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Modulation of stimulatory action of follicle stimulating hormone (FSH) and inhibitory action of epidermal growth factor (EGF) on aromatase activity in Sertoli cells by calcium

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Aromatization of testosterone by cultured Sertoli cells isolated from immature rats was stimulated more than 7-fold by follicle stimulating hormone (FSH) or dcAMP. The effects of FSH and dcAMP could be partly inhibited by epidermal growth factor (EGF) in a dose-dependent manner (ID_{50} 0.5 nM). The phorbol ester 4β-phorbol-12-myristate-13-acetate (PMA) could also inhibit aromatase activity in a fashion similar to EGF. When 3 mM EGTA was present in the culture medium, the inhibitory effect of EGF was abolished but the stimulatory effect of FSH or dcAMP was magnified. These results suggest that EGF exerts a negative control on aromatase via calcium and protein kinase C. The abolishment of the inhibitory effect of EGF and the enhancement of the stimulatory effect of FSH or dcAMP by a calcium deficiency may be an indication that growth factors produced by Sertoli cells negatively control FSH-induced responses in an autocrine fashion.

Aromatization; EGF; Ca^{2+}; Phorbol ester; (Sertoli cell)

1. INTRODUCTION

The Sertoli cells in immature rats are an important source of intratesticular oestradiol [1]. Follicle stimulating hormone (FSH) stimulates the aromatization of testosterone probably via cyclic adenosine monophosphate (cAMP). After addition of serum to Sertoli cells this stimulatory effect of FSH on oestradiol production was inhibited [3]. In a previous paper we have shown that EGF may be one of the active principles in serum responsible for this inhibitory effect since EGF inhibits the FSH-stimulated aromatization in Sertoli cells from immature rats whereas insulin had no effect [4].

The inhibition of FSH-stimulated aromatization by EGF occurred in parallel with the stimulation of lactate production [4]. It is unknown which intracellular mediators are involved in this specific inhibitory effect of EGF on at least one of the many functions of Sertoli cells [5].

It has been shown that addition of EGF or foetal calf serum (FCS) to fibroblasts caused a rise in the cytoplasmic levels of Ca^{2+}, whereas insulin did not modify these levels. Therefore, it was suggested that a rise in the intracellular calcium could function as a mediator of the growth factor signal [6].

It has also been shown that addition of a phorbol ester [7], or a synthetic diacylglycerol [8] to cultured granulosa cells inhibited the production of oestradiol stimulated by FSH. Since both substances are known activators of protein kinase C, which is also calcium-dependent [9], the observed effects of EGF on aromatase activity in Sertoli cells may be (partly) mediated by changes in

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intracellular calcium and/or activation of protein kinase C. Therefore, we have studied the effect of EGF, the phorbol ester phorbol myristate acetate (PMA) and different extracellular calcium concentrations on the FSH- or dcAMP-stimulated aromatase activity in Sertoli cells.

2. MATERIALS AND METHODS

EGF was a generous gift from Dr Rolando Perez (Instituto Nacional de Oncologia y Radiobiologia, Habana) and was obtained from mouse submaxillary glands as described [10]. Eagle's minimal essential medium (MEM), foetal calf serum (FCS) and a solution of non-essential amino acids were purchased from Gibco, Europe. Testosterone, dibutyryl adenosine 3′,5′-monophosphate (dbc-AMP), 3-isobutyl-1-methylxanthine (IBMX), 4β-phorbol-12-myristate-13-acetate (PMA), EGTA, Hepes, deoxyribonuclease and collagenase were obtained from Sigma, London. FSH-NIH-S12 was a gift from the Endocrinology Study Section of the National Institute of Health (Bethesda, MD, USA).

2.1. *Isolation and culture of Sertoli cells*

Sertoli cells were isolated as described by Oonk et al. [11], with slight modifications as reported earlier [4]. Briefly, decapsulated testes from 16-day-old Wistar rats were dispersed twice in phosphate-buffered saline (PBS), containing 0.5 mg/ml collagenase and 20 μg/ml deoxyribonuclease, both times for 30 min at 37°C in a shaking water bath at approx. 80 cycles per min. The suspension of tubular fragments obtained, was washed several times in MEM supplemented with 20 mM Hepes, 1% FCS and antibiotics, until the supernatant was completely clear. The tubular

