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Androgenic Modulation of Cyclic Adenosine Monophosphate (cAMP)-Dependent Meiotic Arrest

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ABSTRACT

This study investigated further our previous finding that testosterone synergizes with dibutyryl cyclic AMP (dbcAMP) to arrest the meiosis of a very high proportion of cumulus-enclosed (intact) pig oocytes in vitro, suggesting that estradiol may have mediated in this synergism. The effects of androgens [testosterone (T), 0.5 μM; dihydrotestosterone (DHT), 0.5 μM], estradiol (E_2), 0.5 μM, gonadotropins [NIH follicle-stimulating hormone (FSH), 0.1 to 10 μg/ml; highly purified (hp) FSH, 0.1 μg/ml; luteinizing hormone (hpLH), 30 ng/ml], and dbcAMP, 1 mM, on the maturation of liberated intact or cumulus-free (denuded) oocytes were investigated. In one series of experiments, intact oocytes were cultured in medium containing T with NIH-FSH (10 μg/ml) with or without the steroid biosynthesis inhibitor, cyanoketone (0.7×10^{-7} M). After culture for 6 to 48 h, oocytes were air dried for cytogenetic analysis, the meiotic stage was scored and in some cases spent media were analyzed by radioimmunoassay for E_2 and progesterone (P_4) content.

After 24 h of culture, FSH and dbcAMP similarly and significantly arrested meiosis of intact oocytes (45% and 43% matured, respectively), but the arresting actions of these compounds were modulated quite differently by androgen; that of dbcAMP was significantly enhanced by T, while that of FSH was significantly depressed by both T and DHT. Nevertheless, FSH with T transiently maintained meiotic arrest since a very high proportion of oocytes remained at the germinal vesicle (GV) stage after 12 h of culture, compared with controls (95% and 45% at GV stage, respectively). No difference was detected between impure NIH-FSH and hpFSH relative to the meiotic progression of intact oocytes and hpLH with T did not duplicate the arresting actions of either FSH preparation. This transient arresting action of FSH with T was mediated by the adherent cumulus cells since the maturation of denuded oocytes was not affected by FSH, T or FSH with T. While substitution of T with E_2 did not duplicate this cumulus cell-mediated androgenic effect, the modulations exerted by DHT on either dbcAMP- or FSH-induced meiotic arrest were not as marked as those exerted by T. Furthermore, analysis of E_2 and P_4 in the spent media of intact oocytes cultured for 6 or 12 h in the presence of FSH with T revealed positive correlations between the proportion of immature oocytes and the E_2:P_4 ratio in the media. Compared with controls, meiotic arrest was maintained for up to 24 h in medium containing cyanoketone with T and FSH, and positive correlations were established between the proportion of GV oocytes and the E_2:P_4 ratio in the spent media.

These results indicate that androgens have the capacity to modulate cyclic AMP-dependent meiotic arrest in vitro and suggest that the expression of this modulation may be regulated by the aromatase system and/or the E_2:P_4 status of the adherent cumulus cells. These findings are considered in light of the two currently popular hypotheses regarding the mechanisms which control meiotic maturation.

INTRODUCTION

Although it is well established that exposure of the mature antral follicle to LH results in the reinitiation of meiosis (Ayalon et al., 1972; Lindner et al., 1974), the mechanisms which mediate this gonadotropic action have not been elucidated (reviewed by Tsafriri, 1979). It is known, however, that while the concentrations in follicular fluid of both estradiol (E_2) and progesterone (P_4) temporarily decline after binding of LH to its receptor protein, the synthesis of P_4 subsequently increases markedly (Thibault, 1977; Eiler and Nalbandov, 1977; Dorrington and Armstrong, 1980). Indeed, it has been reported that in the immature porcine follicle, the ratio of E_2:P_4 is between 1 and 2, but by 18 h after the beginning of estrus this ratio falls to 0.15 (Gérard et al., 1979; Ainsworth et al., 1980). Thus, the concentration of P_4 relative to E_2 increases in follicular fluid approximately at the time of onset of oocyte

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maturation (Thibault, 1977; Gerard et al., 1979).

