Na⁺/K⁺-ATPase α1 isoform mediates ouabain-induced expression of cyclin D1 and proliferation of rat Sertoli cells

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Abstract

Novel roles for the interaction of cardiotonic steroids to Na⁺/K⁺-ATPase have been established in recent years. The aim of this study was to investigate the intracellular signaling events downstream the action of ouabain on Na⁺/K⁺-ATPase in Sertoli cell obtained from immature rats. Treatment of Sertoli cells with ouabain (1 μM) induced a rapid and transient increase in the extracellular signal-regulated kinase (ERK1/2 or MAPK3/1) and phosphatidylinositol 3-kinase (PI3K)/serine–threonine protein kinase (AKT) phosphorylation. Also, ouabain upregulated the expression of cyclin D1 and incorporation of [methyl-³H]thymidine, both of which were dependent on MAPK3/1 but not AKT intracellular cascade, as shown by pretreatment with MEK (MAP2K1/2) inhibitor U0126 and PI3K inhibitor wortmannin respectively. Moreover, the effect of ouabain on these proliferation parameters was completely prevented by phospho-cAMP response element-binding protein (CREB)/CREB-binding protein complex inhibitor KG501 and only partially by nuclear factor κB nuclear translocation inhibitor SN50. Pretreatment with estrogen receptor antagonist ICI 182 780 showed that MAPK3/1 activation by ouabain does not involve this receptor. The Na⁺/K⁺-ATPase α1 isofrom, but not α4, was detected in Sertoli cells, suggesting that ouabain effects in Sertoli cells are mediated via α1. Taken together, these results show a rapid ouabain action in the Sertoli cells, which in turn can modulate nuclear transcriptional events essential for Sertoli cell proliferation in a critical period of testicular development. Our findings are important to understand the role of ouabain in the testis and its possible implications in male infertility.

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Introduction

Na⁺/K⁺-ATPase, besides its ion-pumping role, is now considered a receptor that interacts with many membrane and cytosolic proteins, for example, SRC family of tyrosine kinases, and activates different downstream signaling pathways after bound to cardiotonic steroids (Pierre & Xie 2006). A complex set of cell type-dependent short- and long-term effects has been reported after activation of Na⁺/K⁺-ATPase, such as contraction (Mohammadi et al. 2003), proliferation/viability (Abramowitz et al. 2003, Tian et al. 2009, Li et al. 2010, Quintas et al. 2010), differentiation (Fedorova et al. 2009), secretion (Quintas et al. 2010), and apoptosis (Jiang et al. 2010). The role of cardiotonic steroids becomes more remarkable because, in addition to their therapeutic use as inotropic compounds, they have also been detected endogenously in mammals and are recognized as a new class of steroid hormones (Bagrov et al. 2009). Both cardenolides (e.g. ouabain) and bufadienolides (e.g. marinobufagin) are found in the circulation and their concentrations may increase at distinct physiological and pathological conditions (Bagrov et al. 2009).

In testicular cells, particularly Sertoli cells, Na⁺/K⁺-ATPase function has been poorly investigated. The few available studies show that the pump has an apparent polarized, lateroapical distribution and plays a role on the ionic composition of the seminiferous tubular fluid (Rato et al. 2010) and the intracellular pH of the Sertoli cells (Oliveira et al. 2009). On the other hand, the putative effects of cardiotonic steroids such as the specific sodium pump ligand ouabain have not been analyzed in detail so far. Recent study has shown that ouabain can stimulate SRC-RAF-MAPK cascade and also cAMP response element-binding (CREB) protein in rat-derived Sertoli cell line 93RS2, but the ouabain-induced cellular effects have not been established (Konrad et al. 2011).

Sertoli cells are known to divide in the fetal rat testis, proliferating maximally on day 20 postconception, with a progressive decrease in division until postnatal day 21, when division ceases. The Sertoli cell population remains relatively constant in adult mammals, and each
Sertoli cell supports ~30–50 germ cells at different stages of development in the seminiferous epithelium. Recent studies have also shown that Sertoli cells are capable of dividing even in adult animals, under experimental and physiological conditions. The microenvironment of the seminiferous epithelium, wherein each Sertoli cell supports germ cells at different stages of development, locally produces autocrine and paracrine factors involved with spermatogenesis (reviewed in Lucas et al. (2011)). Thus, a better knowledge about the role of these factors and also of the cardiotonic steroids in the regulation of homeostasis and function of Sertoli cells will be important for the control of male fertility. In this study, we show that different intracellular signaling pathways are activated by ouabain in primary culture of Sertoli cells obtained from immature rats and that MAPK3/1 (extracellular signal-regulated kinase, ERK1/2), CREB, and nuclear factor κB (NF-κB) are involved in ouabain-induced cell proliferation.

