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Calcium/calmodulin-dependent phosphorylation of vimentin in rat Sertoli cells

(trifluoperazine/follicle-stimulating hormone/cytoskeleton)

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ABSTRACT **Ca^{2+}-dependent protein phosphorylation and the role of calmodulin in this process was investigated in subcellular fractions of primary cultures of rat Sertoli cells. Significant Ca^{2+}/calmodulin-dependent protein phosphorylation in Sertoli cells was restricted to the cytosol fraction. The calmodulin dependence of these effects was confirmed by using the calmodulin inhibitor trifluoperazine. One of the Ca^{2+}/calmodulin-dependent phosphoproteins was identified as the intermediate filament protein vimentin, based on the following criteria: (*i*) migration pattern in two-dimensional polyacrylamide gels, (*ii*) Ca^{2+}/calmodulin-dependent phosphorylation of a 58-kilodalton protein present in detergent-insoluble intermediate filament protein extract of Sertoli cells, and (*iii*) peptide mapping of the phosphoprotein. These data support a role for Ca^{2+}/calmodulin-dependent protein phosphorylation in the modulation of Sertoli cell cytoskeletal components.**

Calmodulin, the major calcium-binding protein in nonmuscle cells, modulates several cellular processes in which Ca^{2+} plays an essential role (for review, see refs. 1–4), including Ca^{2+}-dependent protein phosphorylation (5–11). In nonmuscle cells, calmodulin regulates actin-activated myosin ATPase via Ca^{2+}-dependent phosphorylation of the light chain of myosin by myosin light chain kinase (12, 13). Although calmodulin appears to play an important role in microfilament function in several systems (4, 14–16), including Sertoli cells (17, 18), the functional significance of Ca^{2+}/calmodulin-dependent protein phosphorylation in the regulation of cytoskeletal components in the cell remains unknown.

Results of studies from our laboratory (19–21) support a mediatory role for cAMP in hormone-dependent processes in Sertoli cells through the modulation of cytoskeletal components. Both cAMP and Ca^{2+} have been implicated in processes associated with cytoskeletal modifications; however, the relationship between Ca^{2+} and cAMP in these processes is not known. In the present study, we have examined Ca^{2+}-dependent protein phosphorylation in rat Sertoli cells as one approach to assessing the role of Ca^{2+} in functional events related to cyclic nucleotide metabolism and cell secretion. Results of this study show the presence of a Ca^{2+}-dependent protein kinase activity in Sertoli cells that is calmodulin-dependent. Several proteins are phosphorylated in a Ca^{2+}/calmodulin-dependent manner, one of which is the intermediate filament protein (IFP) vimentin.

MATERIALS AND METHODS

Materials. Phenylmethylsulfonyl fluoride, 2-mercaptoethanol, EDTA, EGTA, cGMP, cAMP, Pipes, and snake venom (*Ophiophagus hannah*) were obtained from Sigma. [γ-^{32}P]ATP (2,000–4,000 Ci/mmol; 1 Ci = 37 GBq) was purchased from New England Nuclear. Gel electrophoresis reagents were obtained from Bio-Rad except that molecular weight markers and Ampholines were purchased from Pharmacia. Trifluoperazine (TFP) was a gift from Smith, Kline & French Laboratories. *N*-(6-Aminohexyl)-5-chloronaphthalene-1-sulfonamide (W-7) was supplied by H. Hidaka. *Staphylococcus aureus* V8 protease was obtained from Miles Laboratories.

Purification of Calmodulin. Calmodulin was either obtained from Calbiochem (bovine brain) or purified from bovine brain or rat testis using filtration on Sepharose 4B and affinity chromatography on a W-7 affinity column (22). Calmodulin activity was determined by using the assay for calmodulin-sensitive phosphodiesterase. The purity of the preparations was analyzed by $NaDodSO_4$/polyacrylamide gel electrophoresis.

Purification and Assay of Phosphodiesterase. The procedure for the partial purification of calmodulin-sensitive phosphodiesterase from bovine brain was essentially that described by Cheung and Lin (23). Phosphodiesterase activity was assayed by the method of Thompson and Appleman (24).