In addition to documentation of these steroidal changes which occur within the follicular environment of the maturing oocyte, it is also established that intact immature oocytes (i.e., oocytes with adherent cumulus cells) will spontaneously mature when removed from this environment (reviewed by Tsafriri, 1979). This observation has led to general acceptance of the hypothesis that some component of the follicle synthesizes a maturation inhibitor, the effect of which is overridden by LH. The fact that meiotic resumption by oocytes cultured within their intact follicles only occurs if gonadotropins are added to the medium (Tsafriri et al., 1972; Hillensjö et al., 1976) supports this hypothesis. A follicular peptide of low molecular weight (Stone et al., 1978), estradiol (Richter and McGaughey, 1979), and cyclic AMP (Cho et al., 1974; Dekel and Beers, 1978, 1980), each have been suggested as inhibitory factors. Although contradictory data have been presented for the peptide and steroid inhibitors (reviewed by Racowsky and McGaughey, 1982a,b), agreement does exist for an inhibitory role for cyclic AMP; spontaneous maturation of cumulus-free oocytes has been consistently prevented by the cyclic nucleotide derivative $N^6,0^{2'}$-dibutyryl adenosine 3′,5′-cyclic monophosphoric acid (dbcAMP) (Cho et al., 1974; Magnusson and Hillensjö, 1977; Dekel and Beers, 1978, 1980; Rice and McGaughey, 1981), and by phosphodiesterase inhibitors (Cho et al., 1974; Magnusson and Hillensjö, 1977; Dekel et al., 1981).

The oolemma appears not to possess an active regulatory site for adenylate cyclase (Dekel and Beers, 1978), and it has been proposed that meiotic arrest is maintained by cyclic AMP derived from the adherent cumulus cells and transported to the ooplasm via functional coupling between the oocyte and the somatic cells (Dekel and Beers, 1978, 1980). Although both morphological (Szöllösi, 1975; Amsterdam et al., 1976; Anderson and Albertini, 1976) and biochemical (Gilula et al., 1978; Moor et al., 1981) studies indicate that such coupling exists between these heterologous cells, the mechanism(s) which regulates this proposed transport of cyclic AMP remains to be elucidated. In addition, it has yet to be demonstrated that meiotic arrest is maintained by a direct action of the nucleotide, rather than by some cyclic AMP-dependent factor. Indeed, one report has indicated that FSH stimulates the activity of a granulosa cell peptide which has inhibitory properties (Anderson and Stone, 1980).

The results of a recent study with testosterone and dbcAMP suggest that E_2 may be implicated in the regulation of cyclic AMP-dependent meiotic arrest (Rice and McGaughey, 1981). In that study, it was shown that testosterone synergizes with dbcAMP to arrest the maturation of a very high proportion of intact oocytes. Since the stimulation of aromatase activity by FSH (LaCroix et al., 1974; Moon et al., 1975; Armstrong et al., 1979) is mediated by cyclic AMP (Lindsey and Channing, 1979; Anderson et al., 1979), and since dbcAMP was demonstrated to exert a direct arresting action on the oocyte, these results suggest that E_2 may mediate in the arresting action of the nucleotide. In the experiments presented here, not only has this possibility been examined, but the inducer, FSH, has been substituted for the derivatized mediator of FSH action, dbcAMP, to provide a more physiological system with which to investigate the mechanisms which regulate meiotic maturation.

MATERIALS AND METHODS

Collection of Oocytes

Reproductive tracts were obtained from a local abattoir within a few minutes of slaughter. The ovaries from 4 or more gilts were used in an individual experiment. In experiments with two groups (control and experimental), selected oocytes from one ovary served as the controls for experimental oocytes from the contralateral ovary. For experiments with four groups, the ovaries from each tract were halved, and each half ovary was used to provide oocytes for one treatment group. Oocytes were collected from medium-sized (3–5 mm diameter) follicles, by vigorously puncturing the ovary with an 18-gauge needle, into BMOC-3 medium (Brinster, 1972) modified to contain dextran T-70 (4.5 mg/ml; Pharmacia) and reduced osmolarity as described previously (McGaughey, 1977a). Only those oocytes with an intact, adherent cumulus mass (intact oocytes) were selected for subsequent experiments. Denuded (cumulus-free) oocytes were obtained by mechanically removing cumulus cells from intact oocytes with a small-bore pipet (Hillensjö et al., 1975; McGaughey and Van Blerkom, 1977). All oocytes were washed successively three times in the appropriate medium before culture. The collection of oocytes and preparation of cultures routinely were completed within 1.5 to 2 h.

