Regulation of rat Sertoli cell function by FSH: possible role of phosphatidylinositol 3-kinase/protein kinase B pathway

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Abstract

The FSH molecular mechanism of action is best recognized for its stimulation of the adenylyl cyclase/cAMP pathway via activation of a G protein. Recently, links between cAMP, phosphatidylinositol 3-kinase (PI3K) and protein kinase B (PKB) signaling pathways in thyroid and granulosa cells have been observed. The aim of this study was to investigate the possible role of the PI3K/PKB pathway in FSH regulation of Sertoli cell function. Twenty-day-old rat Sertoli cell cultures were used. An increase in phosphorylated PKB (P-PKB) levels in response to FSH and dibutyryl-cAMP was observed. These increments in P-PKB levels were not observed in the presence of two PI3K inhibitors, wortmannin and Ly 294002. Inhibition of protein kinase A (PKA) by H89 did not decrease FSH stimulation of P-PKB levels. Taken

together, these results indicate that FSH increases P-PKB levels in a PI3K-dependent and PKA-independent manner in rat Sertoli cells. In addition, wortmannin partially inhibited the ability of FSH to stimulate two well-known parameters of Sertoli cell function – transferin secretion and lactate production – at doses equal to or lower than 0.1 µM. Related to lactate production, a decrease in FSH stimulation of lactate dehydrogenase activity and of basal and FSH-stimulated glucose uptake was observed in the presence of wortmannin. These metabolic changes were in most cases accompanied by changes in the levels of P-PKB. Altogether, these results suggest a meaningful role of the PI3K/PKB pathway in the mechanism of action of FSH in rat Sertoli cells.

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Introduction

Androgens and the pituitary gonadotropin follicle-stimulating hormone (FSH) control Sertoli cell function. In addition, their effects are modulated by the action of a large set of locally produced factors that include growth factors and cytokines (Gnessi *et al.* 1997). The complex mechanisms by which cells respond to hormonal stimuli are often dependent on several signal transduction pathways that lead to the activation of specific biological responses. The FSH molecular mechanism of action is best recognized for its stimulation of the adenylyl cyclase/cAMP pathway via activation of a G protein. Elevated cAMP levels lead to the activation of protein kinase A (PKA) that phosphorylates several protein substrates. This cascade has been extensively analyzed for the mechanism of action of FSH and of other pituitary hormones such as luteinizing hormone, thyrotropin (TSH) and adrenocorticotropin. As for the mechanism of action of FSH in Sertoli cells, in addition to the cAMP/PKA pathway, other signaling events that include increased intracellular Ca²⁺ levels (Grasso & Reichert 1989, Gorczyńska & Handelsman 1991), activation of phospholipase A₂ (Jannini *et al.* 1994) and nuclear factor kappa B (NFκB) translocation (Delfino & Walker

1998) have been demonstrated. Even though it has been demonstrated that there is no phosphoinositide turnover in response to FSH stimulation in Sertoli cells (Monaco *et al.* 1988), it has been shown that a protein kinase C (PKC)-dependent pathway can modulate Sertoli cell response to FSH (Lambert *et al.* 1991, Meroni *et al.* 1997).

Protein kinase B (PKB), also referred to as Akt or RAC kinase, is a serine/threonine protein kinase which was cloned by virtue of its homology to the A and C protein kinases, and is the cellular homolog of the product of the *v-akt* oncogene (Bellacosa *et al.* 1991, Coffey & Woodgett 1991, Jones *et al.* 1991). This kinase is activated in response to polypeptides acting through tyrosine kinase receptors such as platelet-derived growth factor, insulin, basic fibroblast growth factor and epidermal growth factor in a pathway involving phosphatidylinositol 3-kinase (PI3K) (Coffey *et al.* 1998). Protein kinase B contains an amino terminal pleckstrin homology domain that binds phosphorylated lipids at the membrane in response to activation of PI3K. In this way, PKB is activated by phospholipid binding and phosphorylation at Thr 308 and Ser 473 by specific kinases (Alessi *et al.* 1996). Activation of PKB by PI3K-independent mechanisms has also been described (Filippa *et al.* 1999).

Recently, links between G-protein-coupled receptors, cAMP, PI3K and PKB signaling pathways have been observed. Cass *et al.* (1999) have shown that TSH upon binding to its respective G-protein-coupled receptor and via cAMP (but not PKA) stimulates the phosphorylation of PKB in a PI3K-dependent pathway in a thyroid cell line. In addition, Gonzalez-Robayna *et al.* (2000) have presented evidence that in granulosa cells FSH increases PKB phosphorylation and activation in a way that is cAMP-dependent, PKA-independent but PI3K-dependent.

