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Non-classical effects of androgens on testes from neonatal rats

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ABSTRACT

The intratesticular testosterone concentration is high during the early postnatal period although the intracellular androgen receptor expression (iAR) is still absent in Sertoli cells (SCs). This study aimed to evaluate the non-classical effects of testosterone and epitestosterone on calcium uptake and the electrophysiological effects of testosterone (1 μM) on SCs from rats on postnatal day (pnd) 3 and 4 with lack of expression of the iAR. In addition, crosstalk on the electrophysiological effects of testosterone and epitestosterone with follicle stimulating hormone (FSH) in SCs from 15-day-old rats was evaluated. The isotope $^{45}Ca^{2+}$ was utilized to evaluate the effects of testosterone and epitestosterone in calcium uptake. The membrane potential of SCs was recorded using a standard single microelectrode technique. No immunoreaction concerning the iAR was observed in SCs on pnd 3 and 4. At this age, both testosterone and epitestosterone increased the $^{45}Ca^{2+}$ uptake. Testosterone promoted membrane potential depolarization of SCs on pnd 4. FSH application followed by testosterone and epitestosterone reduced the depolarization of the two hormones. Application of epitestosterone 5 min after FSH resulted in a delay of epitestosterone-promoted depolarization. The cell resistance was also reduced. Thus, in SCs from neonatal Wistar rats, both testosterone and epitestosterone act through a non-classical mechanism stimulating calcium uptake in whole testes, and testosterone produces a depolarizing effect on SC membranes. Testosterone and epitestosterone stimulates non-classical actions via a membrane mechanism, which is independent of iAR. FSH and testosterone/epitestosterone affect each other's electrophysiological responses suggesting crosstalk between the intracellular signaling pathways.

1. Introduction

The process of Sertoli cell proliferation, during the immature period, is crucial to a normal fertility in adulthood. In fetal life, androgen action is more important for testicular development than follicle stimulating hormone (FSH) action. However, during the postnatal period, FSH becomes essential to proliferation, determining Sertoli cell numbers [1,2]. Furthermore, in several species such as rat, mice and human, intratesticular testosterone concentration is high in the early postnatal period [3–5], although the intracellular androgen receptor (iAR) expression is absent in these cells [4,6,7]. Thus, Sertoli cells cannot respond to the high testosterone concentration via classical mechanism at this age. In the late neonatal period, testosterone concentration decreases and remains low until puberty when the level of testosterone increases [3–5], as well as iAR expression in Sertoli cell [4,6,7]. It was found that testosterone through its binding to iAR suppresses proliferation at the end of the Sertoli cell proliferative phase and regulates the expression of markers associated with maturation of the Sertoli cell [8]. In adults, testosterone produced in testes greatly exceeds the required concentration for iAR binding in humans and rats (approximately 25–125-fold higher than that present in serum) [9]. The physiological importance of the high concentration of testosterone in the testis, especially in neonatal period when there is no iAR in Sertoli cell, is not fully understood. It may be due to its

Abbreviations: [^{14}C]-MeAIB, α-methylaminoisobutyric acid; ARKO, mice lacking iAR-receptors; ATF-1, activating transcription factor-1; FSHRKO, mice lacking FSH-receptors; iAR, intracellular androgen receptor; GPCR, G protein-coupled receptor; HBSS, Hank's Balanced Salt Solution; PBS, phosphate-buffered saline; RT, room temperature; SCs, Sertoli cells; K^+_{ATP}, ATP-sensitive K^+ channel; PIP_2, phosphatidylinositol 4,5 biphosphate; PLC, phospholipase C; Pnd, postnatal day; SCARKO, mice lacking iAR-receptors on Sertoli cells; VDCC, voltage-gated Ca^{2+} channels.

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action through a non-classical mechanism. Therefore, it is necessary to investigate the involvement of a non-classical action of testosterone and the relative contribution of this mechanism of action in the regulation of the testicular tissue development.

Epitestosterone (17alpha-hydroxy-4-androsten-3-one) is the natural 17α-epimer of testosterone. Testicular and adrenal tissues are male sources of epitestosterone [10,11]. As described by Havlíkova et al. [12] and reviewed by Stárka [10], the serum epitestosterone levels, in human, are slightly high than testosterone levels in childhood. Because the epitestosterone/testosterone ratio remains nearly constant in adulthood and epitestosterone concentration is not influenced by exogenous administration of testosterone, this steroid has been used as a natural internal standard for assessing testosterone abuse in sports [10]. Some studies have shown an antiandrogenic action of epitestosterone such as inhibition of 5α-reductase [13], a clear competitive inhibition of the iAR [14], and a decrease in the weight of tissues that increase in response to androgens [15]. However, epitestosterone was not able to inhibit testosterone-stimulated prostatic tumor growth [16].