Preparation of Cell Fractions. Primary cultures of rat Sertoli cells were prepared from testes of 20- to 22-day-old rats as described (25). Cells growing in culture flasks were collected in cold 50 mM Pipes, pH 7.0/10 mM $MgCl_2$/50 mM NaCl with and without 0.1 mM phenylmethylsulfonyl fluoride and dispersed by sonication (2.5–3.5 W) for 15 sec. After centrifugation at 27,000 × *g* for 10 min, portions of the supernatant were used to assay for endogenous protein phosphorylation. When subcellular fractions were required, the cells were collected in 50 mM Pipes, pH 7.0/50 mM NaCl and then homogenized in a glass Dounce homogenizer (small clearance pestle). After homogenization, $MgCl_2$ was added to a final concentration of 10 mM. Subcellular fractions (100,000 × *g* cytosol and particulate fractions) were prepared from homogenates as described by Li and Hsie (26). The crude nuclear fraction obtained after Triton extraction (26) was further purified by centrifugation through sucrose as described by Prashad *et al.* (27). Nuclei were rinsed and resuspended in homogenization buffer prior to assay. Purity of the subcellular fractions was monitored by assaying for lactate dehydrogenase activity (28) and 5′-nucleotidase activity (29) in different fractions. Contamination of particulate and nuclear fractions with soluble proteins was <5% (n = 5). Contamination of the soluble fraction with particulate protein was <5% (n = 3) and of the nuclear fractions with particulate protein was <10% (n = 3).

***In Vitro* Phosphorylation.** Reaction mixtures (150 μl) contained 50 mM Pipes (pH 7.0), 10 mM $MgCl_2$, 0.2 mM EGTA,

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Abbreviations: IFP, intermediate filament protein; TFP, trifluoperazine.

5–25 μM [γ-^{32}P]ATP (4–5 μCi per tube), 20–100 μg of protein with or without 0.1–0.25 mM $CaCl_2$ and/or 1–2 μg of calmodulin. Reactions were run routinely for 10 min at 30°C. Reactions were terminated by spotting an aliquot of the reaction mixture on Whatman 3MM filter paper and immediately dipping it in cold 10% trichloroacetic acid/1% sodium pyrophosphate. The filter paper discs were processed as described by Corbin and Reimann (30). The remaining portion of the reaction mixture was mixed with an equal volume of double-strength $NaDodSO_4$ sample buffer (31) and boiled for 5 min. Aliquots of the samples were then analyzed by $NaDodSO_4$/polyacrylamide gel electrophoresis as described below. For analysis of the labeled extracts by two-dimensional gel electrophoresis, samples were removed from the water bath on completion of the reaction and immediately frozen in a dry ice/acetone bath. Samples were lyophilized and then reconstituted in lysis buffer (31).

Gel Electrophoresis and Autoradiography. One-dimensional $NaDodSO_4$/polyacrylamide gel electrophoresis using a 5–15% acrylamide (linear gradient) slab gel and two-dimensional polyacrylamide gel electrophoresis were carried out as described by O'Farrell (31). Gels were fixed and stained with 25% isopropyl alcohol/10% acetic acid/0.05% Coomassie brilliant blue and then destained in 10% acetic acid. For autoradiography, dried gels were exposed to Kodak XR-2 film at room temperature for 6 to 7 days, and then the films were developed and fixed by standard procedures.

Peptide Mapping. One-dimensional peptide mapping was carried out as described by Cleveland *et al.* (32). Phosphoproteins were excised from the dried gels after autoradiography and then reswollen and equilibrated for 30 min in 0.125 M Tris·HCl, pH 6.8/0.1% $NaDodSO_4$/1 mM EDTA. Gel slices were placed in the wells of the stacking gel (4 cm) and overlayed with *S. aureus* V8 protease. Fragments generated after limited proteolysis were separated on a 15% resolving gel.

