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Follicle-stimulating hormone-dependent phosphorylation of vimentin in cultures of rat Sertoli cells

(testis/cAMP-dependent protein kinases/two-dimensional gel electrophoresis/immunofluorescence)

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ABSTRACT **Endogenous protein phosphorylation was investigated in cultured rat Sertoli cells after treatment with follicle-stimulating hormone (FSH) and pharmacological agents that activate cAMP-dependent protein kinases. In intact Sertoli cells, both phosphorylation and dephosphorylation of proteins occurred in response to treatment with these agents. Studies using cell-free preparations suggest that four phosphoproteins phosphorylated by cAMP or the catalytic subunit of cAMP-dependent protein kinase were also phosphorylated in a FSH-dependent manner in intact cells. These data suggest that FSH-dependent phosphorylation in Sertoli cells occurs through activation of a cAMP-dependent protein kinase. A FSH-dependent phosphoprotein with a molecular weight of 58,000 was identified as the intermediate filament protein vimentin, based on its migration in two-dimensional gels and its peptide map. The cellular distribution of vimentin was monitored by immunofluorescence in Sertoli cells after treatment with FSH. Results of this study support a role for intermediate filaments in FSH-dependent events in Sertoli cells.**

Sertoli cell function is regulated by both testosterone and follicle-stimulating hormone (FSH) (1, 2). The development of techniques for the isolation and culture of Sertoli cells (3–5) has provided an opportunity to study their hormonal regulation. We have previously reported the cellular distribution of immunoreactive androgen-binding protein (6), the temporal sequence of Sertoli cell shape changes (7), and protein synthesis and secretion (8) in cultured Sertoli cells in response to FSH treatment. Results of these studies support a cAMP-mediated mechanism that induces alterations in components of the cytoskeleton that may play a role in the processing of proteins by Sertoli cells.

To further characterize the regulatory mechanisms involved in FSH action on Sertoli cells, experiments were conducted to examine the role of protein phosphorylation in modulating FSH effects. To date, no specific FSH-dependent protein substrates have been identified.

Our findings show that treatment of Sertoli cells with FSH or a cyclic nucleotide analog results in both phosphorylation and dephosphorylation of several polypeptides. One of the proteins phosphorylated in a FSH-dependent manner was identified as the intermediate filament protein (IFP) vimentin, a cytoskeleton component of Sertoli cells (7).

MATERIALS AND METHODS

Materials. Ovine FSH (NIH-FSH-S12) was provided by the Pituitary Distribution Program of the National Institute of Arthritis, Metabolism, and Digestive Diseases. 2-Mercaptoethanol, phenylmethylsulfonyl fluoride, N^6,$O^{2'}$-dibutyryladenosine 3′,5′-phosphate (Bt_2cAMP), *N*-morpholinepropanesulfonic acid (Mops), EGTA, ATP, and cAMP were from Sigma. 1-Methyl-3-isobutyl-xanthine (MeiBuXan, "MIX") was from Aldrich. Fluorescein isothiocyanate-conjugated goat anti-rabbit IgG was from Cappel Laboratories (Cochranville, PA). [γ-^{32}P]ATP (2,000–4,000 Ci/mmol; 1 Ci = 37 GBq) and carrier-free $^{32}P_i$ were from New England Nuclear. Ultrapure urea was from Schwarz/Mann. Gel electrophoresis reagents were from Bio-Rad and Pharmacia.

Cell Culture and Preparation of Cell Fractions. Primary cultures of Sertoli cells were prepared from 20- to 22-day-old rats as described (9). Sertoli cells were maintained in serum-free medium for 18–24 hr prior to the addition of various agents. Attached cells were incubated in a humidified atmosphere at 32°C. Cells were collected in cold 20 mM Mops, pH 7.0/10 mM $MgCl_2$/0.2 mM EGTA/150 mM NaCl, dispersed by sonication for 15 sec (10), and then centrifuged at 27,000 × *g* for 10 min. Portions of the supernatant were used to assay protein kinase activity or endogenous phosphorylation. In some experiments, Sertoli cells were collected in 20 mM Mops, pH 7.0/150 mM NaCl/2 mM 2-mercaptoethanol and homogenized in a glass Dounce homogenizer (small-clearance pestle). After homogenization, $MgCl_2$ was added to a final concentration of 10 mM. The cell homogenate was centrifuged at 600 × *g* for 3 min and supernatants were collected and centrifuged at 100,000 × *g* for 60 min.