The nuclear action of testosterone through iAR is known as the classical mechanism of this hormone. It involves dimerization of the hormone-receptor complex, translocation to the nucleus and binding to specific response elements causing DNA transcription and subsequent protein synthesis [17,18]. The non-classical mechanism of testosterone involves a cellular response to the steroid in seconds or in a few minutes. Several authors have been investigating the non-classical mechanism of testosterone action, even though the receptor for this action has not been identified [9,19–22].

Two non-classical signaling pathways have been described for androgens in Sertoli cells [9]. The first non-classical pathway causes the influx of Ca^{2+} through activation of an unidentified receptor. Testosterone (1 μM) stimulates rapid Ca^{2+} influx in several tissues. In osteoblasts, testosterone acts within 5 s to induce Ca^{2+} influx through voltage-gated calcium channels (VDCC) [23]. Cultured Sertoli cells from 17- to 20-day-old rats rapidly increase their intracellular Ca^{2+} in response to testosterone [24]. This steroid increases $^{45}Ca^{2+}$ uptake within 5 min of incubation in isolated Sertoli cells from immature (12- to 15-day-old rats) as well as from rats aged 20, 30, 45 and 60 days [25–27]. Testosterone (0.1–1 μM) induces rapid depolarization of Sertoli cell membranes from immature rats (aged 10–15 days) [25,26]. The testosterone-induced depolarization is produced by a closing of ATP-sensitive K^+ channels (K^+_{ATP}) and subsequent opening of L-type VDCC [26]. This effect seems to involve a membrane receptor that activates phospholipase C (PLC) once U-73122, a PLC inhibitor, blocks the testosterone effect on $^{45}Ca^{2+}$ uptake and in the membrane potential depolarization. The activation of PLC causes hydrolysis of phosphatidylinositol 4,5 biphosphate (PIP_2) [25]. The lack of PIP_2 reduces negative charges near membranes and produces closing of K^+_{ATP} channels [25,28]. These effects of testosterone in the membranes of Sertoli cells from immature rats were reproduced using nandrolone (1 μM), catechin (1 μM) and epitestosterone (0.5–2 μM) [29,30]. These Sertoli cell responses to androgens were specific because neither estradiol nor progesterone produced any effect on Sertoli cell membrane potential [26]. Epitestosterone (1 μM) as well as testosterone were able to stimulate $^{45}Ca^{2+}$ uptake within 5 min and induced depolarization of Sertoli cell membranes from rats aged 15, 21 and 35 days [30]. The effects of both steroids were not affected by flutamide, an iAR antagonist, suggesting that these non-classical actions occur via a receptor other than iAR [29,30]. The other androgen non-classical pathway seems to involve iAR localization near the plasma membrane. The binding of testosterone with iAR near the plasma membrane allows the receptor to interact with activated Src tyrosine kinase, as reviewed in Smith & Walker [9]. This action results in an increase of phosphorylation of ERK and CREB within 1 min [31].

In contrast, FSH inhibits testosterone-mediated ERK phosphorylation in isolated rat Sertoli cells [32], suggesting a crosstalk in the action of testosterone and FSH. Acting through its G protein coupled receptor (GPCR) exclusively expressed in Sertoli cells [33], FSH also produces changes in the membrane potential of Sertoli cells from immature rats. It causes a rapid hyperpolarization (seconds) followed by a prolonged depolarization (6 min) [34]. The hyperpolarization is blocked in the presence of tolbutamide (a K^+_{ATP} channel blocker) [35] and the depolarization is also blocked by verapamil, a VDCC blocker [34]. Because FSH and testosterone have antagonistic actions on K^+_{ATP} channels, it is also possible that crosstalk exists between the actions of these hormones.

The aim of this work is to investigate the non-classical effects of androgens in Sertoli cells from neonatal rat testes in the absence of iAR. In Sprague Dawley rats on postnatal day (pnd) 5, the presence of iAR in Sertoli cells can already be detected in a relatively weak manner. However, in Wistar rats the iAR ontogeny is controversial [36,37]. It was investigated if iAR is present in Sertoli cells of Wistar rats on pnd 3 and 4 and if testosterone and epitestosterone cause changes in the membrane potential and in $^{45}Ca^{2+}$ uptake. In addition, the influence of testosterone or epitestosterone on the FSH response in the membrane potential of Sertoli cells was investigated in rats aged 15 days. In this age, the FSH receptors are exclusively present in Sertoli cell membranes.

2. Material and methods

2.1. Animals

The experimental animals were male Wistar rats on pnd 3, 4 and 15 with the 24 h following the birth defined as pnd 1. The animals were housed under controlled conditions (approximately 24 °C and a 12 h light/dark cycle). A laboratory diet (Nuvilab, Nuvital CR1, Colombo, PR, Brazil) and water were available to the dams *ad libitum*. The suckling rats were kept with their dams until required for the experiments. The rats were sacrificed by cervical dislocation and the testes were immediately removed by abdominal incision, except for immunohistochemical staining as described below. The study and animal care procedures were reviewed and approved by the Ethics Committee for Animal Research at this University (Universidade Federal do Rio Grande do Sul – UFRGS, www.ufrgs.br, protocol number: 22635).