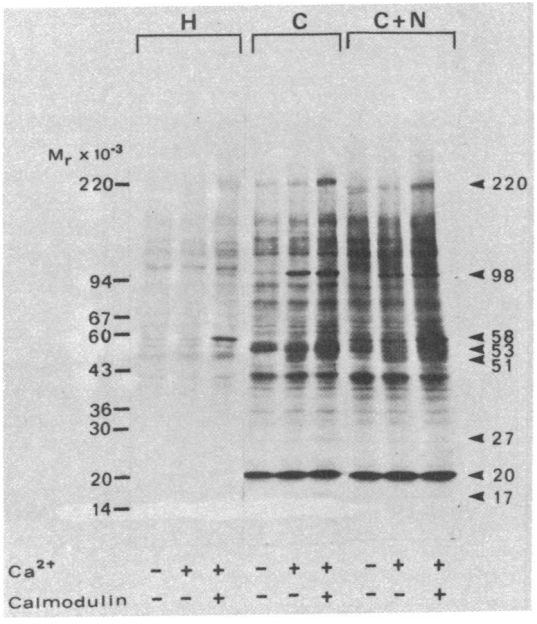
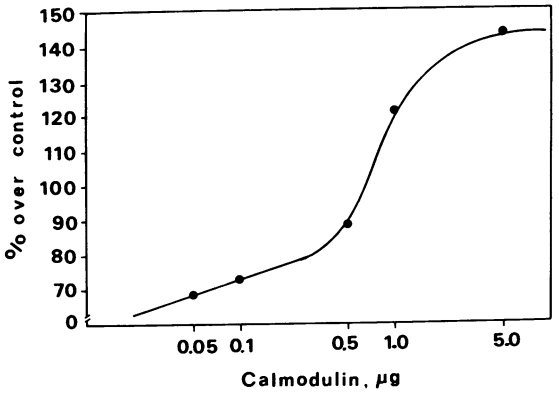


FIG. 1. Ca^{2+}/calmodulin-dependent protein phosphorylation in subcellular fractions of Sertoli cells. Sertoli cell homogenates (H) and subcellular fractions were incubated with 250 μM $CaCl_2$ and [γ-^{32}P]ATP in the presence or absence of 2 μg of calmodulin (Calbiochem). The autoradiograms show protein phosphorylation in a cytosol (C) fraction incubated alone or mixed with an equal volume of nuclear (N) fraction. Arrowheads indicate relevant changes in phosphorylation patterns of proteins (M_r × 10^{-3}) on the autoradiogram.

RESULTS

Ca^{2+}/Calmodulin-Dependent Protein Phosphorylation in Subcellular Fractions of Sertoli Cells. When a whole cell homogenate was incubated in the presence of Ca^{2+} with or without calmodulin, significant phosphorylation was noted for only a protein with a M_r of 58,000 (Fig. 1). However, when a cytosol fraction (100,000 × *g*) was incubated in the presence of Ca^{2+}, enhanced phosphorylation was noted for at least three proteins (M_r, 98,000, 53,000, and 51,000). The addition of calmodulin to the reaction mixture enhanced the Ca^{2+}-dependent phosphorylation of several additional proteins (M_r 220,000, 27,000, 20,000, and 17,000). In the absence of Ca^{2+}, calmodulin had no effect on the protein phosphorylation pattern except that occasionally the M_r 98,000 phosphoprotein showed an enhancement in ^{32}P labeling in the presence of calmodulin alone (data not shown).

When the particulate (100,000 × *g*) and nuclear fractions were assayed for Ca^{2+}/calmodulin-dependent protein phosphorylation, no significant change in activity or protein phosphorylation patterns were observed (data not shown). However,



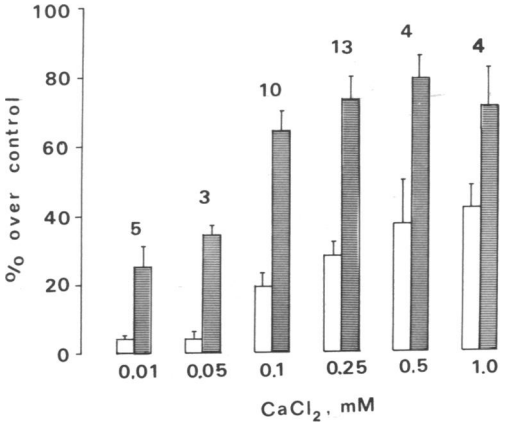


FIG. 2. Calmodulin and Ca^{2+} concentration dependence of Ca^{2+}/calmodulin-dependent protein phosphorylation. Ca^{2+}/calmodulin-dependent phosphorylation was determined in a soluble fraction (27,000 × *g*) prepared from Sertoli cell sonicates. (*Upper*) The reaction mixture contained 250 μM $CaCl_2$ and various amounts of bovine brain calmodulin. Values represent cpm of ^{32}P incorporated and are expressed as percent over control. Each point represents the mean of duplicate determinations. (*Lower*) Phosphorylation was determined in the absence (□) or presence (■) of 1 to 2 μg of bovine brain calmodulin and various concentrations of $CaCl_2$. Values represent cpm of ^{32}P incorporated (mean ± SEM) and are expressed as % over control. Numbers of determinations are indicated above the bars.

