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# Hormonal regulation of protein synthesis, secretion, and phosphorylation in cultured rat Sertoli cells

(follicle-stimulating hormone/androgen-binding protein/vimentin)

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**ABSTRACT** **The accumulation of two polypeptides, SCm1 and SCm2, in the medium of Sertoli cell cultures is enhanced by follicle-stimulating hormone (FSH) but is unaffected by either the cAMP analog, $N^6,O^{2'}$-dibutyryl cAMP or luteinizing hormone. The assigned molecular weights of SCm1 and SCm2 differ from those of androgen-binding protein subunits or any other previously identified Sertoli cell secretory product. Incubation of Sertoli cell cultures with either FSH or $N^6,O^{2'}$-dibutyryl cAMP also stimulates the incorporation of [$^{35}$S]methionine into two intracellular polypeptides, SCc1 and SCc2. In addition, the phosphorylation of three intracellular polypeptides, SCc3, SCc4, and SCc5, is intensified when Sertoli cell cultures are treated with either FSH or $N^6,O^{2'}$-dibutyryl cAMP. Based on these results and on previous work, we conclude that (*i*) SCm1 and SCm2 may, like androgen-binding protein, be secreted by Sertoli cells and function extracellularly while SCc1 and SCc2 are involved in FSH-dependent intracellular activity; (*ii*) SCc3, SCc4, and SCc5 are possible substrates for FSH-stimulated, cAMP-dependent protein kinase activity; and (*iii*) SCc5 is an isoelectric variant of vimentin-type intermediate filament protein presumably involved in FSH- and $N^6,O^{2'}$-dibutyryl cAMP-induced Sertoli cell shape changes.**

The Sertoli cell, the only nongerminal cell type in the seminiferous epithelium, is responsible for the structural support of the germinal cells and the regulation of their development (1). The adaptation of the Sertoli cell to growth in cell culture (2–4) has facilitated the study of several of its functions, including the regulation of androgen-binding protein (ABP) secretion by follicle-stimulating hormone (FSH) (5). In addition to ABP, several factors have been detected in the media conditioned by Sertoli cells in culture. Among these are H-Y antigen (6), a "LHRH-like" factor (7), Müllerian-inhibiting protein (8, 9), plasminogen activator (10), "Sertoli cell factor" (11) [possibly involved in feedback inhibition of FSH secretion by the pituitary and similar to inhibin (12)], and transferrin (13). An *in vivo* correlate for some of these remains to be determined. However, taken together they strengthen the possible role for Sertoli cell secretory products in the interaction with testicular and extratesticular cells.

We have used the high resolving power of two-dimensional polyacrylamide gel electrophoresis (14) and a serum-free, hormone-supplemented medium (15, 16) to study: (*i*) the synthesis and secretion of proteins in Sertoli cell cultures established from 20- to 22-day-old rats, (*ii*) the effect of FSH, $N^6,O^{2'}$-dibutyryl cAMP ($Bt_2$cAMP), and luteinizing hormone (LH) on these processes, and (*iii*) the effect of FSH and $Bt_2$cAMP on the phosphorylation of cellular polypeptides. Our interest in protein phosphorylation stems from biochemical evidence showing FSH activation of cAMP-dependent protein kinases (17) and the need to identify still elusive protein substrates. We demonstrate that FSH stimulates the accumulation of distinct secretory proteins in the medium of Sertoli cell cultures from pubertal rats and that treatment of the cultures with FSH or $Bt_2$cAMP results in the synthesis and phosphorylation of specific intracellular proteins. A preliminary report of these studies has appeared (18).