Results

Ouabain induces MAPK3/1 and AKT phosphorylation in primary rat Sertoli cells

Cardiotonic steroids rapidly activate several intracellular signaling pathways in different cells, including MAPK3/1 (Kometiani et al. 1998, Quintas et al. 2010) and Akt (Liu et al. 2007). Increase in MAPK3/1 phosphorylation was observed with 1 μM ouabain in Sertoli cells (Fig. 1A). Treatment with lower concentrations of ouabain was not able to activate MAPK3/1. The peak of MAPK3/1 phosphorylation occurred at 5 min (2.9 ± 0.5- and 3.6 ± 0.2-fold increase for MAPK3 and MAPK1 respectively compared with control) (Fig. 1B). Similar results were observed for AKT phosphorylation (Fig. 2). No differences were observed in total MAPK3/1 or AKT protein expression under any of these conditions (Figs 1 and 2).

Considering that rat Na+/K+-ATPase α isoforms have distinct sensitivities to the inhibitory activity of cardiotonic steroids (Lopez et al. 2002) as well as to the stimulation of signaling pathways (Pierre et al. 2008), we investigated which α isoforms are present in rat Sertoli cells. The expression of Na+/K+-ATPase α4 isoform was not detected at the protein level in three different batches of primary culture of Sertoli cells, but it was clearly detectable in a human spermatozoa preparation used as a positive control (Fig. 3). Only the housekeeping α1 isoform protein was observed in Sertoli cells, which is consistent with the micromolar ouabain concentration range responsible for activation of signaling pathways.

Ouabain induces cyclin D1 expression and Sertoli cell proliferation through MAPK3/1, CREB, and NF-κB in primary rat Sertoli cells

Ouabain may stimulate cell proliferation via MAPK3/1 and/or AKT in different cells (Dmitrieva & Doris 2003, Khundmiri et al. 2006, Tian et al. 2009), and these protein kinases are involved in the upregulation of cyclin D1 (CCND1) and cell cycle progression (Liang & Slingerland 2003), reviewed in Musgrove et al. (2011). The treatment of Sertoli cells with ouabain (1 μM, 24 h) increased the expression of CCND1 (Fig. 4). 17β-Estradiol (E2) was used as a positive control (Lucas et al. 2010).

Figure 1 Effect of ouabain on MAPK3/1 phosphorylation in rat Sertoli cells. Cells were incubated in the absence (C, control) and presence of increasing concentrations of ouabain for 5 min (A) or in the absence (C, control) and presence of 1 μM ouabain for 2–30 min (B). Total cell lysates (60 μg protein/lane) were resolved in 10% SDS/PAGE, transferred and probed with antibodies specific for phosphorylated (p) MAPK3/1 (top panel) or total (phosphorylation state-independent) MAPK3/1 proteins (bottom panel). The relative positions of pMAPK3/1 and total MAPK3/1 proteins are shown at the right. The data shown are representative of four independent experiments. Right panel: bars represent the densitometric analysis of the western blot assays. Solid bars, pMAPK3; open bars, pMAPK1. Results were normalized to total MAPK3/1 expression in each sample and plotted (mean ± S.E.M.) in relation to control, C (= 1). *MAPK3/1 activation significantly greater than that of control (P < 0.05, Student’s t-test).
In order to evaluate which signaling pathways are involved in the expression of CCND1 in Sertoli cells, inhibitors of MAP2K1/2 (U0126) and phosphatidylinositol 3-kinase (PI3K) (wortmannin) were used. Ouabain-induced CCND1 expression was blocked by pretreatment with U0126 (Fig. 5A). Wortmannin did not significantly change the expression of CCND1 induced by ouabain (Fig. 5A).

Transcription factors, such as CREB and NF-κB, are activated by different intracellular signaling pathways and are also involved in cell proliferation (Witzel et al. 2010, Royer et al. 2012). KG501, the compound that disrupts the phospho-CREB/CREB-binding protein (CBP) complex, blocked the expression of CCND1 induced by ouabain in Sertoli cells (Fig. 5B). On the other hand, pretreatment with NF-κB nuclear translocation inhibitor SN50 produced partial inhibition of CCND1 expression (Fig. 5B). The treatment with U0126, wortmannin, KG501, and SN50 for 30 min, in the absence of ouabain, did not have any effect on CCND1 (data not shown).