Signal transduction pathways for an individual hormone vary from cell to cell. In addition, signal transduction pathways for an individual hormone vary within the same cell type with the maturation state of the cell. It is worth mentioning that MAP kinase (MAPK) activation in response to FSH has been observed in granulosa cells (Cameron *et al.* 1996, Das *et al.* 1996, Moore *et al.* 2001), while in 20-day-old Sertoli cells FSH decreases the levels of activated MAPK (Crepieux *et al.* 2001). These experiments clearly show that even when Sertoli cells are considered the male counterpart of granulosa cells they do not have the same intracellular machinery. A major issue in understanding FSH physiological function in males is to define signaling pathways that allow the hormone to guide its target cells towards differentiation. As mentioned before, it has recently been demonstrated that PI3K/PKB activation is involved in the regulation of granulosa cells by FSH. However, the possibility that a PI3K/PKB pathway may be involved in the mechanism(s) that FSH utilizes to regulate 20-day-old Sertoli cell function has not yet been explored. The aim of the present study was to investigate whether FSH regulation of Sertoli cell function may involve a PI3K/PKB-dependent pathway.

Materials and Methods

Materials

Ovine FSH (NIH-oFSH-S-16) was obtained from the National Hormone and Pituitary Program, National Institute of Diabetes, Digestive and Kidney Diseases (NIDDK), Bethesda, MD, USA. Wortmannin, Ly 294002 and H89 were purchased from Biomol (Plymouth Meeting, PA, USA). [2,6-³H]-2-Deoxy-D-glucose ([2,6-³H]-2-DOG) was obtained from NEN (Boston, MA, USA). Kodak X-Omat S films were purchased from Eastman Kodak (Rochester, NY, USA). All other drugs and reagents were obtained from Sigma-Aldrich (St Louis, MO, USA).

Sertoli cell isolation and culture

Sertoli cells from 20-day-old Sprague-Dawley rats were isolated as previously described (Meroni *et al.* 1999). Briefly, decapsulated testes were digested with 0.1%

collagenase and 0.006% soybean trypsin inhibitor in Hanks balanced salt solution for 5 min at room temperature. Seminiferous tubules were saved, cut and submitted to 1 M glycine–2 mM EDTA (pH 7.4) treatment to remove peritubular cells. The washed tubular pellet was then digested again with collagenase for 10 min at room temperature to remove germinal cells. The Sertoli cell suspension, collected by sedimentation, was resuspended in culture medium which consists of a 1:1 mixture of Ham F12 and Dulbecco's modified Eagle's medium, supplemented with 20 mM HEPES, 100 IU/ml penicillin, 2.5 µg/ml amphotericin B, 1.2 mg/ml sodium bicarbonate, 10 µg/ml transferrin, 5 µg/ml insulin, 5 µg/ml vitamin E and 4 ng/ml hydrocortisone. Sertoli cells were cultured in 6-, 24- or 96-multiwell plates (5 µg DNA/cm²) at 34 °C in a mixture of 5% CO₂: 95% air.

No myoid cell contamination was revealed in the cultures when an immunoperoxidase technique was applied to Sertoli cell cultures using a specific antiserum to alpha smooth muscle actin. Remaining cell contaminants were of germ cell origin and this contamination was below 5% after 48 h in culture as examined by phase contrast microscopy.

Culture conditions

Sertoli cells were allowed to attach for 48 h in the presence of insulin, and medium was replaced at this time with fresh medium without insulin; variable doses of FSH were added on day 3 as indicated in the Figure legends. The 72-h conditioned media obtained on day 6 were used to evaluate transferrin and lactate levels. Cells harvested on day 6 were used to determine gamma glutamyl transpeptidase (γ-GTP) and lactate dehydrogenase (LDH) activities and to evaluate cell viability.

Cells harvested on day 6, pretreated as indicated in the Figure legends, were also used for Western blot analysis of phosphorylated PKB (P-PKB) levels. Cultures obtained under identical conditions were used to analyze intracellular cAMP levels stimulated by FSH. For glucose uptake studies, cells harvested on day 5, pretreated as described in the Figure legends, were used.