## MATERIALS AND METHODS

**Isolation and Culture of Rat Sertoli Cells.** Primary cultures of Sertoli cells were prepared from 20- to 22-day-old rats by the method of Dorrington and Fritz (3) modified as described (19). Aggregates of seminiferous epithelial cells were plated in Eagle minimum essential medium supplemented with the following to the final concentrations indicated: 0.1 mM nonessential amino acids; 4 mM glutamine; 1 mM sodium pyruvate; and 10% (vol/vol) fetal bovine serum (Sterile Systems, Logan, UT). Cells were used for radioisotopic labeling after 6–8 days in culture. This cell culture system has been characterized by morphological and biochemical criteria (FSH responsiveness) (20) and by the immunocytochemical localization of ABP (21). The epithelial-like nature of these Sertoli cells in culture as well as the absence of contaminating peritubular cells has been demonstrated by electron microscopy (19).

**[$^{35}$S]Methionine and [$^{32}$P]Orthophosphate Labeling of Sertoli Cell Cultures and Preparation of Cells and Media for Polyacrylamide Gel Electrophoresis.** Twenty-four hr before labeling, cells were pretreated in serum-free, hormone-supplemented medium (15) supplemented as above, with the omission of serum and the addition of the following to the final concentrations indicated: human transferrin, 5 μg/ml; bovine insulin, 5 μg/ml; epidermal growth factor, 3 ng/ml (Collaborative Research, Waltham, MA); human growth hormone, $6.5 \times 10^{-6}$ IU/ml (AB Kabi, Stockholm, Sweden); retinol, 10 μM; and testosterone, 0.1 μM. Retinol and testosterone were included in the medium as described by Karl and Griswold (16). After 24 hr, the pretreatment medium was removed and replaced with serum-free, hormone-supplemented Eagle minimum essential medium containing either 1/10th the usual concentration of methionine and [$^{35}$S]methionine at 250 μCi/ml (1,100 Ci/mmol; 1 Ci = $3.7 \times 10^{10}$ becquerels) or 1/20th the usual concentration of inorganic phosphate and [$^{32}$P]orthophosphate (carrier-free) at 500 μCi/ml. Ovine FSH (NIH-oFSH-S13), $Bt_2$cAMP (Sigma), and ovine LH (NIH-LH-S20) were added to final concentrations of 5 μg/ml, 0.5 mM, and 5 μg/ml, respectively. FSH and LH were generously provided by the National Institute of Arthritis, Metabolism and Digestive Diseases Pituitary Hormone Distribution Program and A. F. Parlow.

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Abbreviations: ABP, androgen-binding protein; FSH, follitropin (follicle-stimulating hormone); $Bt_2$cAMP, $N^6,O^{2'}$-dibutyryl cAMP; LH, luteinizing hormone; IEP, isoelectric point; SCc1–SCc6, intracellular polypeptides from Sertoli cell lysates; SCm1 and SCm2, polypeptides that accumulate in medium of Sertoli cell cultures.

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After a 6-hr incubation with radiolabeled precursor, media were collected and phenylmethylsulfonyl fluoride was added to a final concentration of 0.1 mM. Media were centrifuged at 12,000 × g for 10 min (4°C), lyophilized, and resuspended in 100 μl of lysis buffer (14) and radioactivity was determined. Attached cells were rinsed three times in ice-cold Hanks balanced salt solution. Cells were resuspended directly into 100 μl of lysis buffer or were scraped off the plastic culture dish into Hanks balanced salt solution and collected by centrifugation at 1,000 × g for 5 min (4°C). The cell pellet was drained carefully and resuspended in 100 μl of lysis buffer.

**Polyacrylamide Gel Electrophoresis.** For one-dimensional electrophoresis, samples of resuspended medium or cell lysates in 1–5 μl of lysis buffer were added to 100 μl of $NaDodSO_4$ sample buffer [10% (wt/vol) glycerol, 5% (vol/vol) 2-mercaptoethanol, 2.3% (wt/vol) $NaDodSO_4$, and 0.0625 M Tris·HCl, pH 6.8 (14)]. Samples were heated for 5 min at 100°C, cooled, and layered into wells of 1.5-mm thick slab gel prepared as for the second dimension of the two-step electrophoretic technique. The separating gel (15 × 14 cm) was a 5–15% exponential gradient of acrylamide and was overlaid with a 4-cm long stacking gel of 4.75% acrylamide. Slab gels were run at 100 V for 14 hr.

