In Vitro Gonadotropin Stimulation of Bovine Sertoli Cell Ornithine Decarboxylase Activity

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The activity of ornithine decarboxylase (ODC) was determined in primary cultures of bovine Sertoli cells after gonadotropin stimulation *in vitro*. A significant increase in both ODC activity and putrescine content was noted 4 hours after FSH stimulation of cells derived from animals 7 to 14 days of age. Response in cells derived from animals 2 months of age was significantly less. Additionally, it was noted that both LH and hCG stimulated the ODC activity in bovine Sertoli cell preparations. These findings suggest that ODC activity in bovine Sertoli cells may be under FSH control and that LH or hCG may have significant FSH-like effects in this system. Also, it is suggested that ODC responsiveness decreases during bovine Sertoli cell maturation.

Key words: ornithine decarboxylase, Sertoli cell, gonadotropins, bovine, calf.

Ornithine decarboxylase (ODC; L-ornithine decarboxy-lyase, EC 4.1.1.17) has been suggested to be one of the enzymes critical for cellular trophic response (Russell et al, 1976). The activity of ODC is elevated under conditions of rapid growth (Janne et al, 1978; Fozard et al, 1980) and, in target organs, as a response to stimulation by either polypeptide or steroid hormones (Levine et al, 1973; Kobayashi et al, 1971; Russell and Taylor, 1971). Studies *in vivo* using either LH or FSH (Macindoe and Turkington, 1973; Reddy and Villee, 1975) have shown stimulation of rat testicular ODC activity and increased production of polyamines.

The purpose of the present study was to extend these observations under defined conditions using Sertoli cells isolated from bovine testis, cultured and stimulated *in vitro*.

Materials and Methods

Animals

Tissue used in all studies was obtained either through local beef producers or a slaughterhouse in Cambridge, Massachusetts. Testes were obtained by castration in adult animals or animals of 2 or 7 months of age and at the time of slaughter in animals 7 to 14 days of age.

Hormones and Reagents

Gonadotropins (NIH-FSH-S-13 and NIH-LH-21) were obtained through the National Pituitary Agency, NIAMDD hormone distribution program. Testosterone (T), human chorionic gonadotropin (hCG), adrenocorticotropic hormone (ACTH), and prolactin (PRL) were purchased from the Sigma Chemical Co. (St. Louis, Missouri). The phosphodiesterase inhibitor, 3-isobutyl-1-methylxanthine (MIX) was obtained from Aldrich Chemical Co. (Milwaukee, Wisconsin); L-[^{14}C]-1-ornithine hydrochloride with specific activity 59 mCi/mmole was obtained from New England Nuclear Corp. (Boston, Massachusetts); 5-Dimethylamino-1-naphthalenesulfonyl chloride (DANS-CL), putrescine, spermidine, and spermine chromatography standards were obtained from Sigma Chemical Co. All materials for tissue culture, including fetal calf serum, glutamine, and Eagles medium #2, were obtained from Grand Island Biological Co. (Grand Island, New York). All cul-

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tures were grown in Costar multiwell plates (Cambridge, Massachusetts) under standard conditions (37 C; atmosphere 5% CO_2: 95% air).

Cell Preparation

Methods for preparation of bovine Sertoli cells were adapted from those used to prepare rat Sertoli cells (Dorrington et al, 1975). Testes were minced into 1-mm fragments and enzymatically digested in 0.25% trypsin for 30 minutes at 37 C. The isolated tubules were collected by filtration over a wire grid and further digested with collagenase (1 mg/ml) for an additional 30 minutes. Single cells were than collected by filtration through nylon mesh, washed and plated at a density of 2.5×10^5 cells/cm^2. Original plating media consisted of Eagles #2 containing 10% fetal calf serum, 4 mM glutamine, and 1% penicillin and streptomycin. After 24 hours, this media was replaced with serum-free media (SFM; Eagles #2 containing 4 mM glutamine and 100 units/ml penicillin and 100 μg/ml streptomycin). All subsequent media were serum-free media. All cultures were maintained for a total of 48 hours prior to study. Cell viability was assessed by trypan blue exclusion at each stage of preparation and at time of stimulation.

Cell Stimulation

Media were removed from culture plates and, following washing, were replaced with SFM containing 0.1 mg/ml MIX either with added hormone (stimulated) or without added hormone (control). Cultures were then incubated for a standard 4-hour period (unless specified otherwise), harvested with a spatula (rubber policeman), and frozen at −70 C until time of assay. Immediately prior to assay, cells were thawed, sonicated for 30 seconds at 4 C, and centrifuged. Supernatants were assayed in duplicate for ODC activity.