Transferrin determination

Rat transferrin (rTF) was measured by RIA as described by Handelsman *et al.* (1989). A polyclonal antibody raised against rTF in rabbits was used (Cappel Laboratories, Cochranville, PA, USA). The cross-reactivity of human transferrin (TF) in this assay is less than 0.003%. Unconditioned medium containing 10 µg/ml human TF did not show any cross-reactivity in this assay. This RIA has a sensitivity of 3 ng/tube and intra- and interassay coefficients of variation were 7% and 16% respectively.

γ -Glutamyl transpeptidase assay

γ -GTP activity was assayed by the method of Orłowsky & Meister (1963), using L- γ -glutamyl *p*-nitroanilide as substrate and glycylglycine as the acceptor molecule. Sertoli cell monolayers were disrupted by ultrasonic irradiation in 0.5 ml reaction buffer (0.1 M Tris buffer, 0.01 M MgCl₂, 0.02 M glycylglycine, pH 9). Adequate aliquots for DNA determinations were saved and 5 mM substrate (L- γ -glutamyl *p*-nitroanilide) were added to the remaining material. The reaction was allowed to proceed for 120 min at 34 °C, and the enzymatic reaction was stopped by addition of acetic acid up to a 1 M concentration. Samples were then centrifuged and absorbances determined in a spectrophotometer at 410 nm. Values were compared against a standard curve with increasing concentrations of *p*-nitroaniline.

Lactate determination

Lactate was measured by a standard method involving conversion of NAD⁺ to NADH determined as the rate of increase of absorbance at 340 nm. A commercial kit from Sigma-Aldrich was used.

Measurement of 2-deoxy-D-glucose uptake

Glucose transport was studied using the uptake of the labeled nonmetabolizable glucose analog 2-deoxy-D-glucose (2-DOG). Cells that had received different treatments for variable periods of time (0.5–48 h), as indicated in the Figure legends, were used. After treatment, culture media were discarded and cells were washed three times with glucose-free phosphate-buffered saline (PBS) at room temperature. Then, Sertoli cells were incubated at 34 °C in 0.5 ml glucose-free PBS containing [2,6-³H]-2-DOG (0.5 μ Ci/ml) for 30 min. Unspecific uptake was determined in incubations performed in the presence of a 10 000-fold higher concentration of unlabeled 2-DOG. At the end of the incubation period, dishes were placed on ice and extensively washed with ice-cold PBS until no radioactivity was present in the washings. Cells were then dissolved with 0.5 M sodium hydroxide, 0.4% w/v sodium deoxycholate and counted in a liquid scintillation spectrophotometer. Parallel cultures receiving identical treatments to those performed before the glucose uptake assay were used for DNA determinations. Results were expressed on a per μ g DNA basis.

LDH activity measurement

After incubation of Sertoli cells in the absence or presence of the different stimuli, culture media were saved and cells were disrupted by ultrasonic irradiation in NaCl 0.9% w/v and centrifuged (15 800 *g*, 10 min). The supernatant was

used to measure total LDH activity. Total LDH activity was determined by a routinely used spectrophotometric method (Randox Laboratories, Crumlin, Co. Antrim, UK).

Cell extracts and Western blot analysis

On day 6, cells cultured on 6-well plates and pretreated for variable periods of time as indicated in the Figure legends were washed once with PBS at room temperature. Then, 200 μ l PBS containing 20 μ l of a protease inhibitor cocktail from Sigma (P-8340) and 2 mM phenylmethylsulfonylfluoride were added to the cells. Cells were then placed on ice and disrupted by ultrasonic irradiation. A 200 μ l volume of 2 $\times$ Laemmli buffer (4% w/v SDS, 20% v/v glycerol, 10% v/v 2-mercaptoethanol, 0.004% w/v bromophenol blue and 0.125 M Tris-HCl, pH 6.8) was added and thoroughly mixed (Laemmli 1970). Samples were immersed in a boiling water bath for 5 min and then immediately settled on ice. Proteins were resolved in 10% SDS-PAGE (10% acrylamide/bisacrylamide for the resolving gel and 4.3% acrylamide/bisacrylamide for the stacking gel) in a Mini Protein II Cell (Bio-Rad, Hercules, CA, USA). After SDS-PAGE, gels were equilibrated in transfer buffer for 10 min and electrotransferred at 100 V for 60 min onto PVDF membranes (Hybond-P, Amersham Pharmacia Biotech, UK) using a Mini Trans-Blot Cell (Bio-Rad). Membranes were probed with a commercial kit (PhosphoPlus Akt Ser 473 antibody kit, New England Biolabs Inc., Beverly, MA, USA) that allows specific recognition of both non-phosphorylated and serine-phosphorylated PKB. The intensities of the autoradiographic bands were estimated by densitometric scanning using NIH Image (Scion Corporation Frederick, MD, USA) software.

