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THE SECOND MESSENGER PATHWAY FOR GERM CELL-MEDIATED STIMULATION OF SERTOLI CELLS

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Summary: Treatment of cultured rat Sertoli cells with FSH or dibutyryl cAMP for 30 min resulted in phosphorylation of the same Sertoli cell proteins. Different Sertoli cell proteins were phosphorylated after calcium ionophore A23187 and 12-O-tetradecanoylphorbol-13-acetate (TPA) treatment. A23187 stimulated the phosphorylation of hsp27, while TPA alone had no effect. TPA plus A23187 resulted in phosphorylation of a 14 kDa protein, in addition to hsp27. The effect of TPA plus A23187 was identical to that of germ cells on Sertoli cell protein phosphorylation. FSH-stimulated cAMP production by Sertoli cells was reduced by prior exposure of Sertoli cells to germ cells. The results indicate that germ cells stimulate Sertoli cells by the inositol trisphosphate/diacylglycerol mediated second messenger pathway. The results also suggest that the germ cell-activated pathway interacts within Sertoli cells to modulate Sertoli cell response to FSH. © 1992 Academic Press, Inc.

For many years it has been accepted that the gonadotropin FSH stimulates Sertoli cell adenylyl cyclase to increase intracellular cAMP, which acts as a second messenger (Means et al, 1976). Investigators have speculated or provided evidence that germ cells also affect Sertoli cell functions either in vivo or in vitro (Parvinen, 1982; Gordeladze et al, 1982; Ritzen et al, 1982; Galdieri et al, 1984; Isomaa et al, 1985; Le Magueresse and Jegou, 1988a, 1988b; Wright, 1988). We have demonstrated that germ cells probably affect Sertoli cells via second messenger-mediated pathways (Ireland and Welsh, 1987), but the identities of the pathways affected in Sertoli cells by germ cells and FSH have not previously been compared nor has the possibility of interaction between the effect of germ cell on Sertoli cells and Sertoli cell response to FSH been examined. We describe here studies of intact Sertoli cells that were treated in a manner that activates specific protein kinases. Dibutyryl

Abbreviations: Dibutyryl cyclic AMP - dbcAMP; 12-O-tetradecanoylphorbol-13-acetate - TPA.

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cAMP (dbcAMP) was used to activate cAMP-dependent protein kinase, calcium ionophore A23187 to increase intracellular calcium ion concentrations and presumably activate calmodulin-dependent protein kinase, and TPA to activate protein kinase C. These agents were used either singly or in combinations in an effort to mimic the changes seen in Sertoli cell protein phosphorylation that result from FSH or germ cell treatment. We also measured FSH-stimulated cAMP production by Sertoli cells to determine if germ cells can modulate Sertoli cell response to FSH. Our results indicate that in Sertoli cells FSH acts via cAMP, that germ cells act via the phosphatidylinositol pathway, and that germ cell stimulation of Sertoli cells may modulate Sertoli cell response to FSH.

Materials and Methods

Sertoli Cell Culture Sprague-Dawley rats, bred and raised in our specific pathogen-free animal colony, of 20-22 day of age were euthanized by CO_2, testes were removed, tunicas were removed, and the tissue was minced. The tissue was digested by sequential treatment with collagenase and pancreatin (Welsh and Wiebe, 1975) to give seminiferous tubule fragments which were then placed into culture as described (Ireland et al, 1986). On day 2, germ cells were removed (Galdieri et al, 1981) and Sertoli cells were used on day 3. Leydig cell contamination was determined by 3 β-hydroxysteroid dehydrogenase (3 β-HSD) enzyme histochemistry (Welsh and Wiebe, 1975), and peritubular cell contamination was determined by immunolocalization of desmin, a peritubular cell cytoskeletal protein absent from Sertoli cells. No germ cells or cells with 3 β-HSD activity were seen, and peritubular cells comprised less than 1 in 5,000 cells. Animal care and use were reviewed and approved by the University of Michigan Committee on the Use and Care of Animals.