2.2. Chemicals and solutions used

Polyclonal rabbit anti-AR antibody (SC-816) was purchased from Santa Cruz Biotechnology, Inc. AR (Dallas, TX, USA). Anti-IgG (R2004), peroxidase anti-peroxidase soluble complex antibody (P1291), and 3,3′-diaminobenzidine tetrahydrochloride (D5637) were acquired from Sigma–Aldrich Co. (St. Louis, MO, USA). Normal goat serum was purchased from Merck KGaA® (Darmstadt, Germany). Hank's Balanced Salt Solution (HBSS) buffer contained: NaCl (145 mM), KCl (4.6 mM), $NaHCO_3$ (5 mM), $MgCl_2$ (1.6 mM), $CaCl_2{\cdot}2H_2O$ (1.6 mM), glucose (5 mM) and HEPES (10 mM) at pH 7.4. The lanthanum chloride ($LaCl_3$) buffer contained: NaCl (127.5 mM), KCl (4.6 mM), $MgCl_2$ (1.2 mM), glucose (5 mM), HEPES (10 mM) and $LaCl_3$ (10 mM), pH 7.4 at 0 °C. All reagents were purchased from E. Merck (Darmstadt, Alemanha), except HEPES and $LaCl_3$ that were acquired from Sigma–Aldrich Co. (St. Louis, MO, USA). Testosterone and epitestosterone were reconstituted in ethanol and diluted in HBSS buffer. The final solution concentration did not exceed 0.1% of ethanol. FSH was reconstituted in distilled water and diluted in HBSS buffer. Testosterone, epitestosterone and FSH were purchased from Sigma–Aldrich Co. (St. Louis, MO, USA). $^{45}Ca^{2+}$ (specific activity 444 GBq/g) was diluted in HBSS buffer at a concentration of 0.2 μCi/sample. The $^{45}Ca^{2+}$ was acquired

from PerkinElmer NEN® (Waltham, MA, USA). OptiPhase HiSafe 3® was purchased from Perkin Elmer, Inc. (Waltham, MA, USA). The drug concentrations for each experiment are reported in the figures and legends.

2.3. Immunohistochemical staining

Immunohistochemistry was used in order to determine if Sertoli cells between 3- and 4-day-old pups expressed iAR. Animals were anesthetized using ketamine (90 mg/kg body weight) and xylazine (10 mg/kg body weight). Under anesthesia, animals were perfused by intracardiac administration of saline followed by buffered 4% paraformaldehyde. Testes were quickly dissected out and immersed in the same fixative solution for 4 h at room temperature (RT). After fixation, tissues were dehydrated and embedded in paraffin wax following standard methods. Sections of 5 μm were obtained using a microtome (Micron), and they were mounted on glass slides. Sections were allowed to air dry before immunohistochemistry. For immunohistochemical demonstration of iAR, dewaxed sections were treated with 3% hydrogen peroxide in 10% methanol (30 min) and were then washed with phosphate-buffered saline (PBS). Antigen retrieval was performed by heating the sections (3 × 3 min) immersed in 0.01 M citrate buffer, pH 5.5 utilizing a microwave oven. Following retrieval, sections were washed in PBS and incubated for 30 min in 3% normal goat serum in PBS containing 0.2% Triton X-100 (PBS-T). Then, slices were incubated for 24 h at 4 °C with a polyclonal rabbit anti-AR antibody at 1:250. Subsequently, sections were incubated with a secondary antibody (anti-IgG,) diluted 1:100 in PBS-T for 1 h 30 min at RT. After washing, sections were incubated with a peroxidase anti-peroxidase soluble complex antibody (1:500) for 1 h 30 min at RT. The peroxidase reaction was developed using a solution of 3,3′-diaminobenzidine tetrahydrochloride (0.06%) and hydrogen peroxide (1 μL/mL) in PBS. Sections were counterstained with hematoxylin and images were acquired using a digital camera connected to a Nikon Eclipse E-600 microscope. Slices from the testes obtained from adult rats were processed together and used as a positive-control of the immunohistochemical procedure. When the primary antibody was omitted, the immunostaining was also abolished.

2.4. Electrophysiological experiments

In each experiment, whole testes from rats on pnd 4 or 15 were decapsulated and carefully stretched with two tweezers, exposing three to ten undisrupted seminiferous tubules.