Cell viability test

A cell viability test was performed in cells cultured on 96-well plates and treated for 90 min or 72 h with H89, LY 294002 or wortmannin. A commercial kit (CellTiter 96 Aqueous Non-Radioactive Cell Proliferation Assay, Promega Corporation) was used.

Other assays

cAMP was determined by RIA using a specific antibody. The RIA has a sensitivity of 25 fmol/tube and intra- and interassay coefficients of variation of 9 and 17% respectively. DNA was determined by the method of Labarca & Paigen (1980).

Statistical analysis

To analyze data from glucose uptake studies, cell viability, lactate, transferrin and cAMP production, and γ -GTP and

Figure 1 FSH increases the levels of phosphorylated protein kinase B (P-PKB) in rat Sertoli cells. (A) Sertoli cells were stimulated for variable periods of time (30, 60 or 90 min) with 100 ng/ml FSH. (B) Sertoli cells were incubated for 60 min in the absence or presence of variable doses of FSH (1, 10 and 100 ng/ml FSH). (C) Sertoli cells were stimulated for 60 min with 100 ng/ml FSH in the absence or presence of two different doses of wortmannin (W; 0.01 and 0.1 μ M). (D) Sertoli cells were stimulated for 60 min with 100 ng/ml FSH in the absence or presence of two different doses of Ly 294002 (Ly; 2.5 and 25 μ M). Cell extracts were prepared at the designated intervals and utilized for Western blot analysis using antibodies specific for PKB (T-PKB) or P-PKB. Numbers at the bottom of each lane in this and subsequent Figures for PKB indicate the fold increase in phosphorylation (ratio of P-PKB to T-PKB in each sample) relative to basal. Results are representative of two reproducible experiments per treatment group.

LDH activity, one-way analysis of variance (ANOVA) followed by the Tukey–Kramer test for multiple comparisons using the GB-STAT version 4.0 statistical program (Dynamic Microsystems Inc., Silver Spring, MD, USA) was performed. Probabilities <0.01 were considered statistically significant.

Results

PKB is phosphorylated in rat Sertoli cells stimulated with FSH and dibutyryl-cAMP (dbcAMP) in a PI3K-dependent manner

Sertoli cell cultures were stimulated for variable periods of time (30, 60 and 90 min) with 100 ng/ml FSH and for 60 min with variable doses of FSH (1, 10 and 100 ng/ml). A low level of P-PKB in basal cultures was observed. Stimulation of the cultures with 100 ng/ml FSH showed

time-dependent increments in P-PKB levels reaching maximal stimulus in 60-min incubations (Fig. 1A). Dose–response studies in 60-min incubations showed a maximal response with 100 ng/ml FSH (Fig. 1B). Pooled data obtained in eight individual experiments performed in 60-min incubations with 100 ng/ml FSH revealed 4 ± 0.8 -fold stimulation (mean $\pm$ S.E.) in P-PKB levels.

In order to evaluate the participation of PI3K in the observed stimulatory effects of FSH on P-PKB levels, cells pre-incubated for 15 min with two well-known PI3K inhibitors, wortmannin and Ly 294002, were stimulated with FSH for 60 min. Figure 1C shows that wortmannin dose-dependently decreased the ability of FSH to increase the levels of P-PKB. Figure 1D shows that Ly 294002 produced similar effects. These inhibitors did not modify the low levels of P-PKB present under basal conditions (data not shown).

Table 1 Effect of different doses of FSH on Sertoli cell intracellular cAMP levels. On day 6, Sertoli cells were stimulated for 60 min with 1, 10 or 100 ng/ml FSH. Intracellular cAMP levels were determined after this incubation period. Results represent means $\pm$ S.D. of triplicate incubations in one representative experiment out of two

Dose of FSH (ng/ml)	Intracellular cAMP (fmol/ μ g DNA)
Basal	142.4 $\pm$ 9.6 ^a
1	254.4 $\pm$ 56.9 ^b
10	442.0 $\pm$ 99.7 ^c
100	2439.5 $\pm$ 566.6 ^d

Different letters indicate statistically significant differences ($P < 0.01$).