Cell Phosphorylation and Treatments On day 3 of culture, Sertoli cells were exposed to culture medium lacking phosphate for 30 min at 37 ^{0} C, then 400 uCi/ml ^{32}P-orthophosphate (Amersham) was added to each dish. This time was determined (Hawkins et al, 1983) to be sufficient to label the ATP pool to equilibrium. At the end of the labeling period, treatments were added and phosphorylation continued for 30 min. Treatments included FSH (1 ug/ml NIH-oFSH-S12, except where otherwise indicted), dbcAMP (100 uM), calcium ionophore A23187 (1 uM), TPA (50 nM), and germ cell-conditioned medium prepared as previously described (Ireland and Welsh, 1987). A23187, dbcAMP, and TPA were obtained from Sigma Chemical Co.(St. Louis, MO).

Processing of Sertoli Cell Proteins for 2-D Gel Autoradiography and Densitometry Cell cultures were rinsed with cold PBS, scraped into 9M urea, 4% CHAPS, 4% 2-mercaptoethanol (Sigma Chemical Co.), and Ampholines (pH 3.5-10.0, Pharmacia LKB, Piscataway, NJ), and sonicated twice for 5 sec while on ice. Equal amounts of radioactive counts were subjected to 2-dimensional polyacrylamide gel electrophoresis and the gels were then processed to produce autoradiograms (Ireland et al, 1986). ^{32}P incorporation into protein spots in gels was quantified as previously described (Ireland and Welsh, 1987).

Assay for Effect of Germ Cells on FSH-Induced Cyclic AMP Accumulation by Sertoli Cells On day 3 of culture, Sertoli cells were rinsed with culture medium and 1 ml aliquots of germ cells, isolated from 40 day old rats (Ireland and Welsh, 1987), were added to some of the cultures while other, control cultures were given 1 ml aliquots of culture medium alone. Sertoli cell were then incubated for 30 min at 37 ^{0}C, and cultures were rinsed with 3 changes of culture medium to remove germ cells. Culture medium (1 ml) containing FSH (from 0.01 to 100 ug/ml, or no hormone) and .25 mM isobutylmethylxanthine was then added to each culture and cells were incubated for 30 min. Cultures were extracted (Zimmerman et al, 1976) and cAMP was then assayed (Gilman, 1970). Treatments and controls were performed in triplicate. Results are expressed as the mean $\pm$ standard deviation of the triplicate measurements.

Results

Cyclic AMP-Mediated Phosporylation of Sertoli Cell Proteins We show an area of interest of the autoradiograms of 2-D gels between molecular weights of 31 kDa down to 13 kDa, and in the P_i range of about 4.8 to 7.0. A typical autoradiogram of protein phosphorylation in untreated cultured Sertoli cells is shown in Figure 1, panel a. We assumed that treating Sertoli cells with dbcAMP would specifically activate cAMP-dependent protein kinase. Panel b of Fig. 1 shows the pattern of protein phosphorylation in Sertoli cells treated with dbcAMP. Four proteins that were not detectable as phosphoproteins in control cells became apparent as phosphoproteins in dbcAMP-treated cells (The proteins are labeled 22a, 22b, 25, and 29 in panel b). The pattern of protein phosphorylation in Sertoli cells treated with FSH, as previously described (Ireland et al, 1986), is shown for comparison in panel c. The same four proteins that were phosphorylated in response to dbcAMP treatment also became evident as phosphoproteins after FSH treatment.

The same proteins showed increased ^{32}P labeling in response to either dbcAMP or FSH, however, relative phosphorylation levels were different for the treatments. Protein 22a showed substantially less ^{32}P labeling after dbcAMP treatment than after FSH treatment and protein 22b showed slightly less labeling with dbcAMP treatment compared to FSH. Protein 29 showed similar labeling with either dbcAMP or FSH, while protein 25 showed more labeling after dbcAMP compared to FSH treatment (compare 1b with 1c).