Table 1. Effects of TFP on Ca^{2+}/calmodulin-dependent phosphorylation of Sertoli cell proteins

Addition(s)	Control		Trifluoperazine	
	Specific activity	% over control	Specific activity	% over control
None	8.14 ± 0.63	0	8.38 ± 1.18	0
Ca^{2+}	9.30 ± 1.41	14	8.20 ± 1.33	0
Ca^{2+}/ calmodulin	11.09 ± 1.92	36	8.87 ± 1.66	6

Ca^{2+}/calmodulin-dependent protein phosphorylation was assayed in soluble fractions prepared from Sertoli cell sonicates. Activity was measured in the presence of 250 μM $CaCl_2$ or of 250 μM $CaCl_2$ and 2 μg of calmodulin in the presence or absence of 20 μM TFP. Specific activity is expressed as pmol of ^{32}P incorporated/mg of protein per min. Values represent the mean ± SEM of four determinations.

if a nuclear fraction was mixed with a cytosol fraction and then analyzed for Ca^{2+}/calmodulin-dependent protein phosphorylation, a M_r 58,000 protein was phosphorylated in the presence of Ca^{2+}/calmodulin (Fig. 1). Addition of a particulate fraction to a cytosol fraction resulted in marked inhibition of overall phosphorylation (data not shown). This latter effect could be due to membrane-associated ATPases present in the particulate fraction and may account for the lack of significant phosphorylation observed in whole cell homogenates.

Ca^{2+}/Calmodulin Concentration Dependence of Protein Phosphorylation. The addition of calmodulin (>0.5 μg) to the reaction mixture in the presence of 250 μM Ca^{2+} resulted in significant enhancement in ^{32}P incorporation over that observed in the presence of Ca^{2+} alone (60% over control) (Fig. 2). Ca^{2+}-dependent phosphorylation was apparent at Ca^{2+} concentrations >100 μM (Fig. 2); however, in the presence of exogenous calmodulin, Ca^{2+}-dependent phosphorylation was observed at 10 μM Ca^{2+}. In the presence of calmodulin alone, only a slight (8%) enhancement in ^{32}P incorporation was noted in these experiments.

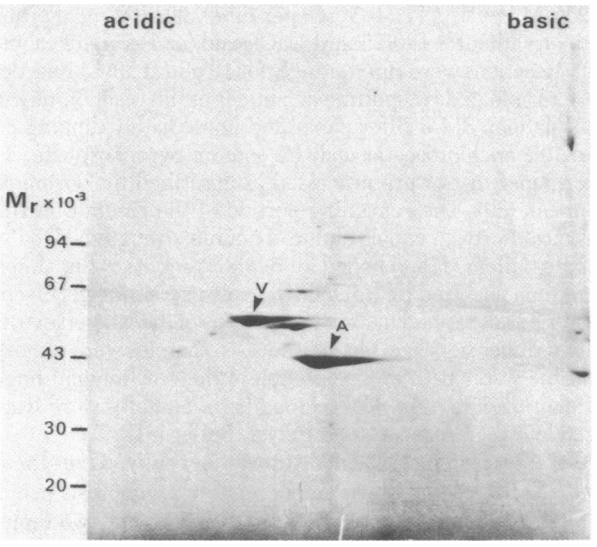


FIG. 4. Two-dimensional polyacrylamide gel electrophoresis analysis of an IFP extract prepared from Sertoli cells. A Coomassie blue staining pattern of the Triton X-100-insoluble extract is shown. The pH range is from 4.5 to 6.8 as determined by measuring the pH of slices of the isoelectric focusing gels. V, vimentin; A, actin.