We next examined whether a cAMP/PKA-dependent pathway mediated the increment in P-PKB levels. Table 1 shows that FSH stimulated intracellular cAMP levels in a dose-dependent manner at doses that also stimulated P-PKB levels. Therefore, we next examined whether a cAMP analog mimicked the effect of FSH. Figure 2A shows that the cAMP analog dbcAMP stimulated the levels of P-PKB. The latter Figure also shows that the effect on the levels of P-PKB produced by the cyclic nucleotide was not present in cells pre-incubated with the two inhibitors of PI3K. In addition, Fig. 2B shows that the specific PKA inhibitor, H89, increased basal levels of P-PKB and it also increased P-PKB levels stimulated by FSH.

Cells incubated for 15 min with H89, wortmannin and Ly 294002 and then stimulated with FSH or dbcAMP for 60 min did not show modifications in cell viability (data not shown).

PI3K activity participates in FSH regulation of Sertoli cell γ -GTP activity and transferrin secretion

The next set of experiments were intended to analyze the relevance of PI3K activity on two Sertoli cell biological responses to FSH largely considered to occur via a cAMP/PKA pathway. The PKA inhibitor, H89, and the PI3K inhibitors, wortmannin and Ly 294002, were added to the cultures 15 min prior to the initiation of a 72-h incubation period with FSH. A cell viability test performed at the end of this 72-h period showed that H89 and Ly 294002 decreased cell viability in long-term incubations (Table 2). As a consequence, for those biological responses obtained in long-term incubations only the effect of wortmannin was analyzed.

Figure 3 shows that while a 1 μ M dose of wortmannin was necessary to partially inhibit the ability of FSH to stimulate γ -GTP activity (A), a dose-dependent decrease in transferrin secretion starting at 0.01 μ M wortmannin was observed (B). Wortmannin did not modify γ -GTP

Figure 2 FSH/cAMP promotes phosphorylation of PKB in a phosphatidylinositol 3-kinase (PI3K)-dependent and protein kinase A (PKA)-independent manner. (A) Sertoli cells were maintained under basal conditions or stimulated for 30, 60 or 90 min with 1 mM dibutyl cAMP (dbcAMP). In addition, cells were stimulated for 60 min with 1 mM dbcAMP in the presence of 1 μ M wortmannin (W) or 25 μ M Ly 294002 (Ly). (B) Sertoli cells were maintained under basal or FSH-stimulated (100 ng/ml) conditions in the absence or presence of 10 μ M H89 for 60 min. Cell extracts were prepared at the designated intervals and utilized for Western blot analysis using antibodies specific for PKB (T-PKB) or phosphorylated PKB (P-PKB). Results are representative of two experiments giving identical results.

Table 2 Effect of wortmannin Ly, 294002 and H89 on Sertoli cell viability. Sertoli cells were incubated for 72 hours (day 3 to day 6) without (Control) or with 1 μ M wortmannin (W), 25 μ M Ly 294002 (Ly) or 10 μ M H89 in the absence (Basal) or presence of 100 ng/ml FSH. The cell viability assay was performed on day 6. Data are expressed as percentage of the control under basal conditions and are presented as means $\pm$ S.D. of triplicate incubations in one representative experiment out of three

	Cell viability (% of control)	
	Basal	FSH
Control	100	97 $\pm$ 7
W (1 μ M)	96 $\pm$ 6	97 $\pm$ 7
Ly (25 μ M)	52 $\pm$ 4*	63 $\pm$ 4*
H89 (10 μ M)	85 $\pm$ 6	72 $\pm$ 5*

* $P < 0.01$ vs Control.

Figure 3 Wortmannin inhibits FSH stimulation of Sertoli cell γ -glutamyl transpeptidase (γ -GTP) activity and transferrin secretion. Sertoli cells were stimulated for 72 h (day 3 to day 6) with 100 ng/ml FSH in the absence or presence of different doses of wortmannin (W). (A) γ -GTP activity was determined in cells harvested on day 6. (B) Transferrin was determined in the 72-h conditioned media recovered on day 6. Results represent means $\pm$ S.D. of triplicate incubations in one representative experiment out of three. Different letters indicate statistically significant differences ($P < 0.01$).

activity and transferrin secretion under basal experimental conditions at any dose tested.

PI3K activity participates in FSH regulation of Sertoli cell lactate production

In addition to the effects of wortmannin on FSH-stimulated γ -GTP activity and transferrin secretion, Fig. 4A shows that this PI3K inhibitor decreased FSH-stimulated lactate production at a 0.1 μ M concentration. Wortmannin did not modify basal lactate secretion at any dose tested.