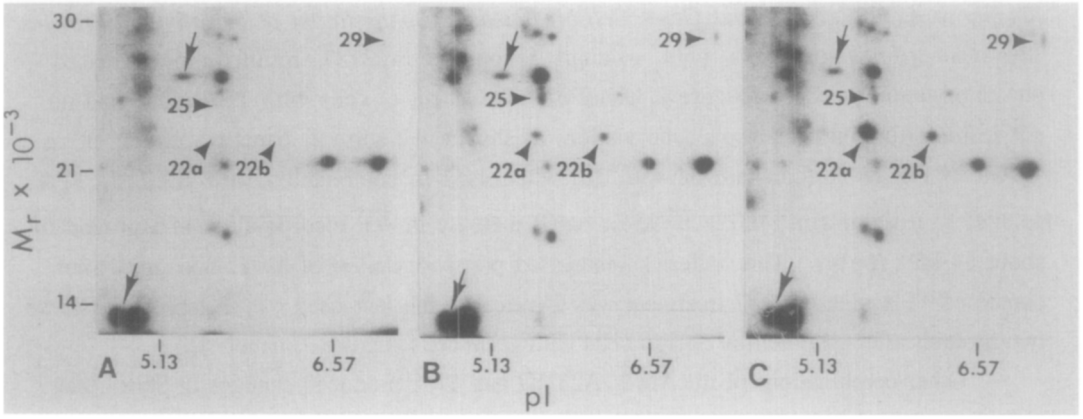

Figure 1. Sertoli cell phosphoprotein patterns in autoradiograms of 2-D polyacrylamide gels: effects of 30 min treatment with dbcAMP or FSH. Panel A: Phosphoproteins in untreated Sertoli cells. Panel B: Phosphoproteins from Sertoli cells treated with 100 μM dbcAMP. Panel C: Phosphoproteins from Sertoli cells treated with 1 μg/ml FSH. Proteins indicated by arrowheads and numbered 22a, 22b, 25 and 29 do not appear as phosphoproteins in control cells, but do appear as phosphoproteins in cells treated with dbcAMP or FSH. Although both dbcAMP and FSH stimulate the phosphorylation of the same proteins, the relative increases in phosphorylation of each protein differ for the two treatments. For purposes of reference, proteins indicated by arrows at MW of 14 and 27 kDa are pp14 and hsp27, respectively, and are addressed in Figures 2 and 3.