ODC Assay

After the method of Osterman and Hammond (1977), 0.1 ml cell sonicate was added to the reaction mixture so that the final composition was 50 mM TRIS-HCl, *p*H 7.2; 0.4 mM L-ornithine; 0.5 μCi [^{14}C]-1-L-ornithine; 0.08 mM pyridoxal phosphate; 2.5 mM dithiothreital; and the final volume was 0.25 ml. Reaction was allowed to proceed for 1 hour at 37 C in sealed vessels which also contained 0.1 ml hyamine hydroxide in a separate well. At the end of the incubation, 0.125 ml 5 *N* H_2SO_4 was added to stop the reaction, and CO_2 evolution was allowed to proceed for an additional 90 minutes. Enzyme blanks were prepared by the addition of H_2SO_4 prior to incubation. Evolved CO_2, captured in hyamine hydroxide, was determined as radioactivity using a Beckman scintillation counter, and cell protein was determined using the BioRad protein assay (Bradford, 1976). Enzyme activity was expressed as pmole CO_2 evolved/mg protein/hour.

Polyamine Concentration

Intracellular putrescine levels were determined by fluorescence of their respective dansyl-Cl derivatives (Seiler, 1971), following separation by thin layer chromatography (TLC). Dansylation was performed using 10 mg DANS-CL in 2.5 ml acetone at room temperature for 16 hours. The products were extracted using toluene and separated by Silica gel TLC with a unidimensional solvent system of triethylamine:ethylacetate (9:1). Dansylated standards were prepared and cochromatographed for identification as well as recovery calculations. Fluorescent spots were identified, scraped, and quantitated using an Aminco fluorometer with a sensitivity of 25 pmole.

cAMP Response

Cultures were stimulated for 30 minutes at 37 C in SFM containing 0.1 mg/ml MIX and specified hormones. Cells were harvested by scraping into 2.0 ml ethanol and were then sonicated. Following centrifugation, the supernatant was removed, evaporated to dryness, and assayed in duplicate for cAMP content using materials purchased from Amersham-Searle Corp. (Arlington Heights, Illinois). Sensitivity of the assay was 0.25 pmole cAMP.

Testosterone Response

Cultures were stimulated for 24 hours at 37 C in SFM containing 0.1 mg/ml MIX and specified hormones. Media were then removed, centrifuged, and assayed in duplicate for testosterone content (Dufau et al, 1972; Abraham et al, 1972). Interassay and intra-assay coefficients of variation were 16.5 and 10.1%, respectively.

Electron Microscopy

Short-term primary cultures were established in either Lab-Tek Flaskettes (Lab-Tek Products, Miles Laboratories, Naperville, Illinois) or glass culture bottles containing standard, sterile 1 × 3 cm glass microscope slides. Within four days, after settling and attachment, these cultures were rinsed twice with Hank's balanced salt solution and fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer, *p*H 7.4, at room temperature for 4 to 18 hours. These cultures were then rinsed in the same buffer, treated in 1% OsO_4 in buffer, dehydrated through alcohols, and embedded in a thin layer of Maraglas resin. This was stripped from the glass substrate after 24 hours of polymerization and stained with toluidine blue. Appropriate areas of cellularity, as determined by light microscopy, were excised and cemented to standard epoxy blocks for electron-microscopic sectioning. Sections (100 nm) were cut parallel to the substrate plane, stained with lead citrate and uranyl acetate, and examined in a Philips EM 400 electron microscope.

Statistics

All data points represent the mean ± SE for duplicate determinations of six replicate plates. Statistical significance was determined using the Student's *t* test (*P* values as stated).

Results

The characteristics of bovine Sertoli cell cultures were first defined. Electron-microscopic examination at low magnification (Fig. 1) revealed a homogeneous population (>95%) of epithelial cells. Rare degenerative cell forms were noted. No fibroblasts or Leydig cells were detected morphologically. A higher magnification of a typical preparation obtained from 7- to 14-day-old bull calves (Fig. 2) demonstrates several features that are characteristic of Sertoli cells but are not observed, for example, in either germ cells or peritubular fibroblasts. These include round to oval nuclei (N) with occasional clefts (not illustrated here) which contain unusually prominent partite nucleoli