Ouabain caused an enhancement of 69% of [methyl-3H]thymidine incorporation after 24-h incubation (Fig. 6). In addition, pretreatment of Sertoli cells with U0126 and KG501 markedly reduced the [methyl-3H]thymidine incorporation into DNA induced by ouabain (Fig. 6). Pretreatment with SN50 only produced partial inhibition of this effect (Fig. 6).

Some cardiotonic steroids, e.g. digitoxin, may interact with estrogen receptors (ERs; Rifka et al. 1976) and have been inferred as putative natural estrogens (Schussheim & Schussheim 1998). Sertoli cells express the classic estrogen receptors (ESR1 and ESR2). The activation of these receptors produces a rapid and transient increase in the phosphorylation of MAPK3/1 (Lucas et al. 2008a, 2008b) and AKT (Royer et al. 2012) and are blocked by the ER antagonist ICI 182 780 (Lucas et al. 2008a, 2008b). Thus, we tested whether ICI 182 780 affected ouabain-induced MAPK3/1 phosphorylation. As shown in Fig. 7, ICI 182 780 did not change MAPK3/1 phosphorylation induced by ouabain, indicating that ouabain did not play a role through ERs. The treatment with ICI 182 780 for 30 min, in the absence of ouabain, did not have any effect on MAPK3/1 phosphorylation (Fig. 7).

We also tested whether ICI 182 780 affected ouabain-induced CCND1 expression. As expected, ICI 182 780 did not change CCND1 expression induced by ouabain (data not shown).

**Discussion**

Cardiotonic steroids have been extensively investigated due to their promising pharmacological, pathological, and physiological properties. It is now well established that cardiotonic steroids bind to Na⁺/K⁺-ATPase and modulate several signaling pathways (Pierre & Xie 2006).

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expression significantly different from control (Doris & Doris 2003, Kulikov of signaling pathways (Kometiani been shown to induce different time courses of activation (Dmitrieva & Doris 2003, Pierre et al 2008). 2007), and in this study, ouabain also induced proliferation, but this appears to be only dependent on MAPK3/1 as inhibition of PI3K/AKT pathway did not affect ouabain-evoked CCND1 overexpression. As a possible explanation, Khundmiri et al (2007) reported that MAPK3/1 is a positive upstream regulator of AKT and it seems to be a specific feature of ouabain signaling. Nonetheless, AKT still depends on PI3K activity to be activated (Khundmiri et al 2007). Thus, the role of AKT phosphorylation by ouabain in Sertoli cells needs further investigation.

In Sertoli cells, CREB is an important transducer of FSH (Walker et al 1995), testosterone (Fix et al 2004, Cheng et al 2007), and E2 (Royer et al 2012) signals to induce gene expression. Ouabain stimulates CREB phosphorylation in the rat Sertoli cell line 93RS2 (Konrad et al 2011), and in this study, ouabain also induced CCND1 expression and proliferation via CREB activity. Activation of CREB in Sertoli cells plays an important role in spermatogenesis. For instance, overexpression of a phosphorylation-defective CREB mutant in Sertoli cells in vivo results in apoptosis and elimination of germ cells (Scobey et al 2001).

Ouabain-induced CCND1 expression and proliferation was partially dependent on NF-κB activity. In rat kidney cells and hippocampus, ouabain stimulates NF-κB causing cell proliferation and cellular protection (Li et al 2006, Kawamoto et al 2012). Thus, both NF-κB and CREB are the mediators of ouabain action in the proliferative activity of Sertoli cells and spermatogenesis.

Using in situ hybridization and immunohistochemistry assays, previous studies reported that the adult testis has the Na⁺/K⁺-ATPase α4 isoform in mature sperm, but not in any other cell types including Sertoli and Leydig cells (Blanco et al 2000, Woo et al 2000). In fact, the α4 isoform (mRNA and protein) was not detected until 4 weeks of age and reached a maximum level of expression (threefold) at 6 weeks, indicating that this

![Figure 4](image-url) Effect of ouabain on cyclin D1 (CCND1) expression in rat Sertoli cells. Cells were incubated in the absence (C, control) and presence of 10 nM and 1 μM ouabain or 0.1 nM 17β-estradiol (E2) for 24 h. Total cell lysates (40 protein/lane) were resolved in 15% SDS/PAGE, transferred and probed with an antibody specific for CCND1. The data shown are representative of five independent experiments. Bars represent the densitometric analysis of the western blot assays. Results were normalized to actin expression in each sample and plotted (mean±S.E.M.) in relation to control, C (=100%). *CCND1 expression significantly different from control (P<0.05, Student’s t-test). E2 was used as a positive control.