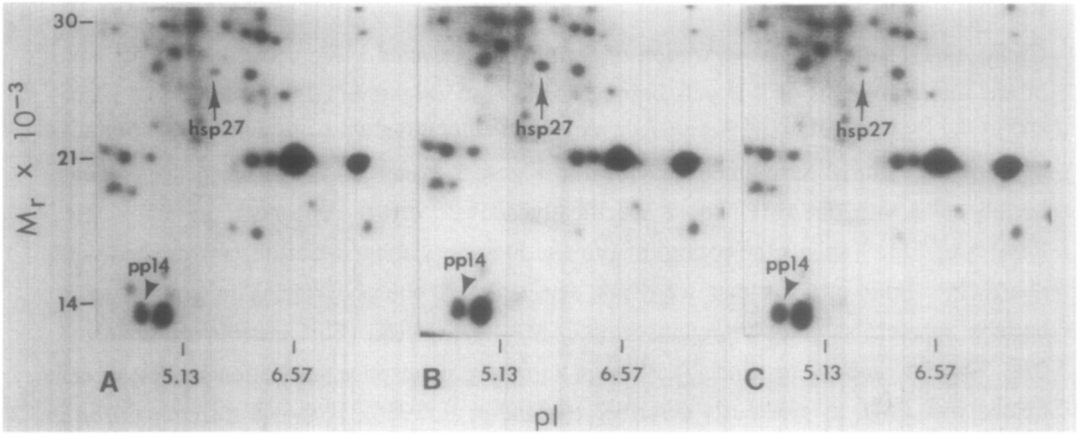

Figure 2. Sertoli cell phosphoprotein patterns in autoradiograms of 2-D polyacrylamide gels: effects of 30 min treatment with calcium ionophore A23187 or TPA. Panel A: Phosphoproteins from untreated Sertoli cells. Panel B: Phosphoproteins from Sertoli cells treated with 1 uM A23187. Panel C: Phosphoproteins from Sertoli cells treated with 50 nM TPA. Treatment with A23187 resulted in a significant increase in phosphorylation of hsp27, but TPA alone resulted in no apparent changes in protein phosphorylation.

Phosphorylation of hsp27 and a 14 kDa protein (pp14), to be further discussed below, was not affected by dbcAMP treatment (after dbcAMP treatment phosphorylation of hsp27 = 117%$\pm$12% (n=3) and pp14 = 104% $\pm$46% (n=3) of controls).

Ionophore A23187, TPA, and Germ Cell-Mediated Phosphorylation of Sertoli Cell Proteins

Treatment of Sertoli cells with calcium ionophore A23187 resulted in increased phosphorylation of hsp27 (Figure 2, panel b). Treatment of cells with TPA alone had no noticeable effect on protein phosphorylation, as shown in Figure 2, panel c. However, as shown in Figure 3, (compare 3a with 3b) when used in combination with A23187, TPA resulted in a significant (460% $\pm$ 15%, n=3) increase in ^{32}P incorporation in a protein of about 14 kDa (pp14). This pattern of increased phosphorylation of hsp27 and pp14 after combined TPA plus A23187 treatment was identical to the increased ^{32}P-labeling of these two proteins after treatment of Sertoli cells with germ cells (Figure 3, panel c).

Other combinations of dbcAMP, A23187 and TPA produced changes in Sertoli cell phosphoprotein patterns that appeared to represent the additive effects of each individual treatment (with the exception of the previously described effect of A23187 plus TPA on pp14 phosphorylation). For example, TPA plus dbcAMP produced changes which were observed for dbcAMP alone (not shown), and A23187 plus dbcAMP resulted in alterations in phosphoprotein patterns that were equivalent to the sum of effects observed with each individual treatment (not shown). Also, although results are presented for a 30 min incubation time, same qualitative changes in phosphorylation could be detected at incubation times of 1 to 5 min.