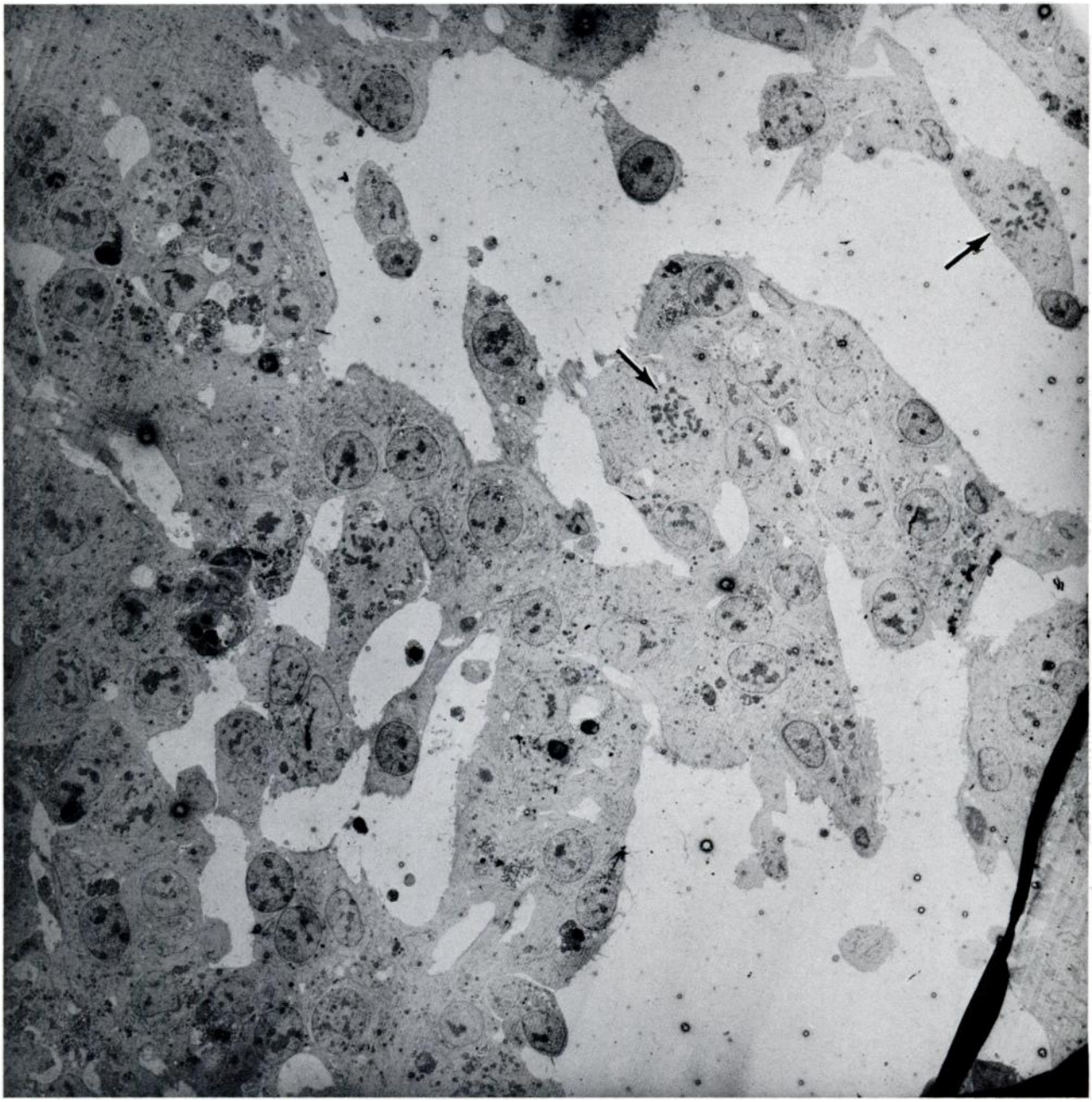

Fig. 1. Low-power electron photomicrograph of bovine sertoli cell preparations. This photomicrograph demonstrates the homogeneity of Sertoli cells in tissue culture from bull calves aged 7 to 14 days. Two of these cells are undergoing mitosis (arrows) (magnification 870).