Two-dimensional electrophoresis of protein samples was performed as described by O'Farrell (14). Cylindrical isoelectrofocusing gels (3 × 13 mm) were run for 13 hr at 400 V and 1 hr at 500 V. Estimates of molecular weights of resolved polypeptide spots were determined by comparison with the positions of purified protein standards. Estimates of isoelectric point (IEP) were made by comparison to the pH gradient in a blank isoelectrofocusing gel after electrophoresis. Gels were processed for fluorography with EN$^3$HANCE (New England Nuclear) according to the procedure of the manufacturer. The dried gels were then exposed to Kodak X-Omat AR film. An equal number of cpm was loaded on gels within the group being compared.

**Immunoprecipitation of ABP from Culture Medium.** Sertoli cell cultures plated in 25-cm$^2$ culture flasks were labeled for 24 hr in 2.5 ml of serum-free, hormone-supplemented medium containing FSH at 5 μg/ml, 1/10th the usual concentration of methionine, and [$^{35}$S]methionine at 50 μCi/ml. After incubation, the medium was collected and centrifuged at 12,000 × g for 20 min (4°C). The medium was dialyzed against deionized water (4°C) in dialysis tubing pretreated with 0.01% (wt/vol) ovalbumin. After dialysis, the medium was lyophilized and the lyophilized material was resuspended in 100 μl of water. Indirect immunoprecipitation was performed as described by Murphy *et al.* (22), except that protein A-bearing *Staphylococcus aureus* cells (23) (Pansorbin, Calbiochem) were substituted for the second precipitating antibody. The specific rabbit anti-rat ABP serum, used at a 1:1,000 dilution in the immunoprecipitation reaction, has been characterized (24).

## RESULTS

**Immunoprecipitation of [$^{35}$S]Methionine-Labeled ABP of Sertoli Cells.** [$^{35}$S]Methionine-labeled proteins that accumulated in the culture medium for 24 hr were immunoprecipitated with rabbit anti-rat ABP serum and analyzed by one-dimensional polyacrylamide gel electrophoresis and fluorography (Fig. 1*C*). Anti-rat ABP serum precipitated a [$^{35}$S]methionine-labeled product consisting of two bands of unequal $M_r$ (47,000 and 41,000) and unequal exposure intensity (heavier component more intense than lighter component). These electrophoretic characteristics are consistent with those described for subunits of ABP purified from rat epididymal cytosol and present there in a 3:1 ratio of heavier to lighter component (24). These data provide direct evidence that ABP is synthesized by cultured Sertoli cells 6–8 days after plating.