Then, the seminiferous tubules were fixed to the bottom of a superfusion chamber and incubated with 1 mL/min HBSS buffer, pH 7.4, at 34 °C. A standard single microelectrode recording was performed according to the method described in von Lebedur et al. [26]. Briefly, the electrophysiological characteristics of neonatal Sertoli cells were analyzed using whole seminiferous tubules, without Leydig cells. As described by Escott et al. [38], the functional integrity of the tubular cells was preserved to maintain the environment as close as possible to its physiological conditions. The bulk of the recorded cells were characterized as Sertoli cells based on the following factors: microelectrode impalement depths; membrane potential stability and membrane potential range according to previously published data [28,30]. Microelectrode borosilicate pipettes were filled with KCl (3 M) and had a tip resistance of 15–25 MΩ. Our previous works showed this tip resistance is appropriate for the impalement of cells of similar size to Sertoli cells. Furthermore, this tip diameter helps to eliminate the impalement of slim cells such as peritubular cells [26]. Intracellular recording was amplified using an intracellular amplifier Intra 767 WPI (World Precision Instruments Inc., Sarasota, FL, USA). Square current pulses of 0.5 nA, 0.5 Hz and 250 ms duration were applied through the intracellular electrode to estimate membrane resistance using the S48 stimulator (Grass Instrument, West Warwick, RI, USA). An oscilloscope (Tektronix, 2 Channel Digital Oscilloscope TDS 210, Beaverton, OR, USA) and Wavestar Lite Version 1.0.10 software were used to record the variation in membrane potential. After the resting potential had stabilized for at least two min, testosterone (1 μM), epitestosterone (1 μM) and FSH (4 mU) were topically administered. Each treatment was repeated at least five times with different cells from different animals. The variations in membrane potential and membrane resistance (R0) were recorded. The results are presented as mean ± SEM.

2.5. $^{45}Ca^{2+}$ uptake experiments

The radioisotope $^{45}Ca^{2+}$ was used as a marker to investigate calcium uptake. Testes were removed and one gonad from each rat, alternately the left or right, was used as the experimental gonad; the contralateral gonad was used as the control. The testes ($n = 5$ in each group) were weighed, decapsulated and pre-incubated in HBSS with $^{45}Ca^{2+}$ (0.2 μCi/sample) for 60 min in a Dubnoff metabolic incubator to equilibrate intra- and extracellular $^{45}Ca^{2+}$ levels until they reached a plateau at 34 °C, pH 7.4.

Following the equilibrium, the gonads were incubated for 5 min in HBSS with $^{45}Ca^{2+}$, with or without testosterone (1 μM) or epitestosterone (1 μM). To end the experiment and stop calcium flux, 0.5 mL of $LaCl_3$ buffer was added to the samples. This cold (0 °C) solution was calcium free.

The supernatant was preserved, and the testes were removed into screw-cap tubes containing 0.5 mL of distilled water and stored at −20 °C for further analysis. The testes were frozen for 24 h and subsequently boiled. Aliquots of 100 μl were taken from each sample and placed in an OptiPhase HiSafe 3® for measurement of radioactivity using an LKB rack beta liquid scintillation spectrometer, model 1215 (LKB – Producer AB, Bromma, Sweden). The results are expressed as pmoles of $^{45}Ca^{2+}$/g tissue. The counting efficiency was 85–90%.

3. Results

3.1. iAR-immunoreactivity

Immunohistochemistry was used to evaluate iAR-immunoreactivity in the testes of Wistar rats during the postnatal period (pnd 3 and 4) and during adulthood (60-day-old rats). Adult iAR immunostaining was used as a positive control of the staining. Immunoreactivity was visualized in the seminiferous tubules of neonatal as well as in adult animals. In the seminiferous tubules of adult animals, iAR-immunoreactivity was observed in different types of cells, including Sertoli cells, characterized by triangular nuclei toward the basal membrane of the tubule, besides spindle-shaped peritubular cells and Leydig cells, localized in the interstitial space (Fig. 1C). The iAR-immunoreactivity in the seminiferous tubules of rats on pnd 3 is shown in Fig. 1A. In these animals, iAR immunostaining was observed in peritubular and Leydig cells. However, we were unable to observe any iAR-positive staining in Sertoli cells on pnd 3. This same pattern was observed in animals on pnd 4, which showed positive staining in peritubular and Leydig cells, with no observed staining in Sertoli cells (Fig. 1B). These results are indicative that Wistar strain pups do not present the iAR in Sertoli cells on pnd 3 and 4.

3.2. Effect of testosterone and epitestosterone on calcium uptake in testicular tissue from rats on pnd 3

To investigate the rapid non-classical action of testosterone and epitestosterone, testes from rats on pnd 3 were incubated with the