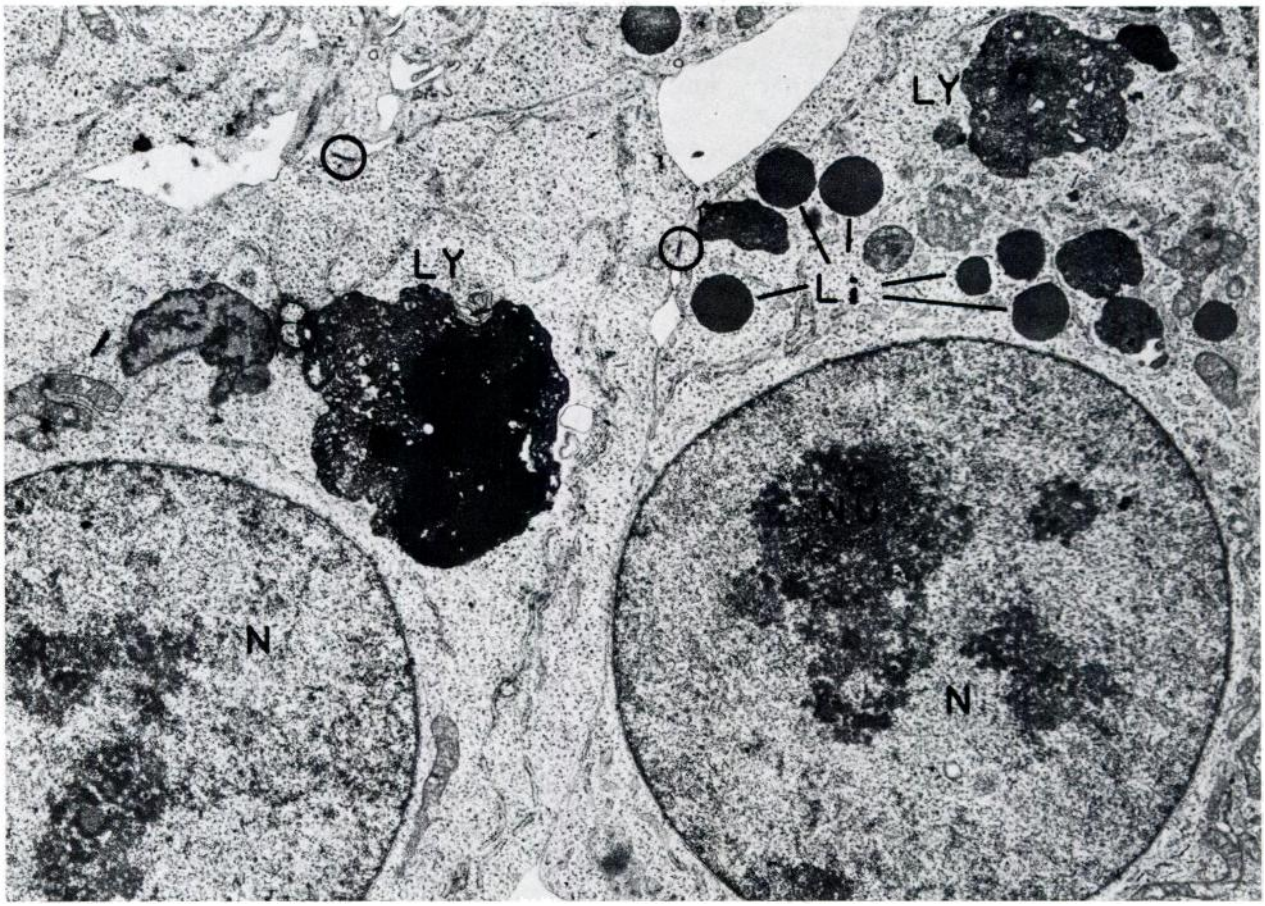

Fig. 2. Ultrastructural examination of bovine Sertoli cell preparations. Two Sertoli cell nuclei (N) with prominent partite nucleoli (NU) are present at the base of this micrograph; unusually prominent phagolysosomes (LY) are seen above the nuclei, a characteristic finding in Sertoli cells that are phagocytic. Lipid droplets (Li), another common finding in Sertoli cells, are also numerous. Membrane specializations (encircled) were generally zonula adherens; highly characteristic gap junctions were inapparent, as was extensive smooth endoplasmic reticulum (magnification 7800).

(NU). Such elaborate nucleoli are not observed in other testicular cell types. Very prominent phagolysosomes (LY; phagocytic vacuoles formed by the fusion of primary lysosomes and phagocytosed cellular debris, frequently of germ cell origin) were frequently found in the cell cytoplasm. Lipid droplets (Li) were commonly observed, as noted previously by others, in Sertoli cells but not in germ cells or fibroblasts.

Most characteristic were the numerous cell-to-cell attachments (shown in circles), generally of zonula adherens types. Microfilaments inserted in these plaques were occasionally noted. The specialized membrane junctions described by others in *in situ* Sertoli cells were not readily demonstrated in these cells, and extensive smooth endoplasmic reticulum was also inconspicuous. In sections close to the cell substrate, numerous actin filament bundles and microtubules were noted, as well as extensive membrane attachments. As expected, no basement membrane was detected following collagenase treatment of the tubules. Several normal mitotic figures and associated mitotic spindles were noted morphologically in these primary cultures; however, growth beyond eight days of these presumed Sertoli cells was not observed, and later cultures consisted solely of fibroblasts. Collagen fibers and fibroblasts were occasionally present; the latter were readily distinguishable from Sertoli cells. The persistent collagen fibers presumably reflect incomplete collagenase digestion.

In addition to ultrastructural data indicating the presence of Sertoli cells, response to gonadotropin stimulation was also measured. Bovine Sertoli cells (derived from 7- to 14-day-old bull calves)

responded to FSH stimulation with a 15-fold rise in cAMP level (Fig. 3). Maximal cAMP response was noted with 1.0 μg/ml FSH. No cAMP response was observed with LH stimulation at concentrations up to 5.0 μg/ml. No testosterone response was seen following stimulation with either FSH or LH (Table 1). Based upon these findings, significant Leydig cell contamination of bovine Sertoli cell preparations was excluded.

In Sertoli cell cultures derived from 7- to 14-day-old bull calves, FSH stimulation (1.0 μg/ml) of ODC activity was maximal between 4 and 6 hours (Fig. 4). By 8 hours, ODC activity returned rapidly to control value.

Putrescine levels were also elevated 4 hours following FSH stimulation (1.0 μg/ml; $P < 0.01$) (Fig. 5). This increase persisted for at least 12 hours and then returned to control level by 24 hours. No increase was noted in spermidine levels following FSH stimulation (Table 2).

A significant elevation in ODC activity was observed following stimulation with FSH (1.0 μg/ml), LH (1.0 μg/ml), and hCG (50 IU/ml) (Fig. 6). No response was seen following stimulation with 1 μg/ml ACTH or PRL nor with testosterone (0.5 μM).

Dose response curves for gonadotropin stimulation of ODC activity show that both FSH and LH significantly stimulated ODC activity over the same range of concentrations (Fig. 7). FSH stimulation of ODC activity was always greater than that of LH (approximately double).

