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# Follicle-Stimulating Hormone Activates a cAMP-Dependent Chloride Conductance in TM4 Sertoli Cells

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**The effect of follicle-stimulating hormone (FSH) on the electrical properties of TM4-Sertoli cells was investigated. Addition of 5 IU/ml FSH caused a dose-dependent and reversible depolarization of the resting membrane potential by +15.3 ± 1.0 mV accompanied by a decrease of the input resistance. The depolarization was completely abolished in chloride-free solutions. The reversal potential of the effect was close to the calculated reversal potential for chloride. We conclude that FSH activates a chloride conductance in cultured TM4 Sertoli cells.** 

Sertoli cells, which are located in the seminiferous tubules of the testis, are involved in the development and maintenance of spermatogenesis as well as in the synthesis and release of proteins, among them inhibin and androgen binding protein. Further they provide nutritional and mechanical support for the germ cells [1]. Follicle-stimulating hormone (FSH), a pituitary glycoprotein hormone, is essential for normal sexual development and reproductive function. It elicits a number of biochemical changes in Sertoli cells of both, immature and adult animals [2]. The initial event is the interaction of FSH with its specific membrane receptor. The receptor-agonist complex is subsequently internalized and metabolized [3]. This reaction is required for the activation of the intracellular signaling pathways, including stimulation of the adenylate cyclase, which catalyzes the production of cyclic adenosine 3′- 5′monophosphate (cAMP) and finally the activating protein kinase A [2,4]. Although the FSH receptor is known to be similar in structure to the luteinizing hormone- (LH) and thyroid stimulating hormone- (TSH) receptors, [5] the FSH receptor is insensitive for these hormones [6].

Previous electrophysiological studies on primary cultures of Sertoli cells have shown, that FSH induces either a hyperpolarization of the resting membrane potential, most likely caused by a calcium-sensitive potassium conductance [7], or starting with a rapid and transient hyperpolarization followed by a depolarization, which could be blocked by verapamil [8]. These findings suggest the involvement of a calcium conductance in primary signal transduction. In order to avoid problems of primary cell cultures, which are known to provide less defined cell material and to further elucidate the primary actions of FSH on Sertoli cells in vitro, we investigated the electrophysiological effects of acute adminstration of FSH on the established Sertoli cell line TM4, which was isolated from an immature mouse testis.

## MATERIAL AND METHODS

*Cell culture.* The TM4 Sertoli cell line was obtained from American Type Culture Collection (ATTC, Rockville, MD). Cells were maintained in 1:1 mixture of Ham's F12 nutrient mixture and Dulbecco's modified Eagle's medium (DMEM), supplemented with gentamycin (20$\mu$g/ml), L-glutamine, 5%(v/v) horse serum,and 2.5% fetal calf serum in an atmosphere with 95% humidity and 5% CO2 at 37°C. For electrophysiological recordings cells were used from passage 15 to 32, plated on coverslips and incubated for 24 hours in serum free medium prior the experiment.

*Solutions for intracelluar recordings.* Extracellular perfusates were composed of (in mM): 114 NaCl; 5.4 KCl; 21 $NaHCO_3$; 1.2 $CaCl_2$; 0.8 $MgCl_2$; 1.2 $Na_2HPO_4$-$NaH_2PO_4$ (4:1, pH 7.4); 5.5 glucose. The solutions were equilibrated with 5% CO2 and 95% air (pH 7.4) and kept at 37°C. In calcium free solutions, $Ca^{2+}$ was replaced by 1mM EGTA. In potassium or chloride-free solutions, potassium was replaced by N-methyl-D-glucamin and chloride by gluconic acid, respectively.

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*Intracellular recordings.* Glass-microelectrodes were filled with 1M KCl and had a resistance of 50-100 M( and tip potentials of 5 mV. Signals were amplified and recorded with a high-impedance preamplifier as described previous in detail [9]. Recording times from single cells were between 5 and 20 minutes. For each series cells from at least five culture dishes were studied.