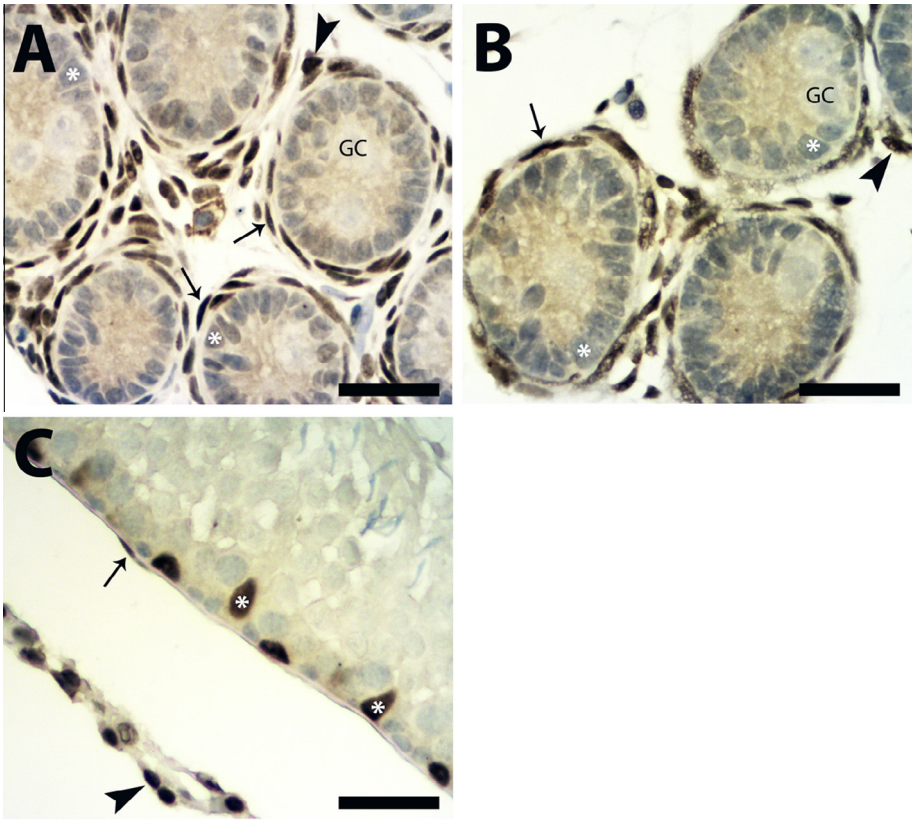

Fig. 1. Photomicrographs of transverse sections of seminiferous tubules comparing the iAR-positive staining pattern on pnd 3 (A), pnd 4 (B) and adult (C) Wistar rats. In adult animals (C), iAR was immunolocalized to peritubular cells (arrow), interstitial Leydig cells (arrowsheads) and tubular Sertoli cells (asterisks). In seminiferous tubules of pups on pnd 3 (A) and pnd 4 (B), no immunostaining was detected in Sertoli cells (asterisks), whereas staining was still observed in peritubular cells (arrow) and interstitial Leydig cells (arrowsheads). Some primordial germ cells are indicated by GC. Bars = 30 μm.

radioisotope $^{45}Ca^{2+}$ and with one of the hormones for 5 min. As expected, testosterone (1 μM) stimulated $^{45}Ca^{2+}$ uptake in the testicular tissue from rats on pnd 3 within 5 min of incubation (Fig. 2A). Similarly, epitestosterone (1 μM) stimulated $^{45}Ca^{2+}$ uptake (Fig. 2A) within 5 min. The stimulatory action of both hormones on calcium uptake was approximately 35% greater than control in rats on pnd 3 and pnd 15 (Fig. 2B).

3.3. Basal electrophysiological values of Sertoli cells in whole seminiferous tubules from rats on pnd 3 and 4

In our experimental conditions, the basal electrical characteristics of the registered Sertoli cells in whole seminiferous tubules from rats on pnd 3 and 4 were as follows: resting membrane potential −32.62 ± 2.32 mV ($n = 8$) and membrane resistance 16.25 ± 0.71 MΩ ($n = 8$). These conditions were stable for at least 5 min before hormone application.

3.4. Testosterone effect on the membrane potential of Sertoli cells in whole seminiferous tubules from rats on pnd 4

Membrane potential depolarization of Sertoli cells in whole seminiferous tubules from rats on pnd 4 was induced by testosterone (1 μM), ranging the basal potential from −32.8 to −28.2 mV (Fig. 3A). This response was significant at 180 s after testosterone application. Fig. 3B shows a typical Sertoli cell register during the administration of testosterone.

3.5. Testosterone and epitestosterone effect on FSH response on the membrane potential of Sertoli cells in whole seminiferous tubules from rats on pnd 15

To ensure that the effect of testosterone and epitestosterone was indeed on the membrane of Sertoli cells, FSH and testosterone or epitestosterone were applied simultaneously, and the effects on the membrane potential were recorded. FSH produces a biphasic effect on the membrane potential of Sertoli cells, a rapid hyperpolarization (30 s) followed by a depolarization (5 min) (Fig. 4A). Testosterone (1 μM) application 30 s after FSH (4 mU) causes prolonged hyperpolarization and reduced the depolarization (Fig. 4A). Epitestosterone (1 μM) application 30 s after FSH (4 mU) produced a similar effect to that observed for testosterone (Fig. 4B). Epitestosterone (1 μM) application at 5 min after FSH (4 mU) delayed the membrane depolarization in response to epitestosterone administration. Epitestosterone alone produced a significant depolarization at 120 s; however, at 5 min after FSH administration, the epitestosterone response was significant only at 300 s (data not shown).