Table 3 shows the effect of FSH stimulation of ODC activity in Sertoli cells derived from older animals (2-month-old bull calves). Both unstimulated and stimulated (1.0 μg/ml FSH) ODC activities were lower ($P < 0.01$) in the older animals. Attempts to isolate Sertoli cells from older animals (7-month-old to adult) using either the technique outlined here or adaptations of a method for preparation of adult rat Sertoli cells (Welsh and Wiebe, 1975) were unsuccessful.

TABLE 1. Testosterone Response to Gonadotropin Stimulation of Bovine Sertoli Cells*

	Testosterone (ng/mg protein ± SE)
Control	21.0 ± 3.0
FSH (1.0 μg/ml)	17.5 ± 2.5
LH (0.5 μg/ml)	23.0 ± 3.0
LH (1.0 μg/ml)	20.0 ± 3.0
LH (5.0 μg/ml)	23.5 ± 4.0

* Bovine Sertoli cells were stimulated for 24 hours at 37 C with specified gonadotropins in SFM containing 0.1 mg/ml MIX. Testosterone in the surrounding media was determined by specific radioimmunoassay, and data were calculated as ng/mg protein ± SE (duplicate determinations of six replicate cultures).

Discussion

The present study defines the response of ODC activity in isolated bovine Sertoli cells to *in vitro* gonadotropin stimulation at 37 C. This temperature was used to coordinate the present study with our interest in Mullerian inhibiting substance secretion in Sertoli cells (Bercu et al, 1981) and has been used in other studies of Sertoli cell function (Tchlokian and Steinberger, 1978; Hagenas et al, 1978). Elevation of ODC activity is observed for 4 to 6 hours after FSH stimulation and rapidly returns to control level. Both FSH and LH or hCG were found to stimulate ODC activity in the Sertoli cell preparations used here. It should be noted that the ultrastructural features described herein unequivocally establish the Sertoli cell linkage of these cultures cells, despite the absence of some features described in other communications, such as specialized cell–cell junctions (tight junctions and gap junctions; Bigliardi and Talluri, 1976), which may have been overlooked. Unlike any descriptions of fibroblasts or germ cells, these cells possessed prominent tri-partite nucleoli, round to oval nuclei, prominent tertiary lysosomes with contained cellular debris, numerous lipid droplets, and numerous cell-to-cell attachments as seen in epithelial cells. Furthermore, groups of cells were clustered together as would be expected of mutually adherent epithelial cells but completely unlike fibroblasts in culture. This array of features could not be found in contaminating fibroblasts or germ cells; those present in culture were in fact readily identified. Thus, the dominant cell population, which was strikingly homogeneous, possessed numerous features of normal Sertoli cells *in vivo*. Furthermore, significant Leydig cell contamination was excluded on the basis of lack of cAMP and T response to LH stimulation. It is possible that the changes in ODC activity reported here represent changes in Sertoli cell ODC activity itself.

Gonadotropin stimulation led to a 15-fold increase in ODC activity which followed a time course of activation and inactivation which is similar to that seen in the ovary (Ostermann and Hammond, 1977). It is noted by comparison that basal ODC activity varies greatly from one cell

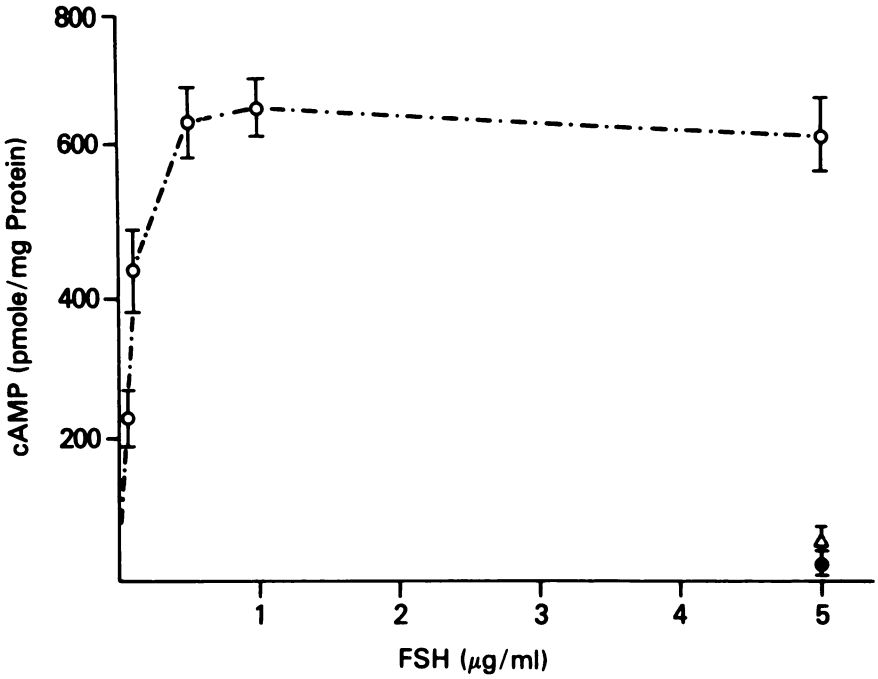

Fig. 3. Gonadotropin stimulation of bovine Sertoli cell cAMP. Bovine Sertoli cells were stimulated for 30 minutes at 37 C with gonadotropin at specified concentrations in SFM containing 0.1 mg/ml MIX. Intracellular cAMP was determined by specific radioimmunoassay, and data were calculated as pmole cAMP/mg protein ± SE (duplicate determinations of six replicate cultures). Control (●); LH, 5.0 μg/ml (△); FSH, (○).