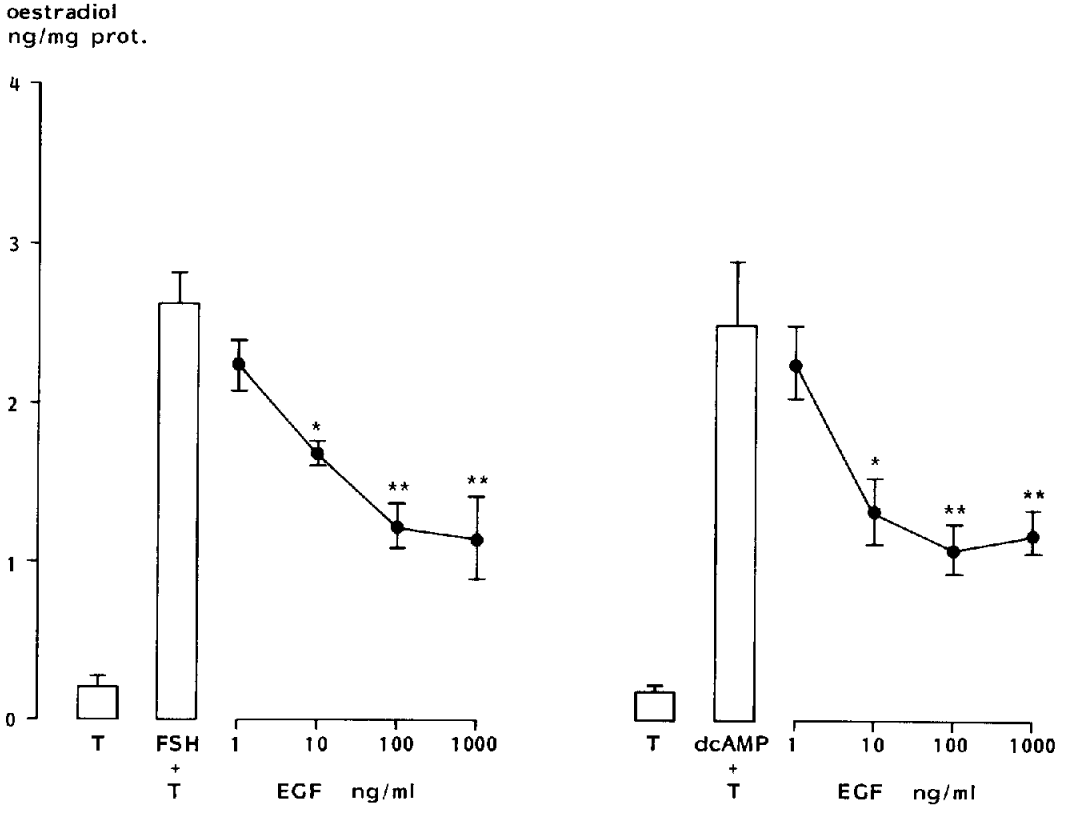

Fig.1. Dose-dependent inhibition by EGF of FSH- and dcAMP-stimulated testosterone aromatization by cultured rat Sertoli cells. Cells were isolated and cultured as described in section 2. On day 5 fresh medium was added containing 200 ng/ml testosterone (T) and either FSH (1 μg/ml) or dcAMP (0.2 mM) together with the indicated concentrations of EGF. Incubations were terminated after 24 h. Each point represents the mean ± SD of 3 different cell preparations.

fragments were resuspended in the same culture medium and plated in 6-well culture dishes (Costar) at an approximate density of 600 μg of cellular protein per well. The cultures were maintained at 37°C in a water vapour saturated atmosphere of 5% CO_2 and after 24 h of culture the dishes were washed and MEM without FCS was added. Washings and renewal of medium were repeated on days 3 and 4 of the culture period. After 4–5 days the cultures were confluent monolayers and consisted of more than 95% Sertoli cells as evaluated by phase contrast microscopy. On day 5 the experiments were started by addition of the appropriate substances. When media-containing EGTA was used the pH was adjusted to 7.4 by addition of NaOH. The ATP content of the cells was measured by the procedure described by Lundin et al. [12]. In experiments where effects of EGTA, or PMA were determined, culture media were collected after various incubation periods and kept at −20°C until assay for oestradiol. 1 M NaOH was added to the dishes in order to dissolve the cells.

2.2. *Measurement of oestradiol and protein*

Oestradiol concentration in culture media was determined following the method of Sufi et al. [13], with the exception that the ether extraction step was omitted after it had been demonstrated that this step had no effect on oestradiol levels measured. For estimation of the protein content of the cells, the procedure of Lowry et al. [14] was used. Experiments were repeated with at least 3 different cell preparations. Student's *t*-test was used to assess the statistical significance of the observed effects.