We have previously shown (Riera *et al.* 2001) that increments in LDH activity and in glucose uptake are part of the mechanisms utilized by FSH to increase lactate production. Therefore, we next examined the possibility that PI3K activity was involved in the regulation of these biochemical steps that are targets for FSH action in Sertoli cells. Figure 4B shows that wortmannin (0.1 μ M) decreased the ability of FSH to stimulate LDH activity. Wortmannin did not modify basal LDH activity.

As for glucose uptake, Table 3 shows that the PKA inhibitor, H89, did not modify glucose uptake under basal or FSH-stimulated conditions. In addition, this Table shows that cycloheximide, a protein synthesis inhibitor, did not modify glucose uptake stimulated by FSH in a 60-min incubation period.

Finally, Fig. 4C shows that wortmannin markedly decreased basal and FSH-stimulated glucose uptake.

Discussion

The gonadotropin FSH plays a key role in the control of Sertoli cell function. FSH determines the number of Sertoli cells that will be present in adult males by its ability to control proliferation at the neonatal stage (Orth 1982). In the rat, Sertoli cell mitosis ceases by day 15 and FSH then takes control of differentiation of these cells that are essential to sustain a qualitatively and quantitatively normal spermatogenesis (Griswold 1993). FSH initiates its action through binding to specific G-coupled receptors that activate adenylyl cyclase resulting in the elevation of intracellular cAMP levels and PKA activation. Other signaling pathways that have been shown to participate in the mechanism of action of FSH have been reviewed in the Introduction. The study of possible signal transduction pathways utilized by FSH different from the classic cAMP/PKA pathway, and the analysis of a possible correlation between their activation and the biological responses elicited are under investigation.

PI3K is a key enzyme implicated in the regulation of a broad array of biological responses including receptor-stimulated mitogenesis, oxidative burst, membrane ruffling and glucose uptake (Rameh & Cantley 1999). At least two types of PI3K, in terms of mode of activation, have been described in mammalian cells. One is stimulated by membrane-bound receptors activating tyrosine kinase (Porter & Vaillancourt 1998), whereas the other is under the direct control of the heterotrimeric GTP-binding proteins (Tang & Downes 1997). Agonist-stimulated PI3K generates specific inositol phospholipids that are recognized by a subset of proteins that contain pleckstrin homology (PH) domains, a specialized lipid-binding module. Protein kinase B was among the first proteins known to contain a PH domain. This PH domain of PKB specifically binds PI3K lipid products, and a firm link between PI3K and PKB signaling has now been established (Vanhaesebroeck & Alessi 2000).

Figure 4 Wortmannin inhibits FSH stimulation of Sertoli cell lactate production, LDH activity and glucose uptake. (A) Sertoli cells were stimulated for 72 h (day 3 to day 6) with 100 ng/ml FSH in the absence or presence of variable doses of wortmannin (W). Lactate was determined in the 72-h conditioned media recovered on day 6. (B) Sertoli cells were stimulated for 72 h (day 3 to day 6) with 100 ng/ml FSH in the absence or presence of variable doses of wortmannin. LDH activity was determined on cells harvested on day 6. (C) On day 5 of culture, Sertoli cells were maintained for 60 min under basal or FSH-stimulated (100 ng/ml) conditions in the absence or presence of different doses of wortmannin. Glucose uptake assay (2 DOG uptake) was performed after this incubation period. Results represent means $\pm$ S.D. of triplicate incubations in one representative experiment out of three. Different letters indicate statistically significant differences ($P < 0.01$).

Table 3 Effect of H89 and cycloheximide on basal and FSH-stimulated glucose uptake. On day 5, Sertoli cells were incubated without (Control) or with 10 μ M H89 or 5 μ M cycloheximide (Chx) in the absence (Basal) or presence of 100 ng/ml FSH for 60 min. Glucose uptake assay was performed after this incubation period. Results represent means $\pm$ S.D. of triplicate incubations in one representative experiment out of three

	Glucose uptake (dpm/ μ gDNA)	
	Basal	FSH
Control	556 $\pm$ 56 ^a	1235 $\pm$ 81 ^b
H89 (10 μ M)	634 $\pm$ 53 ^a	1182 $\pm$ 37 ^b
Chx (5 μ M)	521 $\pm$ 48 ^a	1110 $\pm$ 79 ^b

Different letters indicate statistically significant differences ($P < 0.01$).