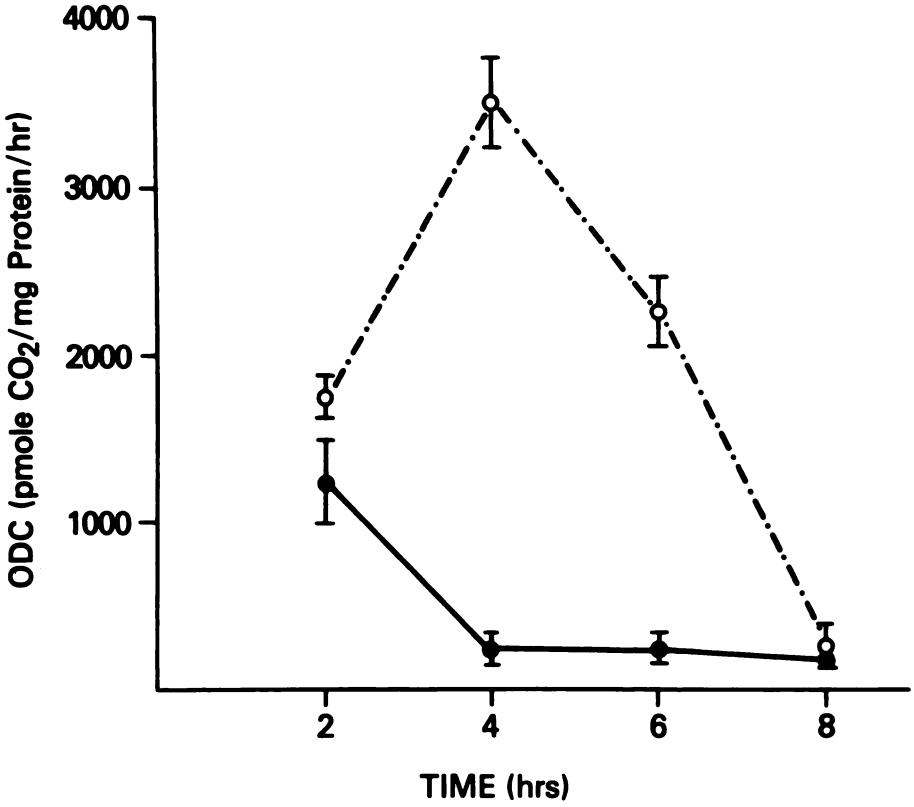

Fig. 4. FSH stimulation of bovine Sertoli cell ODC activity. Bovine Sertoli cells were stimulated with FSH (1.0 μg/ml) for specified periods of time at 37 C in SFM containing 0.1 mg/ml MIX. ODC activity was determined by $^{14}CO_2$ release from 1-hour incubations of cell homogenate in a reaction mixture containing 0.4 mM ornithine. Data were calculated as pmole CO_2/mg protein/hour ± SE (duplicate determinations of six replicate cultures). Control (●); FSH (○).

TABLE 2. Spermidine Levels Following FSH Stimulation of Bovine Sertoli Cells*

	Spermidine (nmole/mg protein ± SE) at		
	4 hr	12 hr	24 hr
Control	140 ± 40	70 ± 20	50 ± 20
FSH	120 ± 45	55 ± 25	35 ± 25

* Bovine Sertoli cells were stimulated with FSH (1.0 μg/ml) for specified periods of time at 37 C in SFM containing 0.1 mg/ml MIX. Intracellular spermidine was determined by fluorescence of the dansylated derivative separated by TLC. Data were calculated as nmole/mg protein ± SE (single determinations of six replicate cultures).

preparation to another. For this reason direct comparison from one cell preparation to another may not be valid. Nevertheless, within any given cell preparation, ODC activity was consistent both in basal and stimulated states. Therefore, valid comparisons are possible only within any given experiment. Response by bovine Sertoli cells appeared to be limited specifically to gonadotropin stimulation. There was no response to ACTH, PRL, or T. It is interesting that FSH, LH, and hCG increased ODC activity. Many studies have proposed the Sertoli cell as the principal site of FSH