3. RESULTS

The addition of EGF to Sertoli cell cultures inhibited the stimulated oestradiol production in a dose-dependent way (fig.1). This effect was observed when aromatization was either stimulated by FSH or dcAMP and the amount of EGF required for the half maximal suppression was approximately the same in both cases (0.9 and 0.5 nM EGF for FSH- and dcAMP-stimulated activity). The aromatase activity was only suppressed to 50% of the original activity at the highest concentrations of EGF.

The inhibitory effect of EGF does not seem to be a result of increased phosphodiesterase activity since addition of the phosphodiesterase inhibitor IBMX had no effect on the actions of EGF (table 1). Addition of EGTA to the culture media further increased the FSH- or dcAMP-stimulated oestradiol production, whereas in the presence of

Table 1

Effect of IBMX on testosterone aromatization by cultured Sertoli cells

Additions	Oestradiol (ng/mg protein)
Test + FSH	2.03 ± 0.30
Test + FSH + IBMX	2.20 ± 0.17
Test + FSH + EGF	1.13 ± 0.15[a]
Test + FSH + EGF + IBMX	1.16 ± 0.05[a]

[a] Significantly different ($p < 0.01$) from cells incubated with testosterone and FSH

Cells were isolated and cultured as described in section 2. On day 5 the indicated substances were added to the cultures to give a final concentration as follows: testosterone (200 ng/ml), FSH (1 μg/ml), EGF (1 μg/ml), IBMX (0.2 mM). The incubations were terminated after 24 h. Values are the mean ± SD of triplicate cultures

Table 2

Effect of EGF in the presence of different concentrations of extracellular calcium on FSH- or dcAMP-stimulated aromatization of added testosterone by cultured Sertoli cells

Additions	Oestradiol (ng/mg protein)	
	FSH (1 μg/ml)	dcAMP (0.2 mM)
–	2.58 ± 0.07	3.31 ± 0.20
EGTA (3 mM)	5.83 ± 0.49[a]	7.57 ± 0.83[a]
EGTA and $CaCl_2$ (5 mM)	3.02 ± 0.31	3.46 ± 0.37
EGF (1 μg/ml)	1.09 ± 0.01[a]	1.50 ± 0.21[a]
EGF and EGTA	5.17 ± 0.46[a]	7.20 ± 0.57
EGF, 1 EGTA and $CaCl_2$	2.43 ± 0.25	–

[a] Significantly different ($p < 0.001$) from cells incubated with testosterone and FSH or dcAMP

For incubation conditions see table 1. Mean values ± SD from 3 different cell preparations are shown