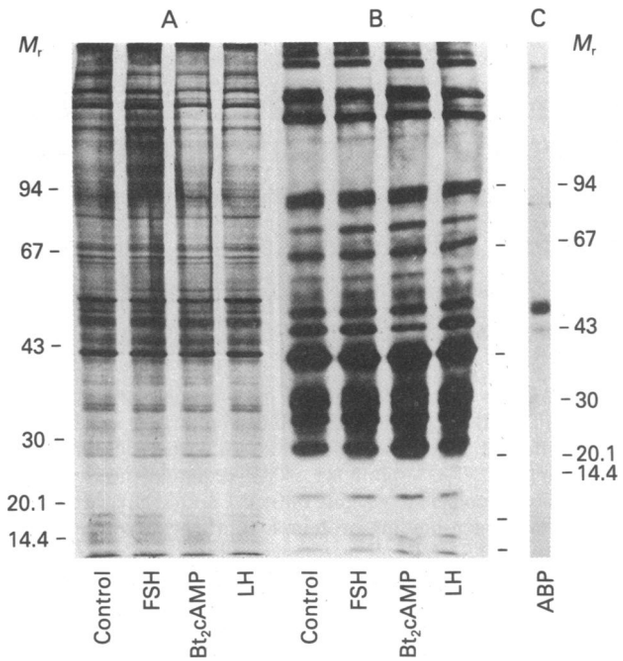


FIG. 1. One-dimensional fractionation of [$^{35}$S]methionine-labeled proteins from Sertoli cell lysates (*A*), recovered from Sertoli cell medium (*B*), or immunoprecipitated from Sertoli cell culture medium (*C*). Sertoli cells were isolated, pretreated, and labeled with [$^{35}$S]methionine as described. In *A*, 100,000 cpm of $^{35}$S-labeled trichloroacetic acid-precipitable material were loaded per well and radiographs were developed after an exposure of 2 wk. In *B*, 20,000 cpm were loaded per well, the gel was prepared for fluorography, and the film was exposed for 2 wk. In *C*, ≈1,000 cpm of immunoprecipitate were loaded per well. The gel was dried and the fluorograph was exposed for 2 wk. The positions of stained molecular weight markers are noted ($M_r \times 10^{-3}$).

**One- and Two-Dimensional Polyacrylamide Gel Electrophoresis of [$^{35}$S]Methionine-Labeled Cellular and Secretory Proteins of Cultured Sertoli Cells.** Proteins in cultured Sertoli cells were labeled with [$^{35}$S]methionine under control conditions and in the presence of FSH, $Bt_2cAMP$, or LH. No differences in the one-dimensional electrophoretic patterns were seen either in proteins in cell lysates (Fig. 1*A*) or in proteins that accumulated in the culture media (Fig. 1*B*). We then used two-dimensional electrophoresis to improve the resolution of newly synthesized and secreted Sertoli cell proteins.

Fig. 2 compares the patterns of [$^{35}$S]methionine-labeled proteins fractionated from Sertoli cell lysates. Two polypeptide spots, not detected in control samples, were very distinct in Sertoli cell samples incubated in the presence of FSH or $Bt_2cAMP$; these intracellular polypeptides have been designated SCc1 and SCc2. The intensity of both SCc1 and SCc2 was greater in $Bt_2cAMP$-treated samples than in FSH-treated ones. These two polypeptides appeared to be weakly labeled in Sertoli cell cultures treated with LH. This last finding was attributed to stimulation induced by FSH contaminants present in the LH preparation. SCc1 and SCc2 have been assigned IEPs of 5.8 and 5.9, respectively, and have similar $M_r$ of 25,000. Although other polypeptide spots in the gels of this experiment were found that varied in labeling intensity, they were not affected by hormone or $Bt_2cAMP$ with the consistency of SCc1 and SCc2.

The two-dimensional polyacrylamide gel electrophoresis technique also allowed the detection of two proteins, designated SCm1 and SCm2, whose accumulation in the medium of cultured Sertoli cells was enhanced by FSH treatment (Fig. 3) as compared to control cultures. The stimulated accumulation of