4. Discussion

The present work reports evidence of the non-classical actions of testosterone and epitestosterone in Sertoli cells through a membrane mechanism that does not require iAR.

iAR-immunoreactivity was evaluated in the testes of Wistar rats during the postnatal period (pnd 3 and 4) and during adulthood (60-day-old rats, used as a positive control of the staining). According to the results, iAR immunostaining was observed in peritubular

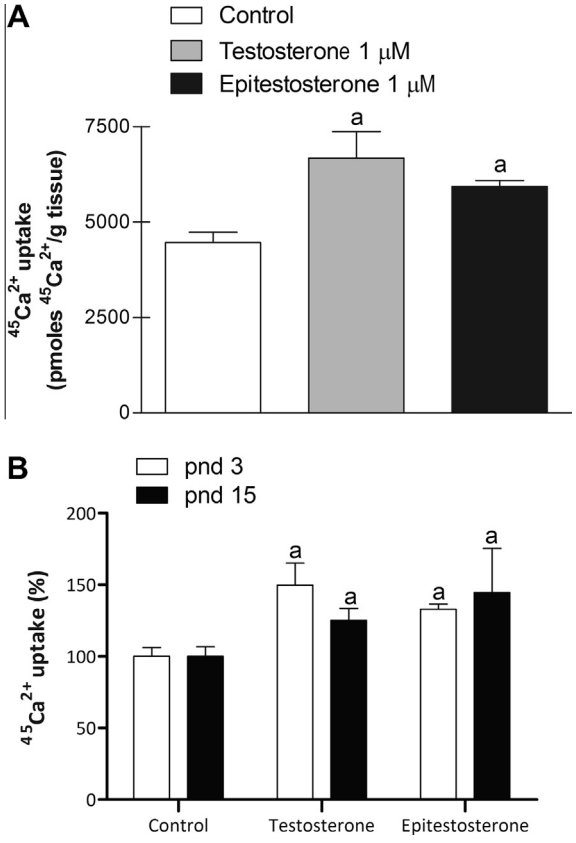

Fig. 2. Testosterone and epitestosterone affect the calcium uptake in whole testes from neonatal rats. (A) Effect of testosterone and epitestosterone on calcium uptake in whole testes from rats on pnd 3. One-way ANOVA, $^{a}p < 0.05$ compared with the control group; $n = 5$ in each group. (B) Effect of testosterone and epitestosterone on calcium uptake in whole testis from rats on pnd 3 and pnd 15 (in percent). Two-way ANOVA, $^{a}p < 0.05$ compared with the control.

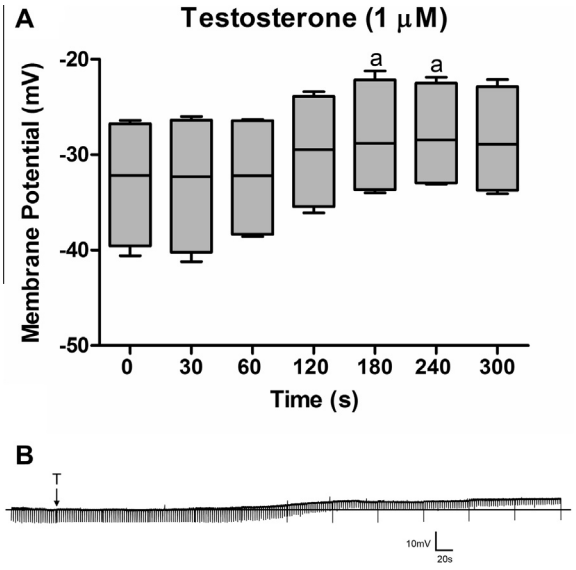

Fig. 3. Testosterone effect on the membrane potential of Sertoli cells from neonatal rats. (A) Testosterone (1 μM) effect on the membrane potential of Sertoli cells from rats on pnd 4. Repeated measure ANOVA, $^{a}p < 0.001$ compared with the resting potential; $n = 4$. (B) Recording of a typical Sertoli cell in whole seminiferous tubules from rats on pnd 4 after administration of testosterone. Vertical traces correspond to the membrane resistance.