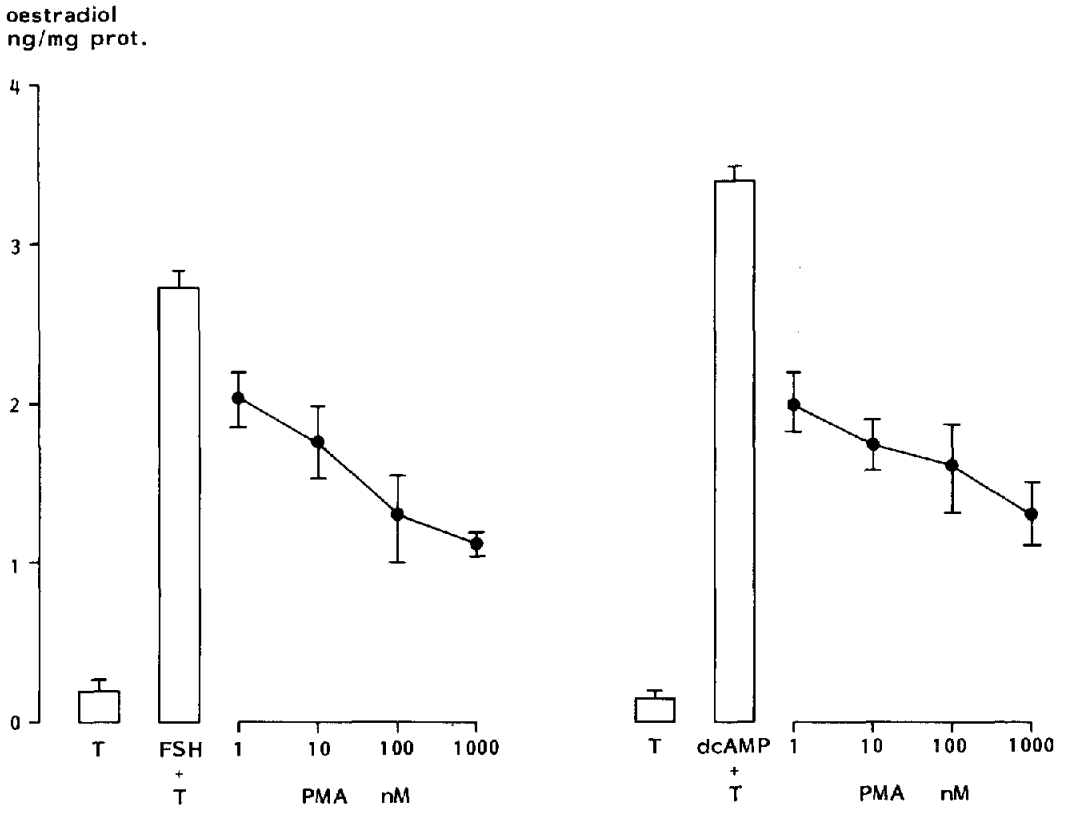

Fig.2. Dose-dependent inhibitory effect of phorbol ester PMA on FSH- and dcAMP-stimulated aromatization of testosterone. Sertoli cells were cultured for 4 days without hormones. On day 5 fresh medium was added containing 200 ng/ml testosterone (T) and either FSH (1 μg/ml) or dcAMP (0.2 mM) together with different concentrations of PMA. Incubations were terminated after 24 h. Each point represents the mean ± SD of 3 different cell preparations. Significantly different (* $p < 0.01$, ** $p < 0.001$) from cells incubated without PMA.

EGTA EGF did not exert any inhibitory effects (table 2). These stimulating effects of EGTA were abolished after addition of $CaCl_2$ (table 2) and could not be shown in the absence of FSH or dcAMP (not shown). When Sertoli cells were incubated in the presence of different doses of the phorbol ester PMA, the FSH- and the dcAMP-stimulated oestradiol production was inhibited in a similar fashion to that with EGF (fig.2). The inhibitory effect of PMA was most probably not caused by cell damage since ATP levels in Sertoli cells incubated with PMA were not different from controls (not shown).

4. DISCUSSION

The results presented in this study show that aromatase activity in Sertoli cells, stimulated by FSH or dcAMP can be partly inhibited by EGF and that these hormonal effects can be modulated by extracellular calcium. The mechanisms involved in these calcium-dependent EGF effects on Sertoli cell function and the possible physiological implications are still unknown, although inhibitory effects of EGF on steroidogenesis in Leydig cells [15], ovarian interstitial cells [16] and granulosa cells [17] have been reported earlier.

Studies on the primary effects of EGF carried out with fibroblasts and A431 cells have shown that EGF causes a transient increase in intracellular calcium levels provided that normal extracellular calcium concentrations are present [6,18]. For EGF action on Sertoli cells similar mechanisms may operate. An important argument

in favour of this hypothesis is that inhibitory effects of EGF could not be demonstrated when extracellular calcium was low. This presumed EGF-induced increase in intracellular calcium could activate a specific calcium-dependent phosphodiesterase [19] or enhance protein kinase C activity.

The first possibility seems unlikely for two reasons: (i) the phosphodiesterase inhibitor IBMX could not reduce the inhibitory effects of EGF and (ii) EGF can inhibit aromatase activity stimulated by an excess of dcAMP.

On the other hand the similarities between the inhibitory effects of EGF and the phorbol ester PMA and the observed effects of manipulations with extracellular calcium reinforce the hypothesis that EGF may activate protein kinase C in Sertoli cells (in part) via regulation of intracellular calcium. This suggestion is further supported by the observation that phorbol esters and EGF can inhibit oestrogen production in granulosa cells [8,16].

Due to the limited number of experiments carried out with gonadal cells, it is unknown which mechanisms may play a role in protein kinase C-mediated regulation of oestradiol production in gonadal cells. However, in KB cells calcium and protein kinase C can reduce the affinity of the EGF receptor for EGF, probably via receptor phosphorylation [20,21]. It has been reported that Sertoli cells can secrete a growth factor that can block binding of EGF to EGF receptors [22]. Therefore it may be possible that under in vitro conditions, and perhaps in vivo as well, this Sertoli cell growth factor can negatively control, in an autocrine fashion, the FSH-induced aromatase activity via protein kinase C and calcium. This model could explain why the stimulation of aromatase by FSH or dcAMP is more pronounced in the absence of calcium. This hypothesis can be tested further by studies on the binding of EGF to Sertoli cell receptors under the influence of EGF with or without calcium present.

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