Sertoli cell plays a key role in spermatogenesis, and a better knowledge about the role of steroid hormones and growth factors in the regulation of the homeostasis and function of this cell will be important for the control of male fertility (reviewed in Lucas et al (2011)).

Immature rat Sertoli cells responded to 1 μM ouabain treatment in 5 min with a significant increase in MAPK3/1 and AKT phosphorylation. The activation of these effector kinases by ouabain at this concentration range has been reported in different rodent cell types and is consistent with the major/exclusive presence of the relatively insensitive Na⁺/K⁺-ATPase α1 isoform (Haas et al 2000, Elkareh et al 2007, Liu et al 2007, Pierre et al 2008, Quintas et al 2010). Ouabain has been shown to induce different time courses of activation of signaling pathways (Kometiani et al 1998, Dmitrieva & Doris 2003, Kulikov et al 2007, Nguyen et al 2007, Pierre et al 2008). Rat Sertoli cells exhibit a very quick and short-lived stimulation profile of the MAPK pathway by ouabain, similar to that reported for other cell types (Dmitrieva & Doris 2003, Pierre et al 2008). This rapid and transient increase in the phosphorylation state of MAPK3/1 by ouabain in Sertoli cells is similar to that observed with E2 (Lucas et al 2008a), G protein-coupled ER 1 (GPER) G-1 agonists (Lucas et al 2010), retinol (Gelain et al 2006), and FSH (Crépieux et al 2001) in these cells. On the other hand, Sertoli cells respond with different kinetics of activation to the nonhydrolyzable androgen agonist R1881, which induces a rapid (1–15 min) and sustained (12 h) increase in the phosphorylation state of MAPK3/1 (Fix et al 2004), or to carbachol, a stable analog of acetylcholine, which also induced a rapid and sustained phosphorylation of MAPK3/1 (1–60 min) (Lucas et al 2008b). Therefore, these results suggest that the time-dependent activation of MAPK3/1 in Sertoli cells is agonist-specific.

Both MAPK3/1 and PI3K/AKT are associated with cell proliferation and viability (Mendoza et al 2011) and ouabain has been shown to produce such effects (Dmitrieva & Doris 2003, Khundmiri et al 2006, Tian et al 2009). Our results demonstrate that in immature rat Sertoli cells, 1 μM ouabain also induced proliferation, but this appears to be only dependent on MAPK3/1 as inhibition of PI3K/AKT pathway did not affect ouabain-evoked CCND1 overexpression. As a possible explanation, Khundmiri et al (2007) reported that MAPK3/1 is a positive upstream regulator of AKT and it seems to be a specific feature of ouabain signaling. Nonetheless, AKT still depends on PI3K activity to be activated (Khundmiri et al 2007). Thus, the role of AKT phosphorylation by ouabain in Sertoli cells needs further investigation.

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isoform is regulated in parallel to the onset of sexual maturity (Woo et al. 2000). The ubiquitous z1 isoform, on the other hand, was detected at a constant level throughout the life of the animal. Wagoner et al. (2005) showed low levels of z4 isoform in 1-week-old rat testis using a preparation enriched in germ cell types by means of unit gravity sedimentation method, thus preventing the interference of other testis cells, and in spermatogonia isolated from 10-day-old rats. In contrast, Konrad et al. (2011) detected the mRNA for Na+/K+-ATPase z1 and z4 isoforms in prepubertal rat-derived Sertoli cell line 93SR2 and in pubertal Sertoli cell line SCIT-C8, both immortalized cells (Jiang et al. 1997, Konrad et al. 2005). It is important to emphasize that, different from primary Sertoli cells, the immortalized cells fail to express androgen receptors and to respond to androgens and FSH (Konrad et al. 2005). The mRNA for Na+/K+-ATPase z4 isoform was also detected in primary culture of Sertoli cell from 19-day-old rat (Konrad et al. 2011); at this age, rat Sertoli cells start differentiation. We were not able to detect the Na+/K+-ATPase z4 isoform at the protein level in Sertoli cells obtained from immature rats (15-day-old), similar to the findings of Woo et al. (2000), and found that the Na+/K+-ATPase z1 present in these cells mediates a rapid ouabain-induced signaling cascade, which is to a great extent similar to the rapid signaling pathway triggered by testosterone and E2, and plays a role in Sertoli cell proliferation. Interestingly, differently from rodents, the Na+/K+-ATPase z1 isoform is sensitive to cardiotonic steroids as ouabain (Touza et al. 2011).