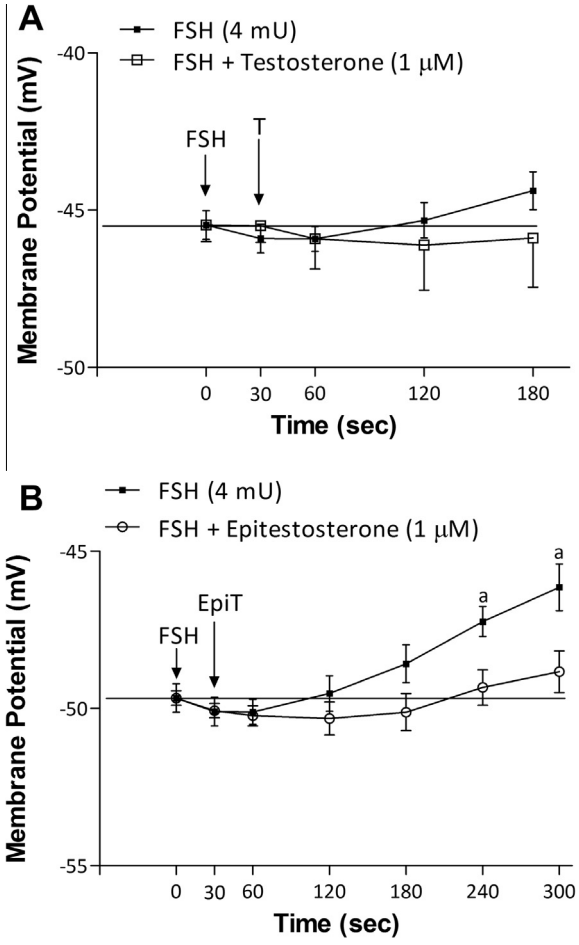

Fig. 4. Crosstalk between electrophysiological actions of testosterone, epitestosterone and FSH. (A) Crosstalk between electrophysiological actions of testosterone (1 μM) and FSH (4 mU) on the membrane potential of Sertoli cells from rats on pnd 15. Repeated measure ANOVA; $n = 7$. (B) Crosstalk between electrophysiological actions of epitestosterone (1 μM) and FSH (4 mU) on the membrane potential of Sertoli cells from rats on pnd 15. Repeated measure ANOVA; $^{a}p < 0.05$; $n = 6$.

and Leydig cells on pnd 3 and 4. However, immunohistochemical analysis showed no iAR-positive staining in Sertoli cells from rats on pnd 3 and 4 (Fig. 1A and B). These results strongly suggest that Wistar strain pups do not present the iAR in Sertoli cells at this age. Nevertheless, testosterone and epitestosterone have triggered an increase in $^{45}Ca^{2+}$ uptake within 5 min of incubation in testes of rats on pnd 3 (Fig. 2). The effect of testosterone increasing $^{45}Ca^{2+}$ is similar to that observed in isolated Sertoli cells from testes of 15-day-old rats [26] and whole testis of 15-, 20-, 30-, 45-, and 60-day-old rats [27,29]. This stimulatory action of testosterone in all ages tested was similar (approximately 35%). Epitestosterone also showed the same result in testes from 12- to 15-day-old rats [30]. In all these works, with different aged rats, an increase of $^{45}Ca^{2+}$ uptake was observed, in a very similar pattern of response using 5 min of incubation in the presence of testosterone or epitestosterone.

The electrophysiological technique is a useful tool for isolating the membrane effect of testosterone and epitestosterone. This technique was first developed for testes of 10- to 15-day-old rats [28]. In younger rats, Sertoli cells are predominantly localized along the basement membrane (Fig. 1A and B). The electrophysiological characteristics of these cells were analyzed using whole seminiferous tubules. Thus, the functional integrity of the tubular cells and of the gap junctions and paracrine factors was preserved;

the tubular environment was maintained as close as possible to its physiological conditions to achieve the registers as reviewed in Loss et al. [28]. The tip of the microelectrode does not stay on the surface of the tubule. Instead, it is introduced a few micrometers deeper in the tubule until reaching a steady negative membrane potential (normally it keeps more than 5 min without changing the resting potential value). Hence, considering the electrode insertion depth and the membrane potential stability we are sure we are not registering peritubular myoid cells [28,30]. However, in the testes of rats on pnd 3 or 4 it was very difficult to stabilize the resting potential. In the few cells that were obtained with rats on pnd 4, as observed in Fig. 3A and B, testosterone was administered and caused depolarization in a very similar pattern to that observed in whole seminiferous tubules from older animals [26,28,29].

These rapid responses of testosterone and epitestosterone described above were observed using whole seminiferous tubules from rats on pnd 3 and 4. At these ages, iAR seems to be present only in Leydig and peritubular cells, but not in Sertoli cells, as observed in Fig. 1A and B. These results indicated that iAR is not involved in this non-classical pathway – mediated by testosterone and epitestosterone on Sertoli cells. In recent study, Shihan and coworkers [39] demonstrated that iAR also do not participate in the testosterone-mediated non-classical activation of Erk1/2, CREB and ATF-1 (activating transcription factor-1) in spermatogenic cell line GC-2. The mechanism of the Ca^{2+} influx stimulated by testosterone may be involved in several physiological processes such as cytoskeleton rearrangement, secretion or even gene transcription [40].