Effects of TFP on Ca^{2+}/Calmodulin-Dependent Protein Phosphorylation. TFP, a potent inhibitor of calmodulin activity (33), was used to examine the calmodulin dependence of the phosphorylation of specific proteins. As shown in Table 1, Ca^{2+}/calmodulin-dependent phosphorylation was significantly reduced in the presence of 20 μM TFP. When the protein phos-

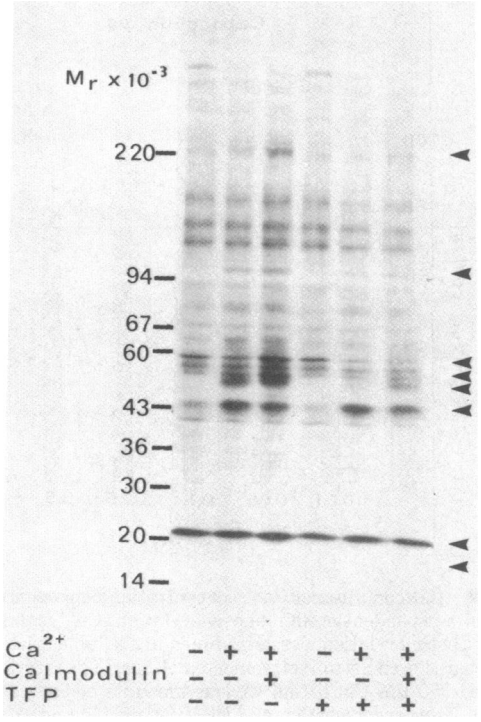


FIG. 3. Effect of TFP on Ca^{2+}/calmodulin-dependent protein phosphorylation. A soluble fraction (27,000 × *g*) was prepared from a cell sonicate and incubated with 250 μM $CaCl_2$ in the presence or absence of 1 μg of calmodulin and 20 μM TFP. Arrowheads indicate relevant changes in ^{32}P-labeled proteins.

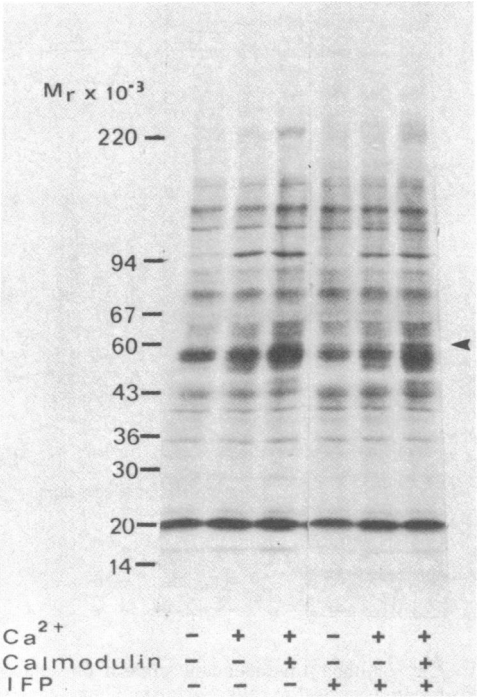


FIG. 5. Phosphorylation of an IFP extract. A cytosol fraction (100,000 × *g*) was prepared from a cell homogenate and incubated with 250 μM $CaCl_2$ with or without 2 μg of calmodulin and/or 10 μg of an IFP extract prepared from Sertoli cells. Phosphorylation of the M_r 58,000 protein present in the IFP extract is indicated by the arrowhead.