*Ca2+ imaging.* Cells were loaded with the Ca2+ sensitive fluorescent dye Fluo3-AM (Molecular Probes, Eugene, OR) in the incubator for 45 minutes, washed and examined with a confocal laser scanning microscope (MRC-600, Biorad) at room temperature. After addition of FSH, fluorescence was monitored up to 1 hour. Patch clamp recordings were done in the perforated patch mode using 240$\mu$g/ml Amphotericin B in the pipette solution. Solutions used in patch clamp experiments (in mM): bath solution 150 NaCl, 5.6 KCl, 2 $CaCl_2$, 2 $MgCl_2$, 10 glucose, 10 HEPES, ph7.3 ; pipette solution 55 KCl, 70 $K_2SO_4$, 7 $MgCl_2$, 5 glucose, 10 HEPES, pH 7.3. For data recording in voltage clamp mode pClamp6 software from Axon Instruments was used running on a pentium computer connected to a EPC-7 amplifier from List electronics (Darmstadt, BRD).

All chemicals used were from Sigma (St.Louis). FSH from porcine pituitary (Sigma, lot #128F0521) was used in all experiments. Contamination with other hormones (e.g. TSH, LH, etc.) were below the detection range of the respective assays. All values are given as mean ±SEM (Standard error of mean).

## RESULTS

The average resting membrane potential of TM4 Sertoli cells was −27.7 ± 0.7 mV and measured a membrane input resistance of 37.5 ± 2.5 M (n = 84) in intracellular recordings. Application of 5 IU/ml FSH to the bathing solution caused a depolarization of +15.3 ± 1.0 mV (n=40), accompanied by a reduction of the cell input resistance by −4 ± 0.6 MΩ within 20 sec. In TM4 cells the depolarization was permanent in the presence of FSH (Fig.1). The half maximal effective concentration was calculated from a dose response

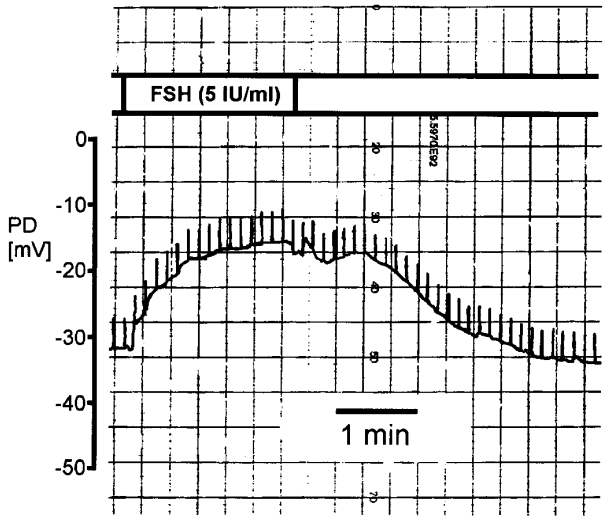


**FIG. 1.** Original tracing of an current clamp experiment. The effect of FSH (5 IU FSH /ml) is shown on the membrane potential (PD= potential difference).

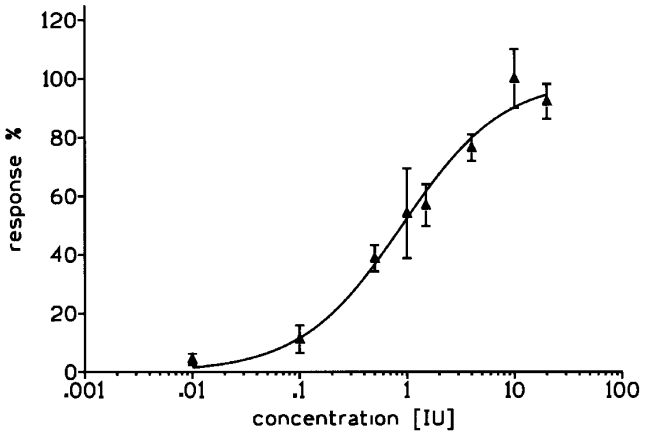


**FIG. 2.** Dose response curve of FSH measured in current clamp mode: The effect of FSH is given as the percentage of the single effect at different concentrations in regard to the maximal effect measured at 10IU.

curve (Fig.2), fitted to a sigmoid according to the equation $Y = 1/[1+(K_D/C)h]$ (were Y represents the normalized response, C=concentration of FSH, h=Hill coefficient, $K_D$=half-maximal concentration) with a $K_D$ of 0.90 IU/ml and a Hill coefficient of 0.923, which is close to 1 indicating a one to one agonist-receptor interaction. The effects of FSH were completely reversible after wash out within 2-3 minutes. In the following experiments a dose of 5 IU/ml FSH was applied.