Although little is known about epitestosterone biological function, its action through iAR suggests an antiandrogenic effect. However, we recently demonstrated that epitestosterone non-classical action shows a pattern very similar to testosterone [30]. Therefore, epitestosterone concentration is slightly higher than testosterone in childhood [12,41]. Thus, epitestosterone non-classical actions could modulate physiological process involved in Sertoli cell development.

In order to prove whether the non-classical response of testosterone and epitestosterone take place in the Sertoli cell membrane, electrophysiological experiments were performed studying the interaction of testosterone or epitestosterone with FSH.

FSH regulates several functions of Sertoli cells by acting through its transmembrane G protein-coupled receptor present exclusively in this cell [35,42]. Some of the effects of FSH occur very rapidly, within seconds to a few minutes, and they are well illustrated by the action of FSH on membrane potential changes and Ca^{2+} uptake through L-type VDCC in the Sertoli cells of immature rat testes [43]. In immature Wistar rats (12- to 15-day-old rats), FSH induces biphasic changes in the membrane potential of Sertoli cells: a very short hyperpolarization (30 s) followed by a prolonged depolarization (more than 6 min) [34,43]. The hyperpolarization is blocked by tolbutamide [35], which is a K^{+}_{ATP} channel-blocker. FSH-induced depolarization and Ca^{2+}-dependent sodium cotransport of α-methylaminoisobutyric acid ([^{14}C]-MeAIB) through system A are nullified by verapamil [34]. Therefore, FSH-induced depolarization and [^{14}C]-MeAIB transport are related to the uptake of Ca^{2+} through VDCC [34,43]. This FSH stimulus on [^{14}C]-MeAIB transport occurs in rats aged between pnd 10 and 20. In younger or older animals, FSH was ineffective [44].

The biphasic response of FSH on the membrane potential of Sertoli cells in 15-day-old rats was changed with the administration of testosterone and epitestosterone to the chamber 30 s after topical administration of FSH (Fig. 4A). In addition, the depolarizing testosterone (Fig. 4A) or epitestosterone (Fig. 4B) effects were completely inhibited when both were added 30 s after FSH. This administration was made during the hyperpolarizing phase of the response to FSH. The FSH hyperpolarizing response was blocked in the presence of tolbutamide, [35]. On the other hand, the testosterone effect is reproduced by glibenclamide or tolbutamide, both of which depolarize Sertoli cell membranes by closing K^{+}_{ATP} channels [26]. The depolarizing effect of testosterone was also blocked by diazoxide, an opener of this channel [26]. Hyperpolarization is the isolated effect of diazoxide in the membrane of Sertoli cells [26]. So, when testosterone or epitestosterone are added 30 s after FSH administration, there was an alteration of the FSH response and a block in the testosterone and epitestosterone response indicating that the steroids and the peptide hormone may act on a common pathway, probably by modulating the K^{+}_{ATP} channel current. Epitestosterone showed a delay in its response when added 5 min after FSH administration (data not shown). The epitestosterone administration was made during the end of the depolarizing phase of the FSH response. The FSH depolarizing phase, as observed previously, was blocked by verapamil, an L-type VDCC blocker [43]. The depolarizing effect of epitestosterone was only partially blocked by verapamil, indicating a current through another channel [30].

The evidence FSH and testosterone/epitestosterone affect each other responses on the membrane potential is very important, proving that the action of these steroids occurs in the membrane of Sertoli cells. It is known that FSH acts exclusively on Sertoli cells, once its receptor is expressed only in these cells [33]. Combined action of FSH and testosterone has been demonstrated to be essential for a full spermatogenesis in adult rats [45]. Even though testosterone action on peritubular cells is crucial for spermatogenesis, apparently androgen action on peritubular cells do not have any effects on Sertoli cells proliferation in immature rats [46].

In addition, the relative importance of FSH and testosterone classical action is usually tested using animals lacking specific hormone receptors, such as iAR (ARKO) and iAR specifically in Sertoli cells (SCARKO), and FSH receptor (FSHRKO), or even the combined action of both hormones using FSHRKO.SCARKO [45]. Nevertheless, all these works evaluate the testosterone effect considering only the classical iAR.

The results presented herein show the effects of testosterone and epitestosterone on Sertoli cell from pnd 3 and 4 pups, and the evidence that testosterone/epitestosterone and FSH affect each other responses on membrane potential of immature Sertoli cells. These results support the following hypothesis: (1) the non-classical effect of testosterone and epitestosterone on Ca^{2+} influx occurs in membranes of Sertoli cells; (2) this non-classical effect is through a membrane mechanism that is independent of iAR. The relative importance of this membrane mechanism must be considered and further evaluated. More studies regarding the physiological importance of androgen-mediated non-classical action in Sertoli cells are necessary.

5. Conclusion

Testosterone and epitestosterone stimulate non-classical actions via a membrane mechanism, which is independent of the iAR in Sertoli cells from neonatal rats. FSH and testosterone/epitestosterone affect the electrophysiological responses of each other, suggesting the presence of a crosstalk between their actions in Sertoli cells from immature rats.

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