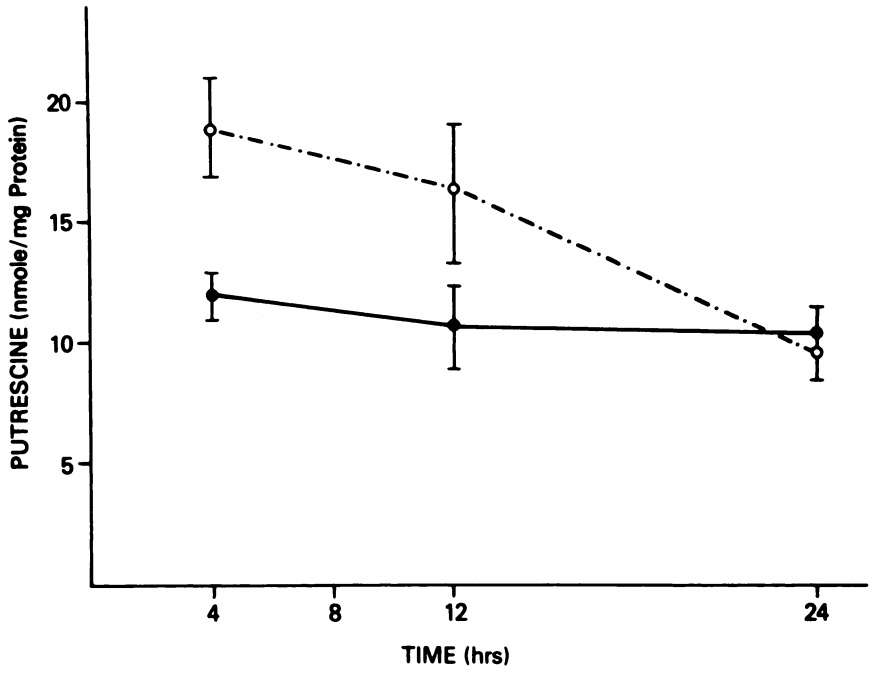

Fig. 5. FSH effect on bovine Sertoli cell putrescine content. Bovine Sertoli cells were stimulated with FSH (1.0 μg/ml) for specified periods of time at 37 C in SFM containing 0.1 mg/ml MIX. Putrescine was determined by fluorescence of the dansylated derivative following TLC separation in triethylamine:ethylacetate (9:1). Data were calculated as nmole/mg protein ± SE (single determinations of six replicate cultures). Control (●); FSH (○).

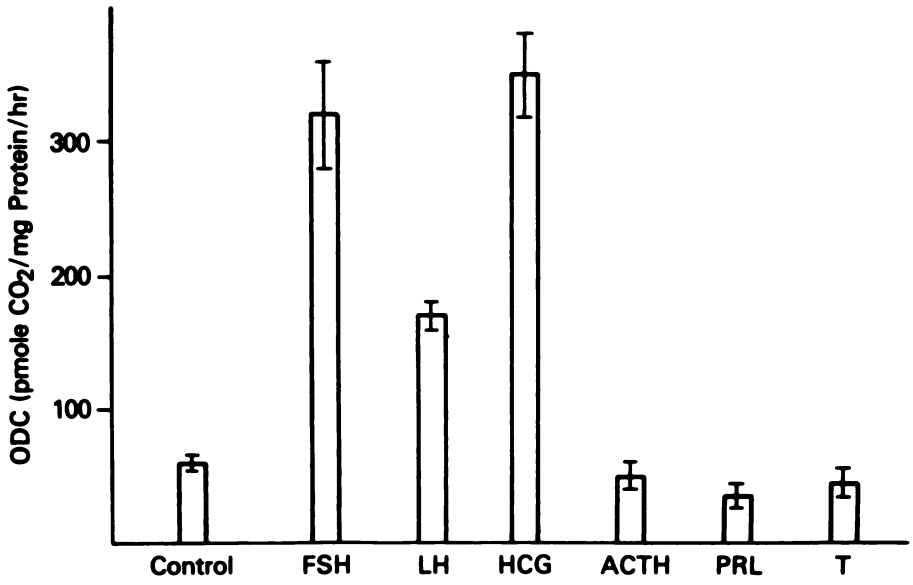

Fig. 6. Specificity of hormonal stimulation of bovine Sertoli cell ODC activity. Bovine Sertoli cells were stimulated for 4 hours at 37 C in SFM containing specified hormones and 0.1 mg/ml MIX. ODC activity was determined by $^{14}CO_2$ release from 1-hour incubations of cell homogenate in a reaction mixture containing 0.4 mM ornithine. Data were calculated as pmole CO_2/mg protein/hour ± SE (duplicate determinations for six replicate cultures). FSH (1.0 μg/ml); hCG (50 IU/ml); ACTH (1.0 μg/ml); PRL (1.0 μg/ml); T (0.5 μM).

TABLE 3. ODC Activity in Sertoli Cells of the Developing Bull Calf*

Experimental Group	ODC (pmole CO_2/mg protein/hr ± SE) at Age	
	7–14 Days	2 Months
Control	200 ± 50	50 ± 25
FSH	5400 ± 600	2300 ± 300

* Bovine Sertoli cells were prepared from animals either 7 to 14 days or 2 months of age. Cells were stimulated with FSH (1.0 μg/ml) for 4 hours at 37 C in SFM containing 0.1 mg/ml MIX. ODC activity was determined by $^{14}CO_2$ release from 1-hour incubations of cell homogenate in a reaction mixture containing 0.4 mM ornithine. Data were calculated as pmole CO_2/mg protein/hour ± SE (duplicate determinations of six replicate cultures).

action and the Leydig cell as the principal site of LH action in the testis (Castro et al, 1972; Dorrington and Fritz, 1974; Castro and Seiguer, 1970; Means et al, 1980; Salhanick and Weibe, 1980; Brandtzaeg et al, 1978; Davies et al, 1979). However, since both LH and hCG contain intrinsic FSH-like activity (Siris et al, 1978; Louvet et al, 1976), it is possible that LH or hCG might have some effect on Sertoli cell cultures. When present in microgram quantities as utilized here, both of these hormones could exhibit significant FSH-like activity. Perhaps this could account for the increased ODC activity observed following LH and hCG stimulation.