Omission of chloride from the perfusing solution abolished the specific FSH response of the membrane potential almost completely + 0.1 ± 0.1 mV (n = 8). In voltage clamp experiments an increase of current was observed after the application of FSH (Fig.3). In the perforated patch clamp studies, the reversal potential of the involved current was found to be −24.1 ± 3.5 mV (n=5), which is close to the theoretical reversal potential for chloride as calculated by the Nernst Equation given the concentrations mentioned above (−21.4mV). The calcium channel blocker verapamil in high concentrations of up to 1 mM had no effect on the FSH induced depolarization in TM4 Sertoli cells. Similar results were obtained with the sodium channel blocker amiloride (1mM), the potassium channel blocker barium (2 mM) or ouabain (1mM).

In experiments utilizing the calcium imaging technique with fluo-3 as calcium indicator, no calcium increase due to the application of FSH could be detected (data not shown). Forskolin, an activator of the adenylate cyclase, depolarized the cell membrane in a dose-dependent manner (+12.8 ±1.3 mV at 1mM forskolin, n=12) (Fig.4). In chloride-free perfusing solutions the administration of forskolin had no depolarizing effect (+0.8±1.3 mV; n = 6) (Fig.4). Application of forskolin and the phosphodiesterase blocker IBMX (1mM) in the same experiment had no significant additive effect on the membrane potential and depolarized the cell mem-

brane by +15.6 ± 1.4 mV (n = 8) (Fig. 4). Application of FSH at this point had no additional depolarizing effect (+13.9 ± 0.6 mV, n= 8) (Fig.4). Perfusion of TM4 cells with 100$\mu$M 8-(4-chlorophenythio)-cAMP, a membrane permeable cAMP analog, caused a slow and sustaining depolarization of the Sertoli cell membrane, but to a smaller extent than FSH (+6.6 ± 1.25mV, n=6).

## DISCUSSION

Our data demonstrate that FSH induced a stable and reversible depolarization of the cell membrane in cultured TM4 Sertoli cells. Full reversibility of membrane resting potential was assumed as an indicator for vital cells. This effect was dose dependent and elicited the maximal depolarization within 20 seconds. It has been shown in many previous studies that FSH increases the intracellular cAMP concentration as a second messenger mechanism [1,2,4,10,11]. The increase of cAMP seems to be the initial reaction to the depolarizing effect as indicated by the experiments with forskolin, an activator of the adenylate cyclase increasing the intracellular cAMP concentration [12],

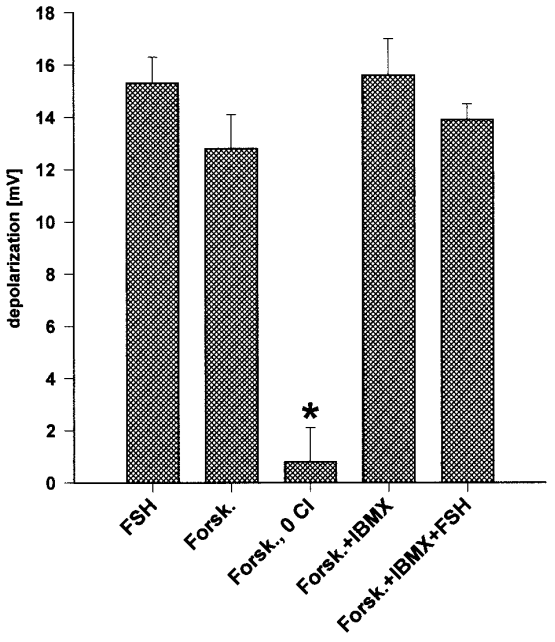


**FIG. 4.** Depolarizing effect of 5 IU/ml FSH added to the bathing solution under various conditions: FSH = control, 0 Cl = chloride free solution, Forsk. = forskolin, IBMX = IBMX.