The concentration of endogenous ouabain (Manunta et al. 2009, Nesher et al. 2009) is lower than that used in this study. In addition to systemic hormones, it is

Figure 5 Signaling pathways involved on cyclin D1 (CCND1) expression induced by ouabain in rat Sertoli cells. Cells were untreated or pretreated with 20 µM MEK1/2 inhibitor (U0126), 1 µM PI3K inhibitor (wortmannin) (A), 25 µM disruptor of the CREB:CBP complex (KG501), or 1 µM NF-kB nuclear translocation inhibitor (SN50) (B) for 30 min. Cells were incubated in the absence (C, control) and presence of 1 µM ouabain for 24 h. Total cell lysates (40 µg protein/lane) were resolved in 15% SDS/PAGE, transferred and probed with an antibody specific for CCND1. The data shown are representative of five independent experiments. Bars represent the densitometric analysis of the western blot assays. Results were normalized to actin expression in each sample and plotted (mean ± s.e.m.) in relation to control. *CCND1 expression significantly different from control (P<0.05, Student’s t-test). **Significantly different from ouabain (P<0.05, Student’s t-test).

Figure 6 Effect of ouabain on rat Sertoli cell proliferation. Cells were initially incubated with 2 µCi/ml [methyl-3H]thymidine for 6 h. Cells were untreated or pretreated with 20 µM MEK1/2 inhibitor (U0126), 1 µM PI3K inhibitor (wortmannin), 25 µM disruptor of the CREB:CBP complex (KG501), or 1 µM NF-kB nuclear translocation inhibitor (SN50) for 30 min. Afterward, cells were incubated in the absence (basal incorporation) and presence of 1 µM ouabain for 24 h. The reaction was stopped, the cells rinsed with ice-cold PBS and 5% trichloroacetic acid, and then solubilized with 0.5 N NaOH. Bound radioactivity was determined and the results were expressed in relation to control, basal levels of [methyl-3H] thymidine incorporation (% above control) as mean ± S.E.M. of five independent experiments performed in triplicate. *[Methyl-3H] thymidine incorporation activation significantly greater than control (P<0.05, Student’s t-test). **Significantly different from ouabain (P<0.05, Student’s t-test).
important to consider the local conversion of steroids and that intra-testicular androgens and estrogens levels do not always mirror systemic levels. The levels of these intra-testicular hormones are dependent on the presence and activity of local steroid-metabolizing enzymes, such as 5α-reductase (Kilian et al. 2003, Pratis et al. 2003) and aromatase (reviewed in Carreau et al. (2011) and Haverfield et al. (2011)), and the concentration of these steroids is very high in intra-testicular level than in plasma. The main source of endogenous ouabain is the adrenal cortex and hypothalamus (Laredo et al. 1995, Hamlyn et al. 1998). Interestingly, endogenous digoxin-like immunoreactivity has been detected in seminal fluid of men (Vadazs et al. 1992). Whether endogenous ouabain and/or other cardiotoxic steroids might be produced by the testis, as the early precursors are present (pregnenolone and progesterone) and the sequential enzymes are unknown, remain to be explored and open questions for further studies.

In conclusion, these results indicate that ouabain-Na+/K+-ATPase α1 may regulate the expression of CCND1 and proliferation in Sertoli cells through MAPK3/1/pCREB obtained from testes of immature rats. Furthermore, NF-κB is also partially involved in this effect. Our findings are important to clarify the role of ouabain in testis and to direct further studies, which may contribute to better understanding of the causes of male infertility.