Recently, a link between FSH binding to its receptor, cAMP and PI3K signaling pathway in granulosa cells at different stages of differentiation has been observed (Gonzalez-Robayna *et al.* 2000). The above mentioned investigation and many others obtained for the action of hormones in different cell types provide increasing evidence indicating that multiple cellular signaling cascades regulate cell function and that these signaling pathways are hormone- as well as cell-specific. The precise molecular and biochemical mechanisms involved in FSH regulation of Sertoli cell function remain largely unknown.

In this paper, we show that FSH increases P-PKB levels in a PI3K-dependent manner in rat Sertoli cells and that this regulation is closely related to Sertoli cell function. We have observed that FSH increases P-PKB levels and that two inhibitors of PI3K, wortmannin and Ly 294002, abolish the effect of FSH. Several speculations can be made on the possible mechanism(s) that may be utilized by FSH to regulate the PI3K/PKB pathway. Firstly, an FSH receptor protein bearing the features of a growth factor type I receptor in the mature testis has recently been cloned and identified (Babu *et al.* 1999). Occupancy of such a receptor by FSH may potentially result in activation of this pathway. Nevertheless, it should be noticed that the presence of this kind of receptor protein has not been unequivocally demonstrated in 20-day-old rat Sertoli cells. Secondly, another possible mechanism that may be involved in PI3K regulation by FSH is the one that utilizes the classic G-protein-coupled receptor and the freed G $\beta\gamma$ subunit. Again, no data are at present available to support the latter hypothesis. Lastly, considering that a similar increase in the levels of P-PKB is observed in cells stimulated with dbcAMP, it is tempting to speculate that the observed phenomenon is a consequence of elevated intracellular cAMP levels produced by FSH post-activation of adenylyl cyclase by G α subunit. In support of the latter hypothesis we have observed parallel increments in cAMP and P-PKB levels produced by FSH stimulation. In addition, the lack of an inhibitory effect of H89 on

FSH-stimulated P-PKB levels and the inhibitory effect of wortmannin and Ly 294002 on dbcAMP-stimulated P-PKB levels suggest that cAMP regulates P-PKB levels in a PKA-independent and PI3K-dependent manner. The exact mechanism by which cAMP regulates PI3K remains largely unknown. However, it is worth mentioning that Richards (2001) proposes that cAMP produced upon binding of several related glycoprotein hormones to their respective G-protein-coupled receptors binds to the cAMP-regulated guanine nucleotide exchange factor (cAMP-GEF) and activates PI3K. Although, at present, a link between cAMP, GEF and PI3K in rat Sertoli cells has not been demonstrated, the data obtained so far support the idea that stimulation of Sertoli cell PI3K by FSH may involve such a mechanism.

In analyzing the consequences of PI3K inhibition on Sertoli cell P-PKB levels and on Sertoli cell response to FSH, several interesting observations were made. Such observations were obtained by evaluating three well-known parameters of Sertoli cell function that increase in response to FSH: γ -GTP activity, transferrin secretion and lactate production.

γ -GTP is a membrane-bound enzyme that is widely distributed in mammalian tissues. It has been suggested that, among other functions, it acts as a transport system for amino acids across the plasma membrane (Tate & Meister 1981). In Sertoli cells its activity is stimulated by FSH and germ cell-secreted products (Schteingart *et al.* 1989). Several signal transduction pathways, not necessarily evoked by FSH, have been analyzed by their ability to regulate γ -GTP activity in rat Sertoli cells (Meroni *et al.* 1997, 1999, 2000). Transferrin is an iron transport protein secreted by Sertoli cells and is vital to the regulation and maintenance of spermatogenesis (Skinner & Griswold 1980). In a similar way to that observed for γ -GTP activity, previous studies have demonstrated that FSH (Skinner & Griswold 1980), growth factors (Skinner & Griswold 1982, Han *et al.* 1993) and germ cell-derived products (Le Magueresse *et al.* 1988) stimulate its secretion. In the present investigation we show that wortmannin, which inhibits PI3K activity and consequently PKB phosphorylation in response to FSH, also partially inhibits FSH stimulation of γ -GTP activity and transferrin secretion. The inhibitory effect of wortmannin on γ -GTP activity was only observed at doses as high as 1 μ M. Even though this dose of wortmannin inhibits neither basal γ -GTP levels nor cell viability in 72-h incubations, results obtained with this dose of the inhibitor should be interpreted with caution. In this respect, it has been demonstrated that high doses of wortmannin also inhibit other signaling enzymes (Fruman *et al.* 1998, Shepherd *et al.* 1998). However, the inhibitory effect of wortmannin on transferrin secretion occurs at doses well within the range of concentrations showing specific inhibitory effects on class I PI3K. This suggests the participation of PI3K and probably of PKB in a down-