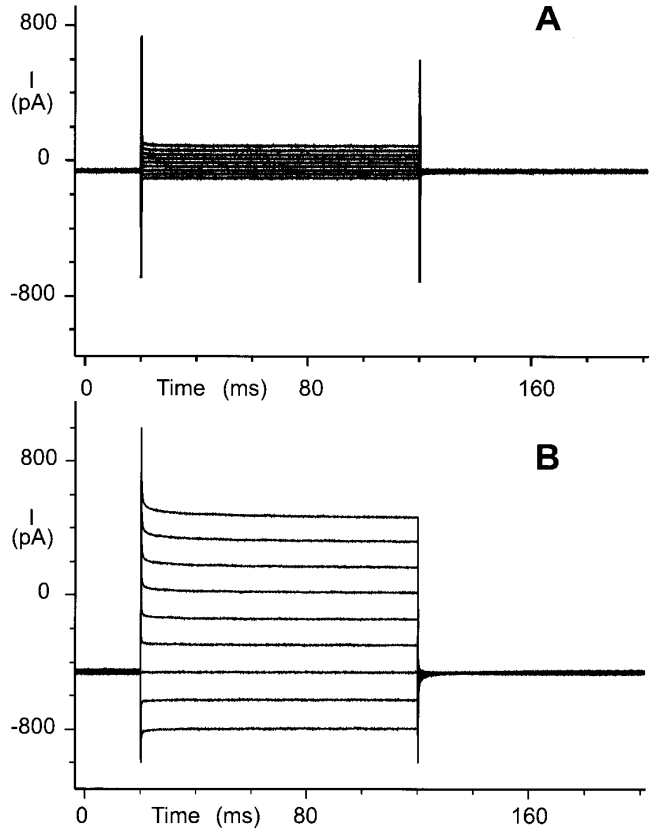


**FIG. 3.** Voltage clamp experiment. A= control conditions, B=after application of 5 IU/ml FSH (holding potential = −60mV, Voltage jumps were from −80mV to 0mV in steps of 10mV) Note the increase of current.

IBMX and membrane permeable cAMP analogs. In our experiments Forskolin mimicked the FSH effect on TM4 cells in respect to the time-course as well as the depolarization of the cell membrane. The depolarizing effect of both, forskolin and FSH was annihilated in the absence of chloride from the perfusing solution, pointing to a similar electrophysiological mechanism for both agents. Electrophysiological studies of the action of FSH on primary culture of Sertoli cells have been published previously [7]. Joffre and Roche [7] observed a hyperpolarization in a dose dependent manner up to −37 mV after incubation of Sertoli cells with FSH. However, in this study FSH was added for 24 to 72 hours. The mechanism of action was described as a cAMP dependent increase of the $Na^+/K^+$ pump and a $Na^+/Ca2^+$ influx through an ionic process further leading to an efflux of $K^+$, through a quinine sensitive $K^+$ channel. In our experiments TM4 Sertoli cells were acutely exposed to FSH and the rapid changes of the cell membrane potential were measured after the application of FSH. The maximal effect was observed within 20 seconds and the depolarizing effect was sustained over a long period of time (up to 30 minutes). Neither changes in the extracellular $K^+$ concentration, nor $K^+$ channel blocks or a block of the $Na^+/K^+$ ATP-ase by ouabain were effective to block the fast depolarizing effect of FSH in TM4 Sertoli cells. These findings suggest that a potassium driven mechanism might not be involved in the primary FSH effect on TM4 cells.

Wassermann et al. [8] described a biphasic effect on the resting membrane potential on Sertoli enriched cell

cultures from rat testes after topical application of FSH. A rapid onset (< 3 s) of a transient hyperpolarization induced by the application of FSH was assumed as the activation of an ATP- dependent potassium channel. The hyperpolarization was followed by a sustained depolarization in these experiments. The depolarizing effect was completely inhibited by the calcium channel blocker verapamil. The authors concluded that a voltage dependent calcium channel might be activated by FSH, which finally activates sodium channels via a second messenger system and together with the stimulation of a sodium amino acid cotransport system might elicit the depolarizing effect of this hormone. However, no data about the reversal potentials of the involved currents were reported, therefore it is uncertain, whether a calcium conductance or a calcium-sensitive chloride conductance is activated by FSH. In contrast we found that in TM4 Sertoli cells verapamil was ineffective to block the depolarizing effect of FSH and in addition the data obtained using the confocal laser microscopy support our observation that no cytosolic calcium increase is involved in the FSH response of TM4 cells.

Omission of chloride from the perfusate did not change the resting membrane potential under control conditions, suggesting that chloride channels contribute only little to the resting membrane potential. However, the FSH and forskolin induced depolarization was completely inhibited in chloride free solutions. In addition the reversal potential of the conductance is near to the calculated reversal potential of chloride.

In conclusion FSH is able to activate a chloride conductance in TM4 cells. The chloride conductance is most likely triggered by an increase of intracellular cAMP which is known to occur after FSH stimulation in Sertoli cells.

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