertoli, peritubular, and spermatogenic cells *in vivo* may be reduced.

The major findings of the present study are: (i) the low binding of ^{125}I -Pin to homogenates of seminiferous tubules isolated from both sexually immature and mature rats, (ii) the time-dependent appearance of ^{125}I -Pin binding sites in cultured Sertoli cells, (iii) the apparent blocking effect of cycloheximide on the development of ^{125}I -Pin binding to β -adrenergic receptors in cultured Sertoli cells, and (iv) the apparent inability of Sertoli cells within the seminiferous tubule to exhibit ^{125}I -Pin binding after a 4-hr incubation—a condition that favors specific binding of this radioligand to cultured Sertoli cells. A possible interpretation of these findings is that, under normal conditions, the β -adrenergic receptor is

probably not present in the cellular components of intact seminiferous tubules. Upon being cultured, Sertoli cells acquire both β -adrenergic receptors and responsiveness to β -adrenergic agonists. This interpretation correlates with recent observations in adult male rat hepatocytes that are normally unresponsive to β -adrenergic receptor agonists but acquire adrenergic responsiveness after culturing (14, 15). Acquisition of β -adrenergic response by cultured hepatocytes was abolished by cycloheximide and α -amanitin (14) and was unaffected by factors present in serum-supplemented culture media. Serum-free, hormone-supplemented culture media did not suppress the β -adrenergic response nor [^{125}I]iodocyanopindolol binding affinity to receptor (15).

Findings reported in our study show the need for correlating cellular responses observed in primary culture with those in the intact tissue. However, the possibility cannot be disregarded that, under pathological conditions that alter testicular functions, Sertoli cells or other testicular cell components may display β -adrenergic responsiveness *in vivo*.

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