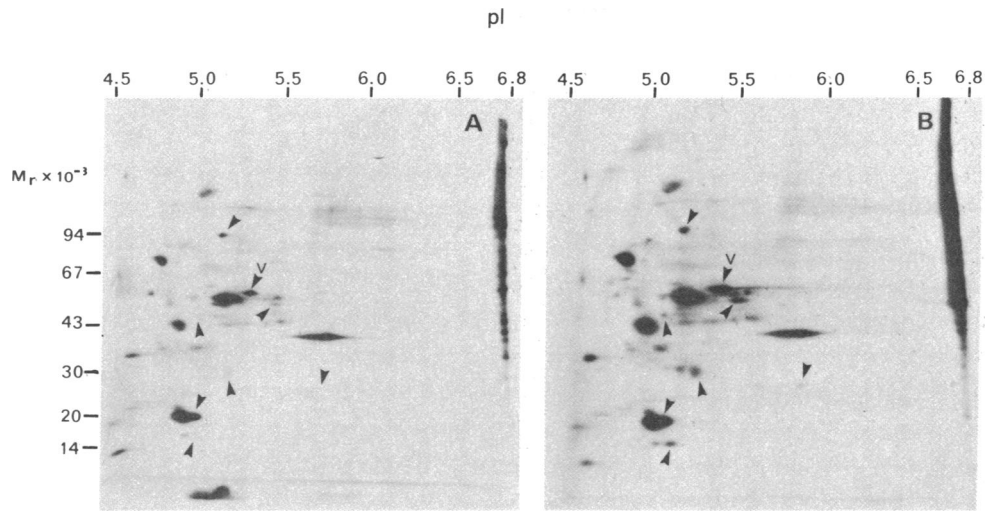


FIG. 6. Two-dimensional polyacrylamide gel electrophoresis analysis of Ca^{2+}/calmodulin-dependent protein phosphorylation. A soluble fraction (27,000 × *g*) was prepared from a sonicate of Sertoli cells and incubated in the absence (*A*) or presence (*B*) of 250 μM $CaCl_2$ and 2 μg of calmodulin (rat testis). Arrowheads indicate relevant changes in ^{32}P-labeling of proteins. The autoradiograms were obtained after a 14-day exposure. V, vimentin.

phorylation patterns were analyzed after incubation in the presence of TFP (Fig. 3), the Ca^{2+}/calmodulin-dependent labeling of several phosphoproteins (e.g., those of M_r 220,000, 98,000, and 58,000) was reduced or abolished but that of the M_r 45,000 protein was not.

Identification of the M_r 58,000 Phosphoprotein. One of the major Ca^{2+}/calmodulin-dependent phosphoproteins present in cultured Sertoli cells is a M_r 58,000 protein. Because of its M_r and subcellular distribution (34, 35), it was possible that this phosphoprotein was the IFP vimentin (36, 37). Intermediate filaments are known to be resistant to nonionic detergents and salt extraction (38, 39); therefore, a detergent-insoluble IFP extract was prepared from cultured Sertoli cells (39). A two-dimensional polyacrylamide gel electrophoresis profile of this extract is shown in Fig. 4. Based on M_r and migration patterns (39), the two major proteins present in the extract corresponded to vimentin and actin. When this extract was added to Sertoli cell cytosol, a protein in the extract with a M_r of 58,000 showed enhanced phosphorylation in the presence of Ca^{2+}/calmodulin (Fig. 5). While it was likely that the M_r 58,000 phosphoprotein detected in one-dimensional gels represented the phosphorylation of vimentin, samples were analyzed by two-dimensional gel electrophoresis to achieve greater resolution. The phosphorylation of several proteins was enhanced in the presence of Ca^{2+}/calmodulin, including a protein (designated V) with a M_r of 58,000 and an pI of 5.3 in urea (Fig. 6). The M_r and pI of this protein are very similar to those reported previously for vimentin (34, 40). In addition, when the autoradiograms were brought into register with the Coomassie blue-stained gels (data not shown), the phosphorylated protein spot was shifted to the acidic side of the stained spot as reported for vimentin phosphorylation in other cell types (34, 41, 42). To aid further in identification of the M_r 58,000 protein, one-dimensional peptide mapping of the phosphoprotein was carried out. Limited proteolysis generated five phosphorylated fragments (Fig. 7) in agreement with the pattern described previously for phosphorylated vimentin in other cell types (43). The same protein fragments showed enhanced phosphorylation in the presence of Ca^{2+}/calmodulin.