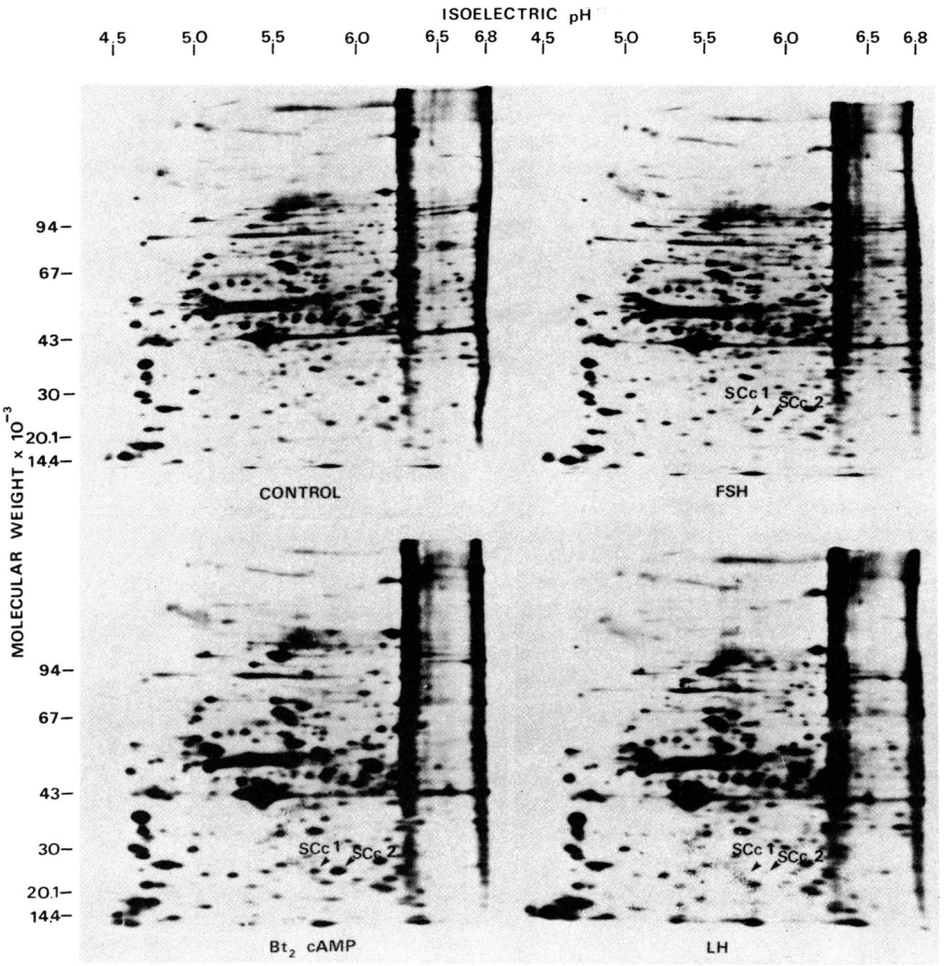


FIG. 2. Two-dimensional fractionation of $^{35}$S-labeled proteins from Sertoli cell lysates. Cultures were incubated for 6 hr in the presence of [$^{35}$S]methionine at 250 $\mu$Ci/ml and FSH at 5 $\mu$g/ml, 0.5 mM $Bt_2$cAMP, or LH at 5 $\mu$g/ml. Lysates of Sertoli cell cultures were prepared as described. The proteins were fractionated on the basis of IEP in the first dimension (horizontal) and on the basis of $M_r$ in the second dimension (vertical). The sample origin is in the upper right corner of each fluorograph. The pH gradient of the first dimension is indicated along the top and positions of molecular weight markers are indicated on the left side of the fluorograph.

SCm1 and SCm2 was unique to Sertoli cell stimulation with FSH and was unapparent after incubation with $Bt_2$cAMP or LH (Fig. 3). SCm1 and SCm2 were assigned IEPs of 5.2 and 5.5, respectively, and have $M_r$ of 110,000 and 80,000, respectively.

**Two-Dimensional Polyacrylamide Gel Electrophoresis of $^{32}$P-Labeled Intracellular Proteins of Cultured Sertoli Cells.** To identify FSH-dependent phosphorylation of intracellular proteins, lysates of Sertoli cells labeled for 6 hr with [$^{32}$P]-orthophosphate were fractionated by two-dimensional polyacrylamide gel electrophoresis. Fig. 4 illustrates protein patterns from control and FSH- or $Bt_2$cAMP-treated Sertoli cells. Polypeptide spots SCc3 (IEP 5.8; $M_r$ 25,000), SCc4 (IEP 5.9; $M_r$ 25,000), and SCc5 (IEP 5.15; $M_r$ 58,000) appeared in FSH-treated samples but were absent in control samples. The same spots were better displayed in radiographs of Sertoli cells incubated with $Bt_2$cAMP. A group of three polypeptide spots (IEP 4.9, 4.8, and 4.7 and $M_r$ 50,000, 48,000, and 46,000, respectively) designated SCc6, descending on a diagonal from SCc5, maximized their $^{32}$P-labeling intensity after $Bt_2$cAMP treatment.

Fig. 4 also illustrates the position of vimentin-type intermediate filament protein in each gel. This identification was based on molecular weight, acidity, and insolubility in Triton X-100 and was confirmed by immunofluorescence and immunoprecipitation (unpublished data) by using an antiserum specific for vimentin-type intermediate filament protein (25). Vimentin is phosphorylated under control conditions and this phosphorylation was enhanced by FSH and even more so by $Bt_2$cAMP. SCc5 has been tentatively identified as an isoelectric variant of vimentin on the basis of its relationship to the identified vimentin spot in the two-dimensional polyacrylamide gel electrophoresis pattern. Polypeptides on the downward diagonal labeled as SCc6 resemble vimentin degradation products (26).

## DISCUSSION

Two-dimensional polyacrylamide gel electrophoresis has allowed the detection of distinct Sertoli cell proteins whose synthesis, secretion, or phosphorylation is affected by FSH and $Bt_2$cAMP. FSH and $Bt_2$cAMP both enhanced the incorporation of [$^{35}$S]methionine into two low molecular weight ($M_r$ 25,000) Sertoli cell intracellular proteins, SCc1 and SCc2. Because of their similar positions in the electrophoretic pattern, it is possible that [$^{35}$S]methionine-labeled proteins SCc1 and SCc2 are related to [$^{32}$P]orthophosphate-labeled proteins SCc3 and SCc4. Supporting this identity is the fact that $Bt_2$cAMP enhanced the synthesis of both SCc1 and SCc2 more than did FSH, just as $Bt_2$cAMP enhanced the phosphorylation of both SCc3 and SCc4 more than did FSH. If SCc1 and SCc2 are identical with SCc3 and SCc4, these proteins represent a distinct pair of intracellular proteins whose synthesis and phosphorylation are both regulated by FSH and $Bt_2$cAMP.

ABP is a specific marker for the characterization of Sertoli cell cultures and the secretion of ABP in culture has been used as an end point for the study of FSH regulation of Sertoli cell function (5). We provide here the additional information that Sertoli cells in culture are capable of *de novo* synthesis of ABP and thus extend the aspects of FSH regulation that can be studied. SCm1 and SCm2 resemble ABP in that they accumulate in the media of Sertoli cell cultures in response to FSH. It is significant to point out that the nominal IEP and $M_r$ assigned to SCm1 and SCm2 do not coincide with any known Sertoli cell

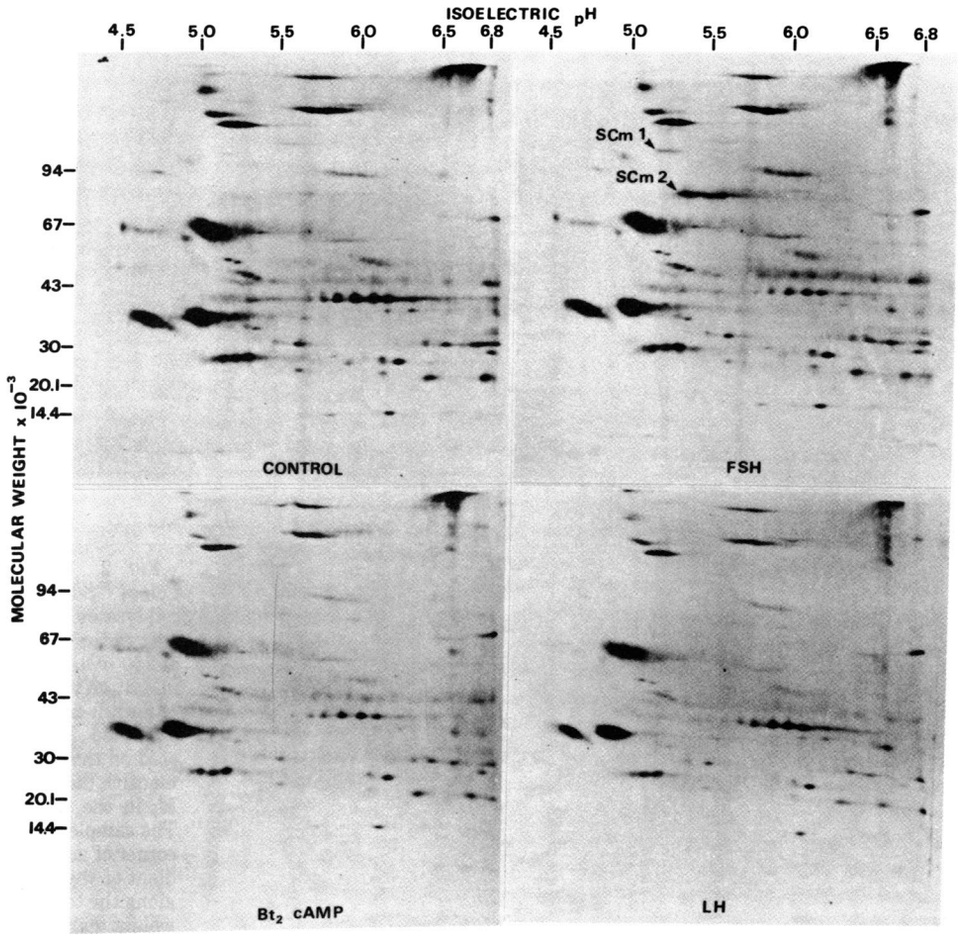


FIG. 3. Two-dimensional fractionation of $^{35}S$-labeled proteins recovered from the medium of Sertoli cell cultures under control conditions and when treated as in Fig. 2 with FSH, $Bt_2cAMP$, or LH. Media from $^{35}S$-labeled cultures were processed as described. Molecular weight markers are indicated on the left side of the fluorograph.

secretory product (Table 1). The two-dimensional patterns of total Sertoli cell secretory proteins shown here are remarkably similar to a two-dimensional polyacrylamide gel electrophoresis map of total Sertoli cell secretory proteins shown previously (32). In this latter study, Sertoli cell secretory proteins were collected from cultures that were not stimulated by FSH and therefore, SCm1 and SCm2 were not evident.

The accumulation of SCm1 and SCm2 in the Sertoli cell culture medium in response to FSH but not in response to $Bt_2cAMP$ is reminiscent of the differential effect of these two agents in eliciting a morphological shape change in cultured Sertoli cells. Although FSH-induced conversion of flat Sertoli

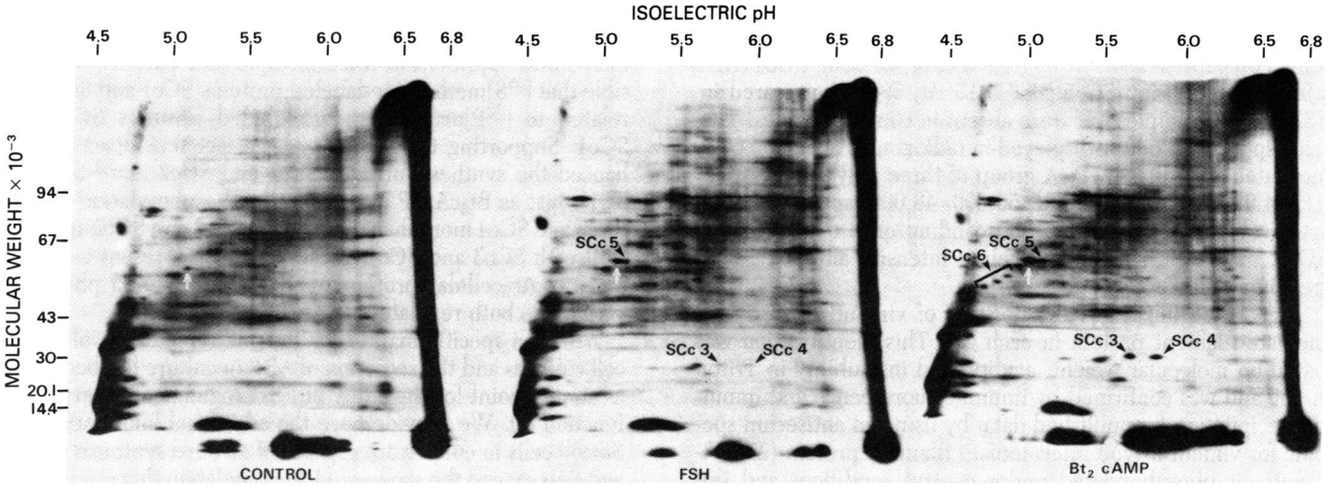


FIG. 4. Two-dimensional fractionation of $^{32}P$-labeled proteins from Sertoli cell lysates. Sertoli cell cultures were labeled for 6 hr with [$^{32}P$]orthophosphate at 500 $\mu$Ci/ml under control conditions and in the presence of FSH at 5 $\mu$g/ml or 0.5 mM $Bt_2cAMP$; 800,000 cpm were loaded on each gel and films were exposed for 3 days. In each instance, the white arrow indicates the position of vimentin. Molecular weight markers are indicated on the left side of the radiograph.

Table 1. $M_r$ and IEP of Sertoli cell secretory polypeptides

| Sertoli cell product | $M_r$ | Ref. | IEP | Ref. |
|---|---|---|---|---|
| ABP | Subunits, 47,000 and 41,000 Dimer, 100,000 | 24 | 4.6–4.7 | 27 |
| H-Y antigen | 16,500 | 28 | NA | — |
| LHRH-like factor | NA | — | NA | — |
| Müllerian-inhibiting protein | 72,000 | 29 | 6.0 | 29 |
| Plasminogen activator | 75,000 | 30 | NA | — |
| Sertoli cell factor | 12,000 | 11 | NA | — |
| Transferrin | 71,000 | 13 | 5.65–5.85 | 31 |
| SCm1 | 110,000 | This work | 5.2 | This work |
| SCm2 | 89,000 | This work | 5.5 | This work |

NA, not available.

cells into a stellate morphology was transient, morphological changes induced by cyclic nucleotide analogs were persistent (20). Fritz (33) has reported that many Sertoli cell effects triggered by FSH can be duplicated by the addition of cAMP analogs to the culture medium. Our results are consistent with a role for cAMP as an obligatory but insufficient intermediate of FSH action. In fact, an important requirement for the secretion of SCm1 and SCm2 is satisfied only by FSH and not solely by the increase in cyclic nucleotide levels.

In addition to SCc3 and SCc4, FSH and $Bt_2$cAMP increase the phosphorylation of vimentin and a group of intracellular proteins, denoted SCc5 and SCc6. Vimentin is the major subunit protein of one type of intermediate filament contributing to the cytoskeleton in a number of cell types (34). Franke *et al.* (35) have shown the presence of immunoreactive vimentin in the cytoplasm of Sertoli cells in the intact rat testis and vimentin-type intermediate filaments have been identified in electron microscopic preparations of cultured rat Sertoli cells (20). The phosphorylation of vimentin *in vitro* by cAMP-dependent protein kinase has been shown (36) as well as the hormone-dependent phosphorylation of vimentin in intact C-6 glioma cells in culture (37). The proximity of SCc5 to vimentin and the similar phosphorylation response of both these proteins to FSH and $Bt_2$cAMP suggest that SCc5 may be an isoelectric variant of vimentin differing from the major form of the protein in degree of phosphorylation (38).

One intriguing aspect of this study is the functional significance of the phosphorylation of vimentin. We have previously suggested (20) that the activation of cAMP-dependent protein kinases could result in the phosphorylation of specific proteins that may activate or integrate the contractile properties of Sertoli cell cytoskeletal proteins. In the intact seminiferous tubules, forces generated by contractile cytoplasmic proteins may lead to both the displacement of germinal cells towards the lumen as they differentiate and the secretion of ABP and other secretory proteins, including SCm1 and SCm2.

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