Protein Kinase Assay. Reaction mixtures (150 μl) contained 20 mM Mops, pH 7.0, 10 mM $MgCl_2$, 0.2 mM MeiBuXan, 0.2 mM EGTA, histones H1 and H2B (1.0 mg/ml), 25–50 μM [γ-^{32}P]ATP (100–200 cpm/pmol), and 20–30 μg of protein with or without 2–10 μM cAMP. Reactions were run for 10 min at 30°C and activity was assayed by spotting on filter disks as described (10).

***In Vitro* Phosphorylation of Cell-Free Extracts.** Sertoli cells were collected and processed as described above except that 0.1 mM phenylmethylsulfonyl fluoride was included in the homogenization buffer. Reaction mixtures (150 μl) contained 20 mM Mops, pH 7.0, 10 mM $MgCl_2$, 0.2 mM MeiBuXan, 0.2 mM EGTA, 1–5 μM [γ-^{32}P]ATP (4 to 5 μCi per tube), 10–20 μM cAMP, and 25–50 μg of protein. Reactions were run for 10 min at 30°C and samples were spotted on filter disks as described (10). The remaining portion of each mixture was mixed with an equal volume of double-strength $NaDodSO_4$ sample buffer (11) and boiled for 5 min.

***In Vitro* Phosphorylation of Intact Sertoli Cells.** Pulse-labeling with ^{32}P was carried out as described by Le Cam *et al.* (12). The agents were added to Sertoli cells in fresh serum-free medium containing 5–10% of the normal phosphate concentra-

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Abbreviations: FSH, follitropin, follicle-stimulating hormone; C subunit, catalytic subunit of cAMP-dependent protein kinase; cAMP/kinase, cAMP-dependent protein kinase; IFP, intermediate filament protein; Bt_2cAMP, N^6,$O^{2'}$-dibutyryladenosine 3′,5′-phosphate; MeiBuXan, 1-methyl-3-isobutylxanthine; Mops, *N*-morpholinepropanesulfonic acid.

tion and $^{32}P_i$ (250–800 μCi/ml) was added to the culture dishes during the last 20 min of incubation with the agents. For one-dimensional polyacrylamide gel electrophoresis, reactions were terminated by removal of media and rinsing the Sertoli cells with phosphate-buffered saline. $NaDodSO_4$ sample buffer was then added to the dishes and the samples were boiled for 10 min. For two-dimensional gel electrophoresis, the cells were rinsed with Hanks' balanced salt solution and centrifuged at 1,000 rpm for 3 min. The salt solution was removed by aspiration and 150 μl of lysis buffer (11) was added to the cells.

Polyacrylamide Gel Electrophoresis and Autoradiography. One- and two-dimensional $NaDodSO_4$/polyacrylamide gel electrophoresis, using a 5–15% acrylamide (linear gradient) slab gel, was carried out as described (11). Gels were processed as described (10).

Peptide Mapping. One-dimensional peptide mapping after limited proteolysis was carried out by the procedure of Cleveland *et al.* (13) as described (10).

Immunoprecipitation. Sertoli cells were labeled for 16 hr in low-methionine (10% of normal) medium containing [^{35}S]-methionine at 100 μCi/ml. The cells were collected, centrifuged at 1,000 rpm for 3 min, and solubilized by boiling for 5 min with 2% $NaDodSO_4$/5% 2-mercaptoethanol.

The samples were diluted (1:20) with 0.5% Triton X-100 in phosphate-buffered saline and centrifuged at 100,000 × *g* for 30 min. Immunoprecipitation was carried out as described by Murphy *et al.* (14) except that *Staphylococcus aureus* protein A absorbent (15) was substituted for the second antibody.

Indirect Immunofluorescence. Immunocytochemical experiments were conducted as described (6, 7). Coverslips were mounted with Elvanol (16). Controls for specificity involved (*i*) omission of the primary antiserum, (*ii*) absorption of the antiserum with IFP, and (*iii*) immunoprecipitation of [^{35}S]methionine-labeled extracts.