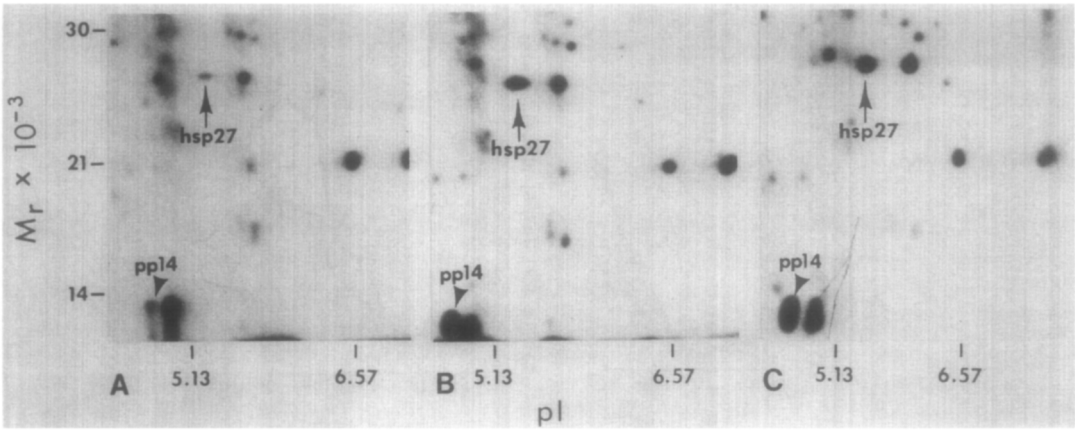

Figure 3. Sertoli cell phosphoprotein patterns in autoradiograms of 2-D polyacrylamide gels: comparison of phosphoproteins from Sertoli cells treated with A23187 plus TPA and cells treated with germ cells. Panel A: Phosphoproteins from control, untreated Sertoli cells. Panel B: Phosphoproteins from Sertoli cells treated with 1 μM A23187 plus 50 nM TPA. Panel C: Phosphoproteins from Sertoli cells treated with germ cells. Treatment of Sertoli cells with A23187 plus TPA resulted in significantly increased phosphorylation of pp14 and hsp27. Similar increases in phosphorylation of hsp27 and pp14 were observed when Sertoli cells were treated with germ cells.

Germ Cell Effects on FSH-Stimulated cAMP Production by Sertoli Cells Because it had previously been reported that treatment of Sertoli cells with TPA would result in a reduced response to subsequent FSH treatment (Monaco and Conti, 1987), we examined whether exposure of Sertoli cells to germ cells would affect Sertoli cell response to subsequent treatment with FSH. Sertoli cells were isolated, cultured and preincubated with germ cells for 30 min, and then germ cells were washed from the Sertoli cells. The amount of cAMP produced by the intact Sertoli cells during a subsequent 30 min incubation with FSH was determined. Pretreatment of intact Sertoli cells with germ cells reduced by 50% the subsequent FSH-stimulated production of cAMP by a Sertoli cells (Figure 4).

Discussion

We present an area on the autoradiograms from MW 13-31 kDa and isoelectric points of from pH 4.8-7.0. We have noted FSH-dependent changes in phosphorylation of proteins in other areas of our gels (Ireland et al, 1986), however, we have chosen to concentrate our research efforts to the area shown. It should be recognized also that results shown in the autoradiograms reveal only net changes in protein phosphorylation; changes in phosphorylation of any protein may reflect both addition of phosphate by protein kinases as well as removal of phosphate by protein phosphatases. Although we present results for proteins labeled for 30 min, similar patterns of protein phosphorylation were seen in cells incubated for 5 min or less. Thus, the events we describe are rapid.

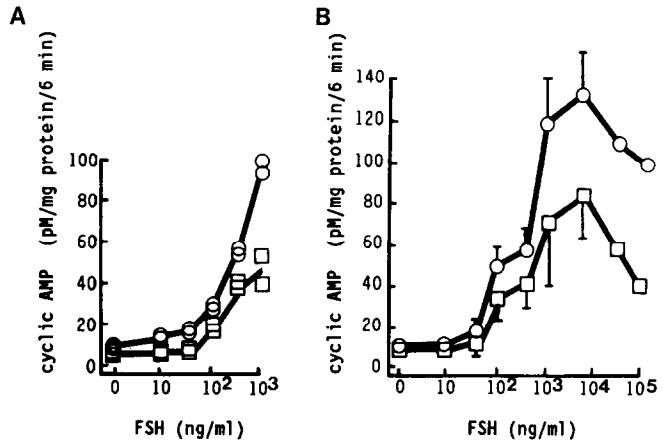

Figure 4. Effect of germ cell treatment on subsequent FSH-stimulated accumulation of cAMP in Sertoli cells. Freshly isolated germ cells were applied to Sertoli cultures for 30 min, after which time germ cells were removed. Sertoli cells were then treated for 30 min with FSH at concentrations indicated. CyclicAMP in Sertoli cells was then measured. Panels A and B show the results of two separate experiments measuring cAMP accumulation as a function of FSH dose. In panel A, duplicate cultures were treated with FSH and assayed for cAMP production. In panel B, triplicate cultures were treated with each FSH concentration except for the two highest concentrations of FSH for which single cultures were assayed. For FSH treatments assayed in triplicate, results are expressed as the mean with the standard deviation being indicated by the vertical bar. Pretreatment of intact Sertoli cells with germ cells resulted in an approximately 50% reduction in cAMP synthesized in response to FSH.

Most of the phosphoproteins detected on the autoradiograms showed no apparent change in intensity of ^{32}P labeling for any treatment. Thus, most phosphoproteins in Sertoli cells appear to be constitutively phosphorylated, although it is likely that the phosphorylation of some other proteins is effected by other regulated protein kinases.

When cells were treated with dbcAMP, four proteins not detectable in autoradiograms of control cells became apparent as phosphoproteins. The same proteins were phosphorylated in response to FSH, although to different degrees compared to dbcAMP treatment. On the basis of these results we conclude, as have others, that FSH acts via the adenylate cyclase/cAMP signal transduction pathway. The differences in protein phosphorylation seen after dbcAMP or FSH treatment may result from differences in access rates to subcellular compartments for exogenous dbcAMP vs. endogenous cAMP produced in response to FSH.

In contrast to the effect of FSH, A23187 plus TPA treatment increased phosphate content of two proteins which were not affected by FSH. We reported previously that treatment of Sertoli cells with A23187 alone resulted in increased phosphorylation of the 27 kDa protein suggesting that it is a substrate for calmodulin-dependent protein kinase (Ireland and Welsh, 1987). We have recently identified this protein as hsp27 (Pittenger et al, 1992). Here we show that pp14 was unaffected by either A23187 or TPA alone, but that

both compounds together resulted in increased phosphorylation of pp14. On the basis of these results, we conclude that pp14 is a substrate for protein kinase C. That A23187 treatment is required to elicit an effect of TPA on pp14 phosphorylation suggests that protein kinase C in Sertoli cells may require higher intracellular calcium ion levels than normally occurs in unstimulated Sertoli cells in culture.

We have previously shown hsp27 and pp14 to be rapidly phosphorylated in intact Sertoli cells in response to germ cells (Ireland and Welsh, 1987). Here we show that hsp27 and pp14 phosphorylation occurs in response to A23187 plus TPA treatment. A23187 would act to increase intracellular calcium ion concentration and activation of calmodulin-dependent protein kinase while TPA would activate protein kinase C. The activation of these protein kinases also occurs in activation of the phosphatidylinositol pathway (Berridge, 1984; Nishizuka, 1984; Majerus et al, 1984; Rana and Hokin, 1990). Thus, the results suggest that germ cells activate the Sertoli cell phosphatidylinositol pathway.

Phosphoprotein patterns shown here suggest that FSH does not acutely affect in Sertoli cells the phosphatidylinositol response pathway, in agreement with studies in which direct measurements of phosphoinositides demonstrated no effect of FSH on Sertoli cell phosphatidylinositol metabolism (Quirk and Reichert, 1988; Monaco et al, 1988).

Other results of these studies support indirectly our conclusion that germ cells affect the phosphatidylinositol pathway in Sertoli cells and, moreover, suggest one function for germ cell interaction with Sertoli cells. Pretreatment of Sertoli cells with TPA has been reported to reduce by 50% Sertoli cell response to FSH (Monaco and Conti, 1987). The decrease in Sertoli cell response to FSH after germ cell treatment *in vitro* measured in our experiments was also about 50%. This is circumstantial evidence for the conclusion that germ cells do stimulate the generation of diacylglycerol (and IP_3) in Sertoli cells. The results also indicate that germ cell-stimulated events in Sertoli cells may modulate Sertoli cell response to FSH. Others have also observed effects of germ cells on Sertoli cells that were interpreted as demonstrating germ cell modulation of Sertoli cell response to FSH (Gordeladze et al, 1982; Galdieri et al, 1984; Le Magueresse and Jegou, 1988a, 1988b).

In summary, our results suggest that germ cells activate the phosphatidylinositol second messenger pathway in Sertoli cells. Pretreatment of Sertoli cells with germ cells was observed to inhibit the subsequent *in vitro* response of Sertoli cells to FSH. Thus, germ cells may be responsible for modulating Sertoli cell response to FSH *in vivo*.

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