Culture Procedures

The oocytes were cultured for up to 24 h in wells of Lab-Tek chamber slides (no. 4838) in 0.25 ml medium under 5% CO_2 in humidified air in a desiccator

placed in an incubator at 37°C. The complex medium of Tsafriri and Channing (1975) was used. This medium (TCM) consisted of Medium 199 with Earle's salts and 12 mM Hepes buffer supplemented with 15% pig serum, 1 mM glutamine, 2.5 mM lactate, 0.03 mM pyruvate, 12.5 mU insulin/ml and 50 μg gentamicin/ml. Stock solutions (X1000) of insulin and sodium pyruvate were prepared in Medium 199, divided into appropriate aliquots and stored frozen at −20°C. The serum was likewise stored in suitable aliquots. Media were made up routinely the evening before an experiment. Prepared TCM medium was supplemented with T (Schwartz/Mann), DHT (Sigma) or E_2 (Sigma), and dbcAMP (1mM; Sigma), impure FSH (NIH-FSH-P1 having a potency 0.76 X NIH-FSH-S1 and LH potency 0.0075 X NIH-LH-S1), highly purified FSH (hpFSH, lot G4-211B) (obtained from Dr. Harold Papkoff, having a potency 75–125 X NIH-FSH-S1 and LH potency <0.0001 X NIH-LH-S1) or highly purified LH (hpLH, lot G3-268DA) (obtained from Dr. Harold Papkoff, having a potency 2.3 X NIH-LH-S1 and devoid of FSH). In one series of experiments, the steroid biosynthetic inhibitor cyanoketone (2-cyano-4,4,17tri-methylandrost-5-en-17-0l-3-one) (Goldman et al., 1965) was added to medium at a concentration of 0.7×10^{-7} M Stock solutions of each compound were prepared: dbcAMP, 1 M; NIH-FSH, 10 mg/ml; hpFSH, 100 μg/ml; and hpLH, 30 μg/ml, were each prepared in water and stored in aliquots at −20°C. Steroids (0.5×10^{-3} M) and cyanoketone (0.7×10^{-4} M) were prepared in absolute ethanol and stored at 4°C. Medium containing the appropriate concentration of dbcAMP or FSH was prepared, sterilized through a Millipore filter (0.45 μM) and an appropriate volume of steroid and/or cyanoketone stock was carefully added aseptically. Steroid-containing media were never filtered since Millipore filters avidly bind steroids. The concentration of ethanol never exceeded 2 μl/ml. An equivalent volume of ethanol was added to media which did not contain steroid. All media were equilibrated to culture conditions for at least 2 h before use. Groups of 10–15 oocytes were cultured in each well. Within an experiment at least two replicates were provided for each treatment, and each experiment was normally repeated at least 3 times. Except where stated otherwise, oocytes were cultured for 24 h. After culture, oocytes were denuded, air dried (McGaughey and Polge, 1971), and stained with 0.29% (w/v) Wright's stain in 2.9% (v/v) glycerol and 97.1% methanol. Oocyte maturation was scored as described by McGaughey (1977b, 1978).

Analytical Methods

Meiotic maturation of examined oocytes was classified as follows: *1*) those remaining at the germinal vesicle (GV) stage (immature), and *2*) those developing beyond the GV stage (diakinesis to metaphase II). The second classification was subdivided into *i*) the proportion of oocytes which reached metaphase I, and *ii*) the proportion of oocytes that were beyond metaphase I. For each culture well, the proportions of oocytes in each of the above classifications were transformed to angles and analyzed by Duncan's multiple range test using the SPSS computer program (Nie et al., 1975; McGaughey, 1978). Significant differences among experimental and control groups were approximated at alpha=0.05. Group means and their 95% confidence intervals were transformed back to percentages for tabulation. Duncan's multiple range test was used because, in comparison to other statistical tests, it employs decreasing significant differences and protection levels based on degrees of freedom (Duncan, 1955).

Estimation of Steroid Synthesis

Aliquots of medium were assayed for E_2 and P_4 content without extraction, using validated assay systems. Volumes of medium ranged from 25 to 100 μl for the E_2 assay and from 10 to 100 μl for the P_4 assay. The accuracy of direct assay was assessed by comparing values obtained for aliquots from the same samples before and after ether extraction. The correlation coefficients for the two methods were 0.99 ($P<0.001$; $Y=0.91x + 0.0003$) and 0.99 ($P<0.001$; $Y=0.88X + 0.005$) for the E_2 and P_4 assays, respectively. The antibodies were obtained from Dr. Gordon Niswender [#244 anti-E_2-6-BSA serum: thoroughly characterized by Korenman et al. (1974); #337 anti-P_4-11-BSA serum: thoroughly characterized by Gibori et al. (1977)]. The minimum amounts of steroid that could be distinguished from medium blanks were 5 pg and 12.5 pg for the E_2 and P_4 assays, respectively; however, the limits of sensitivity were set at 10 pg and 25 pg, for the E_2 and P_4 assays, respectively, since it was at these concentrations that the curves became linear. The within and between assay coefficients of variation, derived from 11 E_2 assays were, respectively, 5.3% and 10.7% as calculated from the standards, and 3.8% and 10.1% as determined from quality control samples run with each assay. The within and between assay coefficients of variation, derived from 10 P_4 assays were, respectively, 5.2% and 7.4% as calculated from the standards, and 4.3% and 10.2% as calculated from quality control samples run with each assay.

RESULTS

The first series of experiments was carried out to determine the effects of T or DHT with FSH, as compared with dbcAMP, on the maturation of intact oocytes. Oocytes were cultured in the presence or absence of dbcAMP (1 mM) or NIH-FSH (10 μg/ml), with or without T (0.5 μM) or DHT (0.5 μM). Neither androgen, with or without FSH, caused a significant decrease in the proportion of maturing oocytes compared with the maturation of oocytes cultured in unsupplemented TCM (Fig. 1). In the presence of dbcAMP, however, both androgens suppressed the proportion of maturing oocytes, the suppressive action of T being significant. There was no significant difference between the proportion of maturing oocytes cultured in the presence of dbcAMP as compared with FSH, although the maturation of both groups of oocytes was suppressed compared with that of oocytes cultured in

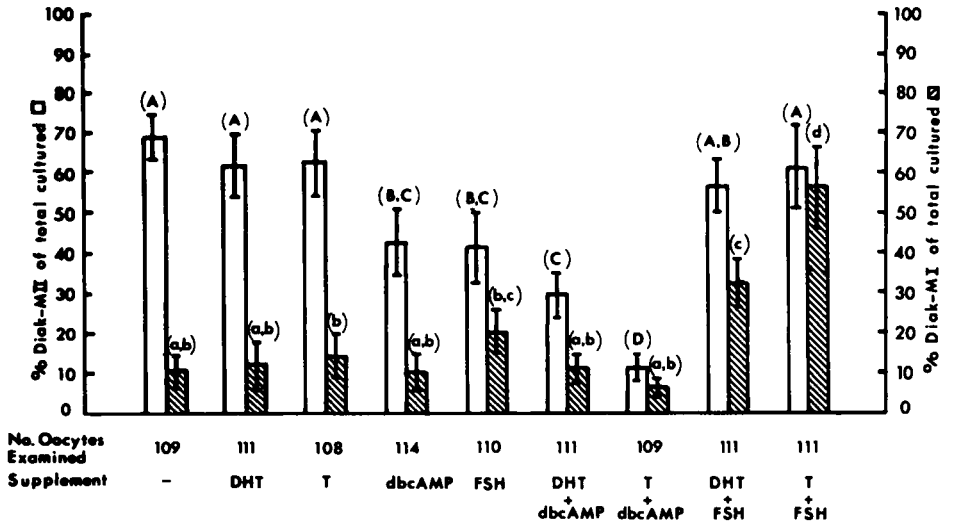

FIG. 1. Effects of androgens, dbcAMP and FSH on the maturation of intact oocytes during 24-h culture. Each histogram represents the mean ± SEM of nine replicate cultures. The letters in parentheses represent the subsets to which the data were assigned by Duncan's multiple range test analysis. Different letters indicate statistically significant differences at α=0.05.

unsupplemented TCM. There were no significant differences among the proportions of oocytes that had not progressed beyond metaphase I in the groups maintained in unsupplemented medium or in medium containing either of the androgens or dbcAMP alone, or dbcAMP with T or DHT. Compared with these groups, however, a significantly greater proportion of oocytes cultured in medium containing FSH alone had not progressed beyond metaphase I; this proportion was significantly greater in those oocytes cultured in the presence of FSH with DHT, and most marked when the gonadotropin was present with T. After 48 h of culture,

TABLE 1. The effect of E_2 with FSH or dbcAMP on the maturation of intact oocytes cultured for 24 h.

Medium supplement	No. oocytes	Mean % oocytes which matured	
		Category I[a] diakinesis-MII	Category II[b] diakinesis-MI
None[c]	66	63.3* (57.7–69.5)	11.8† (2.4–31.1)
dbcAMP[d]	60	40.8** (33.7–48.0)	18.8† (5.3–40.4)
FSH[e]	63	48.9** (32.2–53.0)	20.3† (8.8–41.9)
E_2[f]	69	55.0* (42.0–67.2)	13.6† (2.6–33.2)
E_2 + dbcAMP	72	38.5** (22.8–46.3)	7.7† (2.7–16.0)
E_2 + FSH	75	44.3** (30.9–53.8)	24.3† (11.4–38.0)

[a]Percent of oocytes cultured.

[b]Percent of oocytes in Category I.

[c]Medium containing 1 μl ethanol/ml.

[d]*dbcAMP*; 1 mM.

[e]*FSH*=NIH-FSH, 10 μg/ml.

[f]E_2=estradiol, 0.5 μM.

*,**,†Figures in parentheses are the 95% confidence intervals for the means. Groups with different symbols within each category are significantly different. α=0.05.

however, there was no significant difference between the proportion of oocytes that had progressed beyond metaphase I when cultured in the presence of T and FSH compared with that of oocytes cultured in unsupplemented TCM (90% and 88%, respectively).

The demonstrated effects of T with either dbcAMP or FSH on the meiotic maturation of intact oocytes cultured for 24 h could not be duplicated by substitution of T with E_2 (0.5 μM; Table 1). Furthermore, the meiotic progression of denuded oocytes cultured for 24 h in medium containing T, FSH, or T with FSH was not significantly different from that of oocytes maintained in unsupplemented medium (Table 2).

To investigate further the observed effect of T with FSH on the maturation of intact oocytes, oocytes were cultured for 24 h in medium containing a constant concentration of FSH (hpFSH, 0.1 μg/ml) and varying concentrations of T (0 to 0.5 μM), or a constant concentration of T (0.5 μM) and varying concentrations of hpFSH (0 to 3.2 μg/ml). While the proportion of oocytes that had not progressed beyond metaphase I was unaffected by the concentration of T present in the medium, the proportion of matured oocytes increased with increasing concentrations of androgen (Fig. 2A). In contrast, however, there were no significant differences among the proportions of oocytes maturing in medium containing varying concentrations of FSH, although there was a significant dose-dependency revealed in the

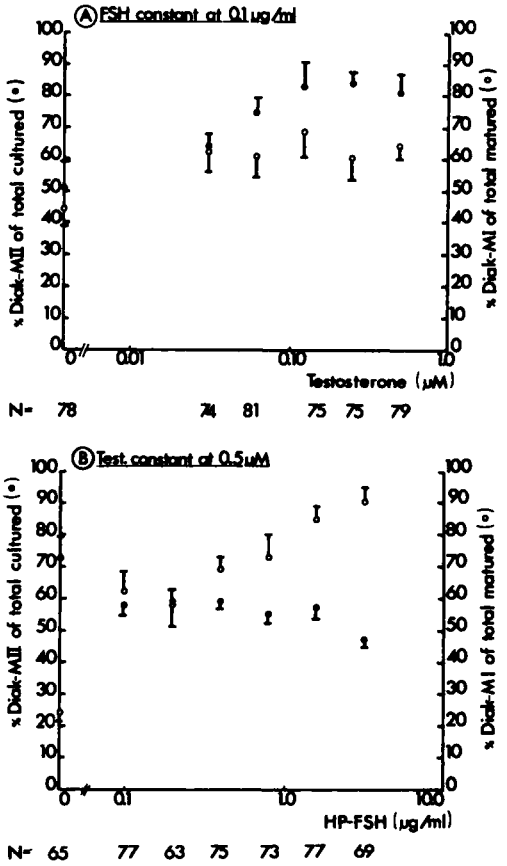

FIG. 2. Dose-response relationships between meiotic maturation of oocytes cultured for 24 h and either testosterone (*A*) or highly purified FSH (*B*). The data are presented as the mean ± SEM of six replicate cultures. *N*=number of oocytes examined.

TABLE 2. The effect of testosterone and FSH on the maturation of denuded oocytes cultured for 24 h.

Medium supplement	No. of oocytes	Mean % oocytes which matured: Category I[a] diakinesis-MII (%)	Mean % oocytes which matured: Category II[b] A1-MII (%)
None[c]	48	73.4* (55.2–88.2)	73.4†,†† (53.6–84.5)
T[d]	43	66.5* (48.2–70.9)	68.8†† (56.2–80.2)
FSH[e]	45	69.3* (49.4–82.4)	82.0† (60.0–95.1)
T + FSH	45	66.9* (41.3–75.8)	85.6† (60.0–95.1)

[a]Percent of oocytes cultured.

[b]Percent of oocytes in Category I.

[c]Medium containing 1 μl ethanol/ml.

[d]*T*=testosterone, 0.5 μM.

[e]*FSH*=NIH-FSH, 10 μg/ml.

*,†,††Figures in parentheses are the 95% confidence intervals for the means. Groups with different symbols within each category are significantly different, α=0.05.

proportions of oocytes which had not progressed beyond metaphase I (Fig. 2B).

A series of time experiments was carried out to determine the basis for the effect on meiotic progression of intact oocytes cultured in medium containing T and FSH. Oocytes were cultured for 6, 12 or 24 h in unsupplemented medium or in medium containing T and FSH. After 6 h, there was no significant difference between either group in the proportion of oocytes at the GV stage (Fig. 3A). After 12 h, however, maturation had been initiated in a significant proportion of oocytes cultured in control medium while it remained completely arrested in the experimental group. After an additional 12 h of culture, however, there was no significant difference between the control and experimental groups in the proportion of oocytes that had matured. There were no significant differences in the proportion of matured oocytes that had reached metaphase I among either group after 6 or 12 h culture, or in the experimental group after 24 h (Fig. 3B); significantly fewer matured oocytes in the control group, however, were at metaphase I after 24 h.

Analysis of the concentrations of E_2 and P_4 in the spent culture media (Figs. 4C and D) revealed that during the 24-h culture, compared with the control groups, the cumulus cell exposed to T and FSH secreted significantly more E_2 during each time period (6 h, $P<0.01$; 12 h, $P<0.01$; 24 h, $P<0.01$; paired t test; Fig. 4C) and between 0 and 12 h, this secretion was proportional to the time of culture. In contrast, P_4 secretion by the cumulus cell exposed to T and FSH was not increased compared with the control values after either 6 or 12 h, but after 24 h had evidenced a tremendous stimulation ($P=0.001$; paired t test) (Fig. 4D).

Comparisons between the proportions of GV stage oocytes in each of the groups that were maintained in the T and FSH system with the ratio of E_2:P_4 in the appropriate spent media (Figs. 4A and B, respectiveiy) indicated that when the E_2:P_4 ratio was high, so was the proportion of oocytes at the GV stage (i.e., after either 6 or 12 h of culture). Linear regression analysis indicated a significant correlation between the proportion of immature oocytes and the E_2:P_4 ratio in the T and FSH medium at 6, 12 and 24 h (Table 3). These correlation coefficients were positive at 6 and 12 h, and negative at 24 h; however, no significant correlations were established between the

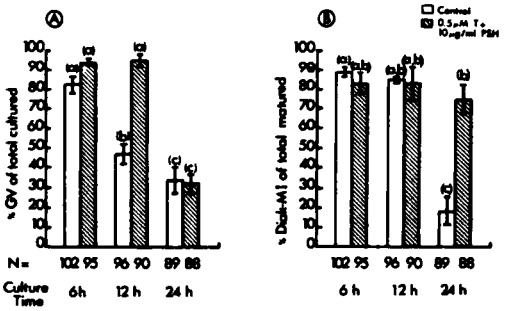

FIG. 3. Effect of FSH and testosterone on the progression of meiotic maturation during 24-h culture. Each histogram represents the mean ± SEM of eight replicate cultures. The letters in parentheses represent the subsets to which the data were assigned by Duncan's multiple range test analysis. Different letters indicate statistical significant differences at $\alpha=0.05$. N=number of oocytes examined.

proportion of immature oocytes and the E_2:P_4 ratio in the medium for any of the control groups.

To determine whether the contaminating LH in the NIH-FSH preparation was responsible for either the increased synthesis of P_4 or the release from meiotic arrest after 12 h, oocytes were cultured for 12 to 20 h in TCM containing

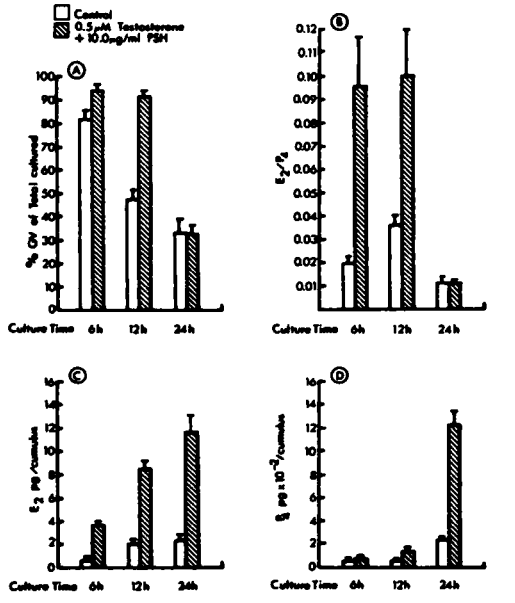

FIG. 4. Effect of FSH and testosterone on meiotic maturation and cumulus cell steroid secretion during 24 h culture. Each histogram represents the mean ± SEM of eight replicate cultures.

TABLE 3. The relationship between arrest of meiosis and the estradiol to progesterone ratio in the medium.

Group	Time cultured (h)	Mean % oocytes[a] at GV stage ± SEM	Mean E_2/P_4[b] in medium ± SEM	Regression equation	Correl. coeff.	*P* value
Control[c]	6	81.09 ± 4.17	0.020 ± 0.003	y= 323.7x + 63.2[d]	r= 0.09	NS[e]
	12	47.36 ± 4.76	0.036 ± 0.005	y= −446.3x + 63.6	r= 0.51	NS
	24	33.00 ± 6.15	0.011 ± 0.003	y= −428.7x + 37.5	r=−0.20	NS
T + FSH[f]	6	94.65 ± 2.24	0.096 ± 0.023	y= 63.15x + 100.7	r= 0.65	<0.05
	12	91.79 ± 3.47	0.100 ± 0.025	y= 88.92x + 82.9	r= 0.63	<0.05
	24	32.91 ± 4.26	0.012 ± 0.001	y= −3655x + 76.77	r=−0.76	<0.01

[a]Percent of total oocytes cultured.

[b]E_2=estradiol, pg/cumulus; P_4=progesterone, pg/cumulus.

[c]Medium containing 1 μl ethanol/ml.

[d]*y*=% GV; *x*=E_2/P_4 in medium.

[e]*NS*=not significant.

[f]*T*=testosterone, 0.5 μM; *FSH*=follicle-stimulating hormone, 10 μg/ml.

T alone or with impure or highly purified FSH. In addition, oocytes were cultured for 24 h in medium containing T and each of the FSH preparations, highly purified LH, or highly purified FSH with LH.

There was no significant difference in the release from arrest and progression of maturation between those oocytes cultured in medium containing impure and highly purified FSH (Fig. 5). Furthermore, there were no significant differences among any of the groups in the proportion of oocytes which had been released from meiotic arrest (*Category I*, Table 4). In the absence of any gonadotropin, however, significantly more matured oocytes had progressed beyond metaphase I than in any of the other groups, although the presence of LH alone resulted in a significantly increased proportion of oocytes beyond metaphase I as compared with that of either of the FSH preparations (Category II). Compared with