RESULTS

cAMP-Dependent Protein Kinase (cAMP/Kinase) Activity in Sertoli Cells. Because activation of cAMP/kinase provides an important regulatory mechanism for many cellular processes through protein phosphorylation (17), we examined the effects of FSH and pharmacological agents that elevate intracellular cAMP in cultured Sertoli cells. Incubation of Sertoli cells with FSH or the phosphodiesterase inhibitor MeiBuXan for 30 min resulted in moderate activation of soluble cAMP/kinase. However, treatment of Sertoli cells with FSH/MeiBuXan or with Bt_2cAMP for 30 min significantly stimulated cAMP/kinase activity (Fig. 1). These findings agree with a previous report (18). Activation of cAMP/kinase occurs within 5 min after exposure of Sertoli cells to FSH in the presence of MeiBuXan (19) and is maintained up to 1 hr after treatment (activity ratios: 0.89 vs. 0.67).

Endogenous Protein Phosphorylation in Cell-Free Extracts. We next investigated *in vitro* protein phosphorylation following treatment with agents that activated cAMP/kinase on the assumption that cAMP/kinase activation in intact Sertoli cells in response to hormone could be maintained and examined *in vitro* by incubation in the presence of [γ-^{32}P]ATP. When protein phosphorylation patterns in soluble fractions (27,000 × *g*) were examined under these conditions (Fig. 2), previous treatment with Bt_2cAMP or FSH/MeiBuXan enhanced phosphorylation of at least nine proteins (M_r, >330,000, 140,000, 98,000, 85,000, 73,000, 58,000, 39,000, 27,000, and 24,000). However, treatment with FSH or MeiBuXan alone only moderately altered the protein ^{32}P-labeling patterns when compared with control samples.

When a whole cell sonicate was incubated with cAMP or the catalytic subunit of cAMP/kinase (C subunit), the phosphorylation of at least six proteins (M_r, >330,000, 140,000, 85,000, 39,000, 27,000, and 24,000) increased (Fig. 3). In addition, three other proteins (M_r, 58,000, 45,000, and 41,000) were phosphorylated in the presence of the C subunit but not in the presence of cAMP.

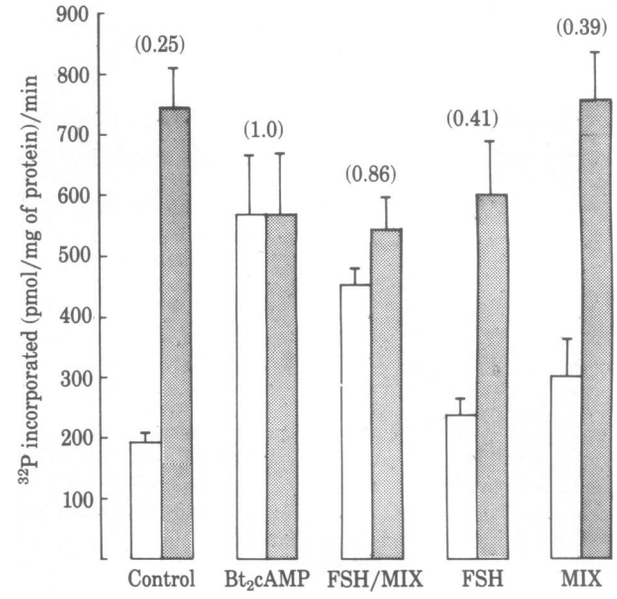

FIG. 1. cAMP/kinase activity in Sertoli cells after treatment with FSH (10 μg/ml), MeiBuXan (MIX; 0.4 mM), FSH/MeiBuXan, or Bt_2cAMP (0.5 mM) for 30 min. Activity ratios (in parentheses) reflect protein kinase activities measured in the absence (□) or presence (▨) of 10 μM cAMP. Results represent mean ± SEM of five determinations.

Endogenous Protein Phosphorylation in Intact Sertoli Cells. While several proteins present in cell-free extracts were phos-

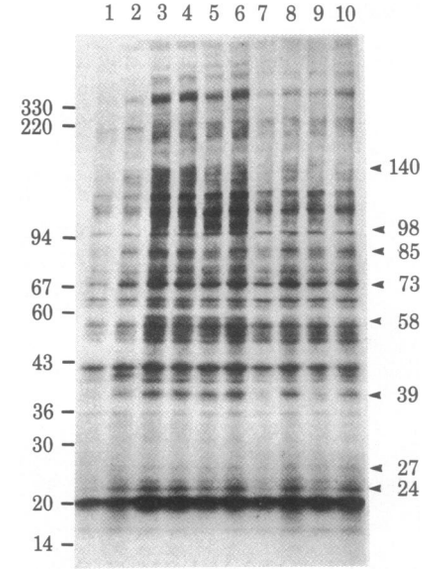

FIG. 2. Effect of previous treatment with various agents on protein phosphorylation of cell-free extracts *in vitro*. Sertoli cells were incubated for 30 min with 0.5 mM Bt_2cAMP (lanes 3 and 4), FSH at 20 μg/ml (lanes 7 and 8), 0.1 mM MeiBuXan (lanes 9 and 10), or FSH/MeiBuXan (lanes 5 and 6) and the 27,000 × *g* soluble fraction prepared from cell sonicates (lanes 1 and 2: control cells) in the presence (lanes 2, 4, 6, 8, and 10) or absence (lanes 1, 3, 5, 7, and 9) of 20 μM cAMP. The following amounts of protein were loaded on the gels: lanes 1 and 2, 32 μg; lanes 3 and 4, 33 μg; lanes 5 and 6, 40 μg; lanes 7 and 8, 36 μg; lanes 9 and 10, 34 μg. Numbers on the left and right represent $M_r \times 10^{-3}$.

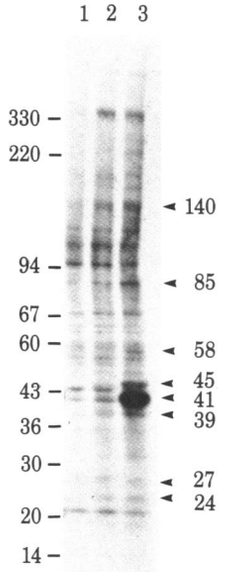

FIG. 3. Endogenous protein phosphorylation in cell-free extracts of Sertoli cells: Autoradiogram showing the protein phosphorylation patterns in cell sonicates incubated with exogenous 20 μM cAMP (lane 2) or 2 μg of C subunit (lane 3). Lane 1: control cells. Numbers are as in Fig. 2.

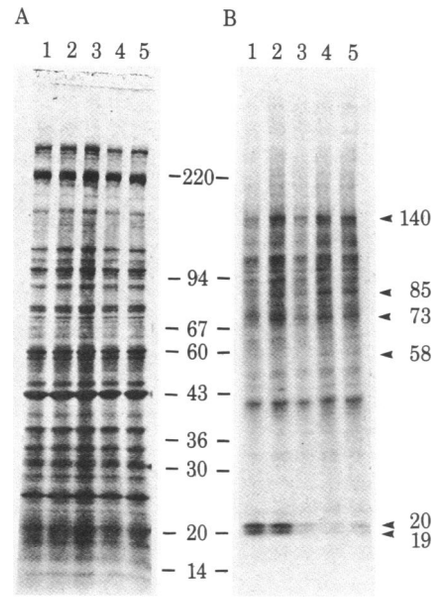

FIG. 4. Endogenous protein phosphorylation in intact Sertoli cells. Cells were incubated with FSH at 10 μg/ml (lanes 2), 0.25 mM MeiBuXan (lanes 3), FSH/MeiBuXan (lanes 4), or 0.5 mM Bt_2cAMP (lanes 5) for 120 min and pulse-labeled with $^{32}P_i$ for 20 min. Lanes 1: control cells. (*A*) Coomassie blue staining pattern. (*B*) Autoradiogram of the stained gel. Phosphorylation/dephosphorylation changes are noted by arrowheads. Numbers on the right of the gels are as in Fig. 2.

phorylated *in vitro* by cAMP or the C subunit or after treatment with FSH or Bt_2cAMP, we examined endogenous protein phosphorylation in intact Sertoli cells because the loss of compartmentation in cell-free extracts could allow access to nonphysiological substrates (20). The protein phosphorylation pattern in intact Sertoli cells pulse-labeled with $^{32}P_i$ during a 2-hr incubation period with various agents is shown in Fig. 4. Both phosphorylation and dephosphorylation of specific proteins were apparent as early as 20 min after treatment and persisted after 3 hr of exposure to the agents (data not shown). Three proteins (M_r, 140,000, 73,000, and 85,000) showed enhanced phosphorylation after treatment with all the agents tested, whereas a M_r 58,000 protein showed moderate enhancement in ^{32}P labeling only after exposure to Bt_2cAMP and FSH/MeiBuXan (Fig. 4). While changes in ^{32}P labeling were not quantitated, there appeared to be differences in labeling intensities depending on the treatment. Interestingly, two proteins (M_r, 20,000 and 19,000) showed a striking decrease in ^{32}P labeling after treatment with FSH/MeiBuXan, MeiBuXan, or Bt_2cAMP but not with FSH alone.

cAMP-Dependent Phosphorylation of Vimentin. In a previous study (10), we showed that the IFP vimentin was phosphorylated in a Ca^{2+}/calmodulin-dependent manner in cell-free preparations of Sertoli cells. Vimentin has been shown to be phosphorylated in a cAMP-dependent manner in other cell types (21, 22). It became apparent during the course of this study that the M_r 58,000 protein phosphorylated by the C subunit (Fig. 3) and by previous treatment of intact Sertoli cells with FSH or Bt_2cAMP (Fig. 2) was probably vimentin. When a Triton X-100-insoluble IFP extract was prepared from Sertoli cells as described (10) and incubated with the C subunit, a M_r 58,000 protein in the preparation was phosphorylated. Peptide maps of this phosphoprotein (Fig. 5) were identical to those reported for phosphorylated vimentin (10, 23).

Hormone-Dependent Phosphorylation of Vimentin. Since vimentin was phosphorylated in a cAMP-dependent manner in cell-free extracts and a M_r 58,000 protein was phosphorylated in intact Sertoli cells after treatment with FSH or Bt_2cAMP (Fig. 4), we used two-dimensional polyacrylamide gel electrophoresis to determine whether the hormone-dependent phosphoprotein was vimentin. Numerous changes in ^{32}P-labeling intensity were noted after 1 hr treatment with FSH (Fig. 6*C*) or Bt_2cAMP (Fig. 6*D*) when compared with control Sertoli cells (Fig. 6*B*). In general, changes in phosphorylation observed in response to FSH were mimicked by response to Bt_2cAMP. When the autoradiograms were brought into register with Coomassie blue-stained gels, one polypeptide spot on the autoradiogram (M_r, 58,000; pI, 5.1) (designated V in Fig. 6) appeared shifted to the acidic side of the corresponding stained spot. This phosphorylation pattern has been reported for vimentin phosphorylation in other cell types (24, 25). This particular behavior of the protein, in addition to its M_r and pI, support its identity as vimentin.

Immunocytochemical Localization of Vimentin in Sertoli Cells. Using an antiserum that crossreacts with vimentin-type intermediate filaments (26), we examined the distribution of

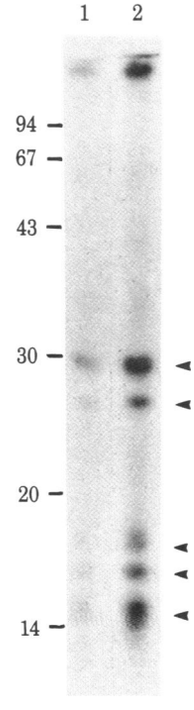

FIG. 5. One-dimensional peptide map of phosphorylated vimentin. An IFP extract prepared from Sertoli cells was added to a soluble fraction (27,000 × *g*) of a Sertoli cell sonicate and incubated in the presence (lane 2) and absence (lane 1) of 0.7 μg of C subunit. Digestion was carried out with 0.02 μg of *S. aureus* V8 protease. Proteolytic fragments are indicated by arrowheads. Numbers on the left are as in Fig. 2.

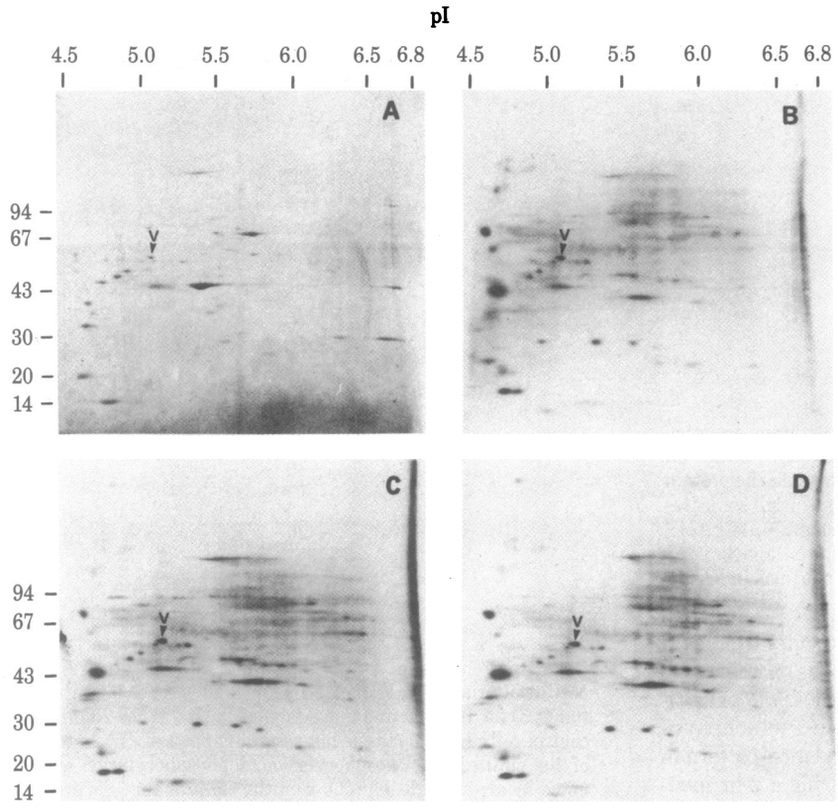

FIG. 6. Two-dimensional polyacrylamide gel electrophoresis analysis of protein phosphorylation patterns in intact Sertoli cells. Cells were incubated with FSH at 10 μg/ml (*C*) or with 0.5 mM Bt_2cAMP (*D*) for 60 min, pulse-labeled with $^{32}P_i$ for 20 min, and autoradiographed. (*A* and *B*) Coomassie blue staining pattern of control Sertoli cells and the corresponding autoradiogram of the ^{32}P-labeling pattern, respectively. Autoradiograms were obtained after a 14-day exposure. V, vimentin. Numbers on the left are as in Fig. 2.

vimentin in cultured Sertoli cells after exposure to FSH or Bt_2cAMP by indirect immunofluorescence. Immunoprecipitation of [^{35}S]methionine-labeled extracts of Sertoli cells confirmed crossreactivity of the antiserum with Sertoli cell vimentin (Fig. 7). Immunoreactive vimentin was localized in the cytoplasm of control Sertoli cells as a diffuse filamentous network (Fig. 8 *A* and *B*). The fluorescence was abolished by absorption of the antiserum with IFP prepared from Sertoli cells (data not shown). Sertoli cells treated with FSH for 60 min displayed the characteristic flat–stellate transition (Fig. 8*C*). Vimentin immunoreactivity in morphologically responsive Sertoli cells appeared restricted to the long cytoplasmic processes and the perinuclear region. Treatment with Bt_2cAMP (Fig. 8*D*) resulted in similar fluorescence patterns except that the morphological response was more rapid and extensive in agreement with a previous report (7). These results confirm the observation of Franke *et al.* (27) that intermediate filaments in Sertoli cells are of the vimentin type.

DISCUSSION

We have examined cAMP/kinase activity and the phosphorylation of intracellular proteins in cultured rat Sertoli cells as an approach to the understanding of FSH regulation of Sertoli cell function. cAMP/kinase activation in response to FSH precedes the development of morphological changes (7), supporting a possible relationship between the phosphorylation of intracellular proteins and alteration in the cytoskeleton. When protein phosphorylation patterns were examined *in vitro* after exposure of Sertoli cells to various agents, previous treatment with FSH/MeiBuXan or Bt_2cAMP significantly enhanced protein phos-

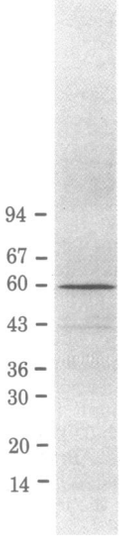

FIG. 7. Immunoprecipitation of [^{35}S]methionine-labeled extracts with vimentin antiserum. Minor labeled bands represent nonspecific adsorption since the background was not reduced by further dilution of antiserum. The autoradiogram was obtained after a 10-day exposure. Numbers are as in Fig. 2.

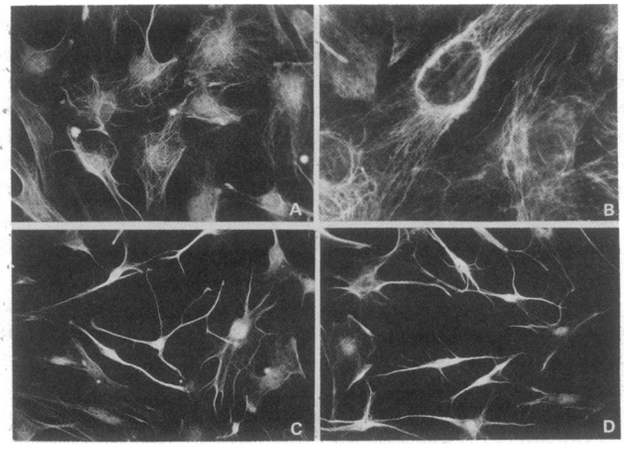

FIG. 8. Immunocytochemical localization of vimentin in cultured Sertoli cells. (*A* and *B*) Distribution of vimentin in control Sertoli cells. (*A*, ×160; *B*, ×480.) (*C*) Distribution after 60 min of treatment with FSH at 10 μg/ml. (×130.) (*D*) Distribution after 60 min of treatment with 0.5 mM Bt_2cAMP. (×120.)

phorylation whereas previous treatment of Sertoli cells with FSH or MeiBuXan alone resulted in moderate phosphorylation changes. This agent-dependent effect on protein phosphorylation correlated well with a similar agent-dependent activation of cAMP/kinase (Fig. 1). The differences in cAMP/kinase activity and protein phosphorylation observed between treatment with FSH or MeiBuXan alone versus FSH/MeiBuXan or Bt_2cAMP treatment may be related to high concentrations of a cAMP/kinase inhibitor present in Sertoli cells of 20- to 22-day-old rats (28). High levels of cAMP may be required to overcome cAMP/kinase inhibition, a requirement that is even more important in cell-free extracts because of the loss of cell compartmentation.

Based on similarities in M_r, results from experiments using intact Sertoli cells suggest that the four major FSH-dependent phosphoproteins were the same proteins phosphorylated in a cAMP-dependent manner in cell-free extracts. These observations support FSH-dependent protein phosphorylation in Sertoli cells as being mediated by cAMP/kinase activation. Of the four major hormone-dependent phosphoproteins, our interest focused on one, the M_r 58,000 phosphoprotein. This protein was phosphorylated in a FSH-dependent manner in both cell-free extracts and intact cells. Based on the following criteria, this protein was identified as the IFP vimentin: (*i*) its M_r of 58,000 and pI of 5.1, (*ii*) the similarity of the peptide maps of the M_r 58,000 phosphoprotein and of phosphorylated vimentin, (*iii*) the shift of the M_r 58,000 phosphoprotein to the acidic side of Coomassie blue-stained protein on two-dimensional gels, and (*iv*) immunoprecipitation of a M_r 58,000 protein from [^{35}S]methionine-labeled extracts with a vimentin antiserum.

Both cAMP and Ca^{2+} are thought to play important roles as intracellular regulators of Sertoli cell function (2, 29). The results of the present study suggest that phosphorylation of vimentin in Sertoli cells, triggered by FSH, occurs in a cAMP-dependent manner. However, since vimentin in Sertoli cells can also be phosphorylated in a Ca^{2+}/calmodulin-dependent manner under certain conditions (10), a role for Ca^{2+}/calmodulin in FSH-dependent processes cannot be ruled out. While the C subunit and calmodulin (10) appear to phosphorylate residues on the same proteolytic fragments, further analysis is required to determine whether the same residues are phosphorylated.

One intriguing question is the possible role of vimentin phosphorylation in Sertoli cells. In C-6 glioma cells, vimentin phosphorylation occurs after treatment with norepinephrine (30). In response to norepinephrine, glioma cells display a transient alteration in cell shape (31) and synthesize and release nerve growth factor (32). In Sertoli cells, FSH induces transient changes in cell morphology (7) and promotes the synthesis and release of several proteins (8, 33, 34). Because vimentin is phosphorylated in both glioma cells and Sertoli cells in response to hormones that induce cell shape changes, it is attractive to suggest a role for intermediate filaments in the hormonal induction of an altered cell morphology. Our immunocytochemical studies show that vimentin-type intermediate filaments in cultured Sertoli cells are not disrupted as Sertoli cells modify their shape from a flat to a stellate morphology in response to FSH. If FSH-induced phosphorylation of vimentin in cultured Sertoli cells can be related to the induction of cell shape changes, it is possible that a similar hormone-mediated mechanism acts on the elaborate cytoskeletal network in Sertoli cells *in vivo*. A dynamic modification of cytoskeletal components in Sertoli cells in the seminiferous epithelium may be required for a variety of processes related to spermatogenic events (7, 9).

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