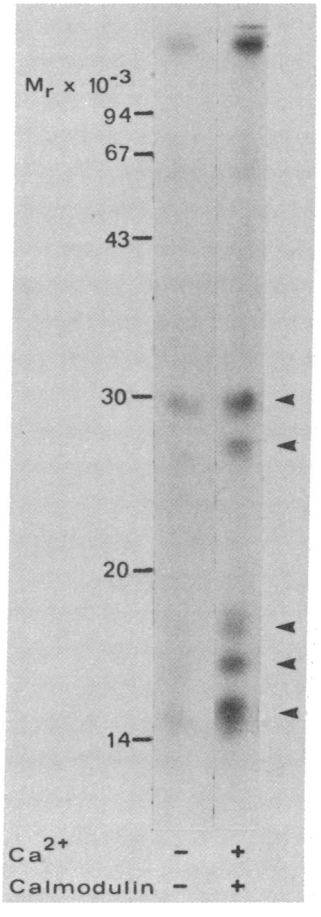


FIG. 7. One-dimensional peptide map of phosphorylated vimentin after limited proteolysis. An IFP extract was added to a soluble fraction (27,000 × *g*) of a sonicate of Sertoli cells in the presence or absence of 250 μM $CaCl_2$ and 2 μg of calmodulin (rat testis). Digestion was carried out with 0.02 μg of *S. aureus* V8 protease. The phosphorylated proteolytic fragments generated are indicated by arrowheads.

DISCUSSION

We have examined Ca^{2+}-dependent protein phosphorylation in cultured Sertoli cells as one approach to assessing the functional role of Ca^{2+} and calmodulin in this cell component of the seminiferous epithelium. When protein phosphorylation was analyzed in subcellular fractions, major Ca^{2+}-dependent effects were restricted to the cytosol fraction, except for the phosphorylation of a M_r 58,000 protein associated with the nuclear fraction. Several proteins showed increased phosphorylation in the presence of Ca^{2+} and their phosphorylation was further enhanced by exogenous calmodulin. Using the calmodulin inhibitor TFP, we found that the phosphorylation induced by Ca^{2+}/calmodulin was blocked or significantly reduced except for that

of a M_r 45,000 protein. The latter protein appears to be phosphorylated by Ca^{2+} in a calmodulin-independent manner.

While several Sertoli cell proteins were phosphorylated in a Ca^{2+}/calmodulin-dependent manner, attention was focused on the identification of one of the phosphoproteins, a M_r 58,000 protein. Based on the following criteria, the M_r 58,000 phosphoprotein was identified as the vimentin-type IFP: (*i*) a M_r 58,000 protein present in detergent-insoluble IFP extracts was phosphorylated in the presence of Ca^{2+}/calmodulin; (*ii*) two-dimensional polyacrylamide gel electrophoresis revealed Ca^{2+}/calmodulin-dependent phosphorylation of a protein with a M_r and pI comparable with those reported for vimentin (34, 40); (*iii*) peptide maps of the M_r 58,000 protein yielded identical patterns to those reported for vimentin (43); and (*iv*) the M_r 58,000 phosphoprotein, like vimentin, was found associated with the nuclear fraction (34, 35).

Vimentin is phosphorylated in both cAMP-dependent and -independent manners (37, 44). However, the functional role of phosphorylation in the modulation of intermediate filaments is not known. The results of the present study indicate that vimentin can also be phosphorylated in a Ca^{2+}/calmodulin-dependent manner. Data from a separate study (21) indicate that vimentin in Sertoli cells can be phosphorylated in a cAMP-dependent manner. Because of the complex relationship between cAMP and Ca^{2+} in a variety of cellular processes, the phosphorylation of vimentin in Sertoli cells may be regulated in a coordinate fashion by Ca^{2+}- and cAMP-dependent protein kinases. Multisite phosphorylation could provide a regulatory mechanism allowing cellular responses to diverse physiological stimuli to take place according to different control pathways.

Intermediate filaments, as components of the cytoskeleton, are thought to contribute to the compartmentation and mechanical integration of intracellular processes (for review, see ref. 37). Both Ca^{2+} and cAMP can affect the integrity of cytoskeletal components and these alterations may provide a mechanism for regulating secretory processes in the cell (4, 14, 16). In several cell systems, studies have suggested that Ca^{2+}-dependent phosphorylation plays a role in secretory process (4, 9, 45–47). In Sertoli cells both Ca^{2+} (17, 18) and cAMP (17–19) have been implicated in mediating follicle-stimulating hormone-induced responses and some of these effects may occur by rearrangement of the cytoskeleton. Hormone-dependent phosphorylation of vimentin in cells that undergo morphological changes and secrete specific proteins in response to hormones (21, 48) could support a role for intermediate filaments in hormone-induced processes.

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