Materials and Methods

Sertoli cell culture

Primary cultures of Sertoli cell were obtained from 15-day-old male Wistar rats housed in the Animal Facility at Instituto de Farmacologia e Biologia Molecular (INFAR), Universidade Federal de São Paulo – Escola Paulista de Medicina (UNIFESP-EPM), and maintained on a 12 h light:12 h darkness lighting schedule, at 23 °C, with food and water ad libitum. The experimental procedures were conducted according to guidelines for the care and use of laboratory animals as approved by the Research Ethical Committee from UNIFESP-EPM (protocol 121/08). The testes were removed and decapsulated and Sertoli cells were prepared as described previously (Lucas et al. 2004, 2008a, 2008b, 2010). The cells (4.5×10⁶ cells/ml) were plated at a density of 4×10⁶ cells/9 ml in 100 mm dish (about 5×10⁵ cells/cm²) to allow the formation of monolayer in phenol red-free Ham F12/DMEM (1:1, Gibco, Invitrogen) containing 0.02 g/l gentamicin (Sigma Chemical Co.), pH 7.2–7.4, and supplemented with 10 μg/ml insulin, 10 μg/ml transferrin, 10 ng/ml sodium selenite, and 10 ng/ml epidermal growth factor (Sigma). Cells were grown in a humidified atmosphere of 5% CO₂—95% air at 35 °C and, after 48 h, they were treated with 20 mM Tris–HCl, pH 7.4, to lyse residual germ cells (Galdieri et al. 1981) and allowed to grow for another 24 h. Culture medium was replaced by another one without supplements 20 h before the experiments with Sertoli cells. At this stage, cells were 90–95% confluent and viable cells in each culture was more than 90% as determined by trypan blue exclusion. The presence of other cell types in the primary culture of Sertoli cells was evaluated by several criteria that confirmed the absolute predominance of Sertoli cells in the primary culture (Lucas et al. 2008a, 2008b).

Human spermatozoa homogenates

Semen sample was collected by masturbation from a healthy adult donor enrolled in the Setor de Reprodução Humana e Infertilidade (Instituto de Ginecologia, Universidade Federal do Rio de Janeiro) with normal spermogram. The procedure was approved by the Institutional Research Ethics Committee and a written informed consent was obtained. The sample was diluted 1:1 with cryoprotectant medium (test yolk buffer with glycerol) frozen in liquid nitrogen and stored at −80 °C. Subcellular homogenate was performed according to

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**Figure 7** Effect of antiestrogen ICI 182 789 on MAPK3/1 phosphorylation induced by ouabain in rat Sertoli cells. Cells were untreated or pretreated with estrogen receptor (ESR1 and ESR2) antagonist ICI 182 780 (ICI, 0.1 nM) for 30 min. Afterward, cells were incubated in the absence (C, control) and presence of 1 μM ouabain for 5 min. Total cell lysates (60 μg protein/lane) were resolved in 10% SDS/PAGE, transferred and probed with antibodies specific for phosphorylated (p) MAPK3/1 (top panel), or total (phosphorylation state-independent) MAPK3/1 proteins (bottom panel). The relative positions of pMAPK3/1 and total MAPK3/1 proteins are shown at the right. The data shown are representative of four independent experiments. Right panel: bars represent the densitometric analysis of the western blot assays. Solid bars, pMAPK3; open bars, pMAPK1. Results were normalized to total MAPK3/1 expression in each sample and plotted (mean±S.E.M.) in relation to control, C (=1). *MAPK3/1 activation significantly greater than that of control or ICI 182 780 (P<0.05, Student’s t-test).
Western blot for detection of phospho- and total MAPK3/1 and AKT, CCND1, and Na\(^+\)/K\(^+\)-ATPase α isoforms

Primary Sertoli cell cultures were incubated in the absence (control) or presence of 0.1 nM–1 μM ouabain (Sigma) for 5 min and 1 μM ouabain for 2–30 min at 35 °C for detection of phospho- and total MAPK3/1 and/or AKT (Lucas et al. 2008a, 2008b). For CCND1, Sertoli cells were incubated with 1 μM and 10 nM ouabain and/or E\(_2\) (0.1 nM) for 24 h at 35 °C (Lucas et al. 2008a, 2008b). In some experiments, the cells were pretreated for 30 min with the ER antagonist ICI 182 780 (0.1 nM, AstraZeneca, Macclesfield, UK), MAP2K1/2 inhibitor U0126 (20 μM, Cell Signaling Technology, Beverly, MA, USA), PI3K inhibitor wortmannin (1 μM, Sigma), disruptor of the CREB:CBP complex KG501 (25 μM, Sigma), or translocation inhibitor of the NF-κB active complex into the nucleus SN50 (1 μM, Calbiochem, San Diego, CA, USA).