Following FSH stimulation, a rise was seen in putrescine levels without a concomitant rise in spermidine. Such observations are similar to those of Macindoe and Turkington (1973). As suggested by them, cooperativity between different testicular cells might be required for completion of spermidine biosynthesis. Also, either the time course or temperature dependence of spermidine biosynthesis might be significantly different from those of putrescine. Alternatively, maturation of polyamine biosynthesis might occur in stages, with putrescine synthesis occurring at an earlier stage than spermidine or spermine. We evaluated maturational changes of ODC activity in Sertoli cells from bull calves up to 2 months of age. At that age ODC activity was less responsive than at 7 to 14 days of age. We were unable to obtain viable Sertoli cells from older animals. These observations, although limited, extend that of Macindoe and Turkington (1973), who found a decrease in the specific activity of testicular ODC during development in the rat and attributed this decrease to a lower percentage of Sertoli cells in the older testis. Our present findings extend these observations under defined *in vitro* conditoins. Sertoli cell cultures derived from 2-month-old animals still respond to FSH. No quantitative comparison of relative responsiveness can be derived to compare the responsiveness of these cells relative to that of younger calves. One could postulate that either

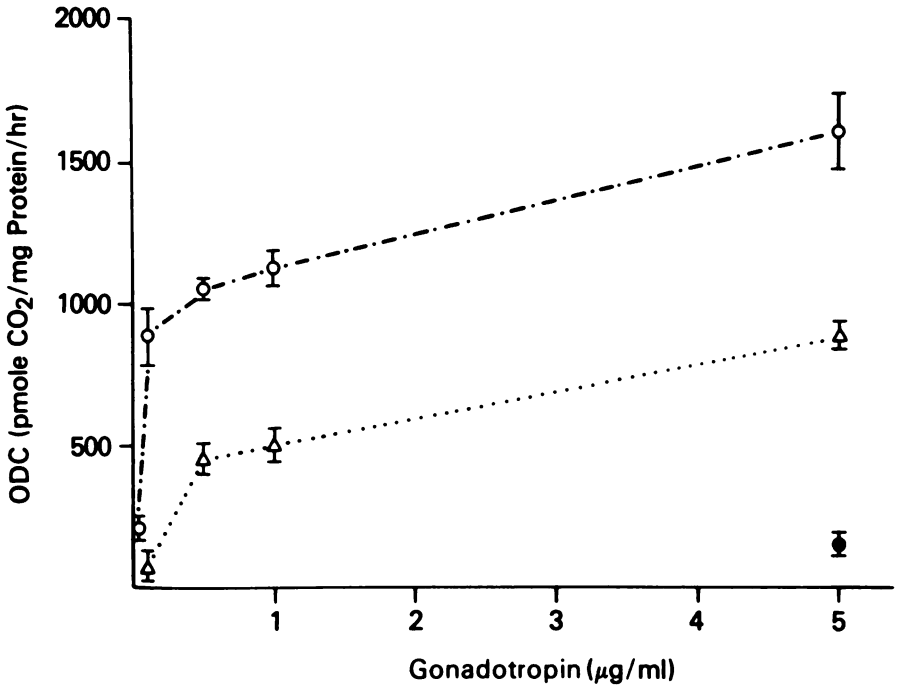

Fig. 7. Gonadotropin stimulation of bovine Sertoli cell ODC activity. Bovine Sertoli cells were stimulated for 4 hours at 37 C in SFM containing specified gonadotropins and 0.1 mg/ml MIX. ODC activity was determined by $^{14}CO_2$ release from 1-hour incubations of cell homogenate in a reaction mixture containing 0.4 mM ornithine. Data were calculated as pmole CO_2/mg protein/hour ± SE (duplicate determinations of six replicate cultures). Control (●); LH (△); FSH (○).

changes in Sertoli cell number, purity of cell preparation, or sensitivity to FSH could account for the lower response in these cells from 2-month-old calves. Attempts were made to evaluate maturational changes in rat Sertoli cell ODC activity as well (unpublished observations). Although viable Sertoli cells were readily obtained from pups ranging in age from 1 to 60 days, no stimulation of ODC activity with etiher FSH or LH (hCG) was shown. Such a lack of stimulation of ODC activity was also reported for the rat ovary (Osterman and Hammond, 1977). This suggests that such differences between the rat and other species may either be due to an intrinsic difference in the rat or be related to the use of a heterologous hormone system. For this reason, the bovine Sertoli system may offer additional insights into Sertoli cell function beyond those already described for the rat.

Acknowledgments

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