stream signaling event for the regulation of transferrin secretion by FSH. Ly 294002 and H89 decreased cell viability in 72-h incubations. For this reason these inhibitors could not be assayed to evaluate an alternative PI3K inhibitor and the relevance of the cAMP/PKA pathway respectively in the regulation of γ -GTP activity and of transferrin secretion. Several events downstream of PI3K/PKB activation are related to protein synthesis (Vanhaesebroeck & Alessi 2000) and increments in γ -GTP activity and in transferrin secretion involve *de novo* protein synthesis. In this way, it is not surprising that activation of this signaling pathway may be somehow related to the metabolic changes observed.

Carbohydrate metabolism in Sertoli cells presents some unique characteristics. Studies on glucose metabolism have shown that Sertoli cells actively metabolize glucose but the majority of it is converted to lactate and is not oxidized via the citric acid cycle (Robinson & Fritz 1981, Grootegoed *et al.* 1986). In addition, germ cells, particularly post-meiotic germ cells, are unable to use glucose for their energetic metabolism and they do prefer lactate as an energy source (Mita & Hall 1982). The importance of lactate for normal spermatogenesis has been demonstrated in a recent *in vivo* study (Courten & Plöen 1999). These observations have led to the conclusion that one of the most important Sertoli cell nurse functions is to provide lactate for the production of energy in spermatocytes and spermatids. It must be noticed that several biochemical steps may lead to increments in lactate production. Some of them like glucose transport and LDH activity play essential roles at the beginning of the process, providing the substrate, and at the end of the process, interconverting pyruvate and lactate. We have previously shown that FSH stimulates both processes in rat Sertoli cells (Riera *et al.* 2001); however, the signaling molecules involved in these actions of the hormone had not previously been investigated. The present study shows that FSH stimulation of lactate production and LDH activity are partially blocked in cells pre-incubated with the PI3K inhibitor, wortmannin. Again, doses of wortmannin showing the ability to decrease FSH action on these parameters are well within the range of concentrations considered to be specific for inhibition of PI3K. These results suggest that, in a similar way to that observed for transferrin secretion, a PI3K/PKB signaling pathway participates in the FSH regulation of these parameters.

As for the regulation of glucose transport by FSH the relevance of a cAMP/PKA pathway was evaluated due to the fact that H89 does not change cell viability in 60-min incubations. Results obtained showing a lack of inhibitory effect of H89 on FSH-stimulated glucose uptake suggest that the cAMP/PKA pathway is not involved in glucose transport in Sertoli cells. On the other hand, the inhibition of glucose uptake induced by wortmannin under basal conditions and the ability of wortmannin to inhibit FSH stimulation of glucose uptake points to the fact that a

PI3K-dependent pathway is essential to the process of glucose transport through the plasma membrane. These results are not surprising, as the relationship between a PI3K pathway and glucose transport has been extensively analyzed (Okada *et al.* 1994, Huppertz *et al.* 1996, Czech & Corvera 1999). Nevertheless, the latter studies refer mainly to translocation to the plasma membrane of the glucose transporter GLUT4 and are referred to the mechanism of action of insulin and growth factors but not to that of FSH. Fewer studies have analyzed GLUT1 translocation to the plasma membrane but it apparently does occur (Samih *et al.* 2000). In rat Sertoli cells glucose transport is mediated mainly by the glucose transporter GLUT1, the only glucose transporter demonstrated so far in these cells (Ullisse *et al.* 1992). Our results suggest that the mechanism of action of FSH in rat Sertoli cells involves translocation of GLUT1 to the plasma membrane. The latter suggestion is based mainly on two observations that are somehow related: (a) changes in glucose uptake induced by FSH are observed after a short-term exposure to the hormone and (b) the protein synthesis inhibitor cycloheximide does not inhibit FSH stimulation of glucose uptake.

Altogether, the results presented in this investigation suggest an important role of a PI3K/PKB pathway in the mechanism of action utilized by FSH/cAMP to regulate Sertoli cell metabolism. In addition to PKB, other signaling pathways downstream of PI3K may be partly responsible for the observed effects of FSH on Sertoli cell function. However, as in most cases the metabolic changes were accompanied by changes in the levels of P-PKB it is tempting to speculate that this serine/threonine kinase plays a meaningful role in the mechanism of action of FSH/cAMP in rat Sertoli cells.

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