Afterward, the medium was removed, cells were washed with ice-cold PBS, and lysed in ice-cold lysis buffer (20 mM HEPES, pH 7.5, 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1 mM EDTA, and homogenized using a glass–glass homogenizer. Protein concentration was determined by Bio-Rad protein assay, using BSA as standard (Bio-Rad Laboratories).

After washing in TBST, membranes were incubated with rabbit polyclonal antibodies against MAPK3/1 (p44/p42 MAP kinase, ERK1/ERK2, Cell Signaling Technology), phospho-MAPK3/1 (Thr202/Tyr204, Cell Signaling Technology), phospho-AKT (Ser473, Cell Signaling Technology), CCND1 (cyclin D1, Cell Signaling Technology) or β-actin (Sigma), rabbit MAB against AKT (AKT (pan), Cell Signaling Technology), mouse MAB against Na\(^+\)/K\(^+\)-ATPase α1 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), and mouse polyclonal antiserum against Na\(^+\)/K\(^+\)-ATPase α4 (generously provided by Dr Gustavo Blanco, University of Kansas Medical Center, USA) diluted in blocking solution overnight at 4 °C. After incubation with donkey anti-rabbit (Amersham Biosciences, Piscataway, NJ, USA) or rabbit anti-mouse (Promega, Madison, WI, USA) HRP-conjugated secondary antibodies diluted in TBST for 1 h at room temperature, proteins were visualized by enhanced chemiluminescence reagent (ECL, Amersham Biosciences). Apparent molecular weights of these proteins were determined from molecular weight standards (New England Biolabs, Ipswich, MA, USA).

Band intensities from individual experiments were quantified by densitometric analysis of linear-range autoradiograms using Epson Expression 1680 scanner (Epson America, Inc., Long Beach, CA, USA) and Quick Scan 2000 WIN software (Helena Laboratories, Beaumont, TX, USA). Results were normalized based on total MAPK3/1, total AKT, or β-actin expression in each sample and plotted (mean ± S.E.M.) in relation to control.

[Methyl-\(^3\)H]thymidine incorporation assays

For proliferation assays, Sertoli cells were prepared as described earlier and used for the experiments when the cultures reached a confluency between 50 and 60%. Incorporation of [methyl-\(^3\)H]thymidine into cell DNA was estimated as described by Guizzetti et al. (1996) with slight modifications (Lucas et al. 2004, 2008a). In previous studies, we determined that incorporation of [methyl-\(^3\)H]thymidine (2 μCi/ml, specific activity 79.0 Ci/mmol, GE Healthcare Life Science, Pittsburgh, PA, USA) in cultured Sertoli cells was time dependent and linear from 2 to 10 h of incubation. All studies were performed after incubation with [methyl-\(^3\)H]thymidine for 6 h (Lucas et al. 2004, 2008a). On day 4 of culture, primary Sertoli cells were incubated with 2 μCi/ml [methyl-\(^3\)H]thymidine for 6 h at 35 °C. Incubation was continued in the absence (basal incorporation) and presence of ouabain (1 μM, Sigma) for 24 h at 35 °C. The cells were also untreated or pretreated with MEK1/2 inhibitor U0126 (20 μM, Cell Signaling Technology; 30 min), PI3K inhibitor wortmannin (1 μM, Sigma; 30 min), disruptor of the CREB:CBP complex KG501 (25 μM, Sigma; 30 min), or NF-κB nucleus translocation inhibitor SN50 (1 μM, Calbiochem; 30 min). Afterward, the cells were stimulated with ouabain (1 μM, for 24 h) at 35 °C. The reaction was stopped by cooling the cells at 0 °C. The medium was aspirated and the cells rinsed twice with ice-cold PBS and 5% trichloroacetic acid (Sigma). The cells were then solubilized with 0.5 M NaOH, collected with cotton swabs, and transferred to 5 ml OptiPhase HiSafe-3 scintillation liquid (PerkinElmer Life Science Products, Boston, MA, USA). Bound radioactivity was determined in a scintillation β counter (LS 6000 IC, Beckman Coulter, Inc., Palo Alto, CA, USA). Results were expressed in relation to control, basal levels of [methyl-\(^3\)H] thymidine incorporation (absence of ouabain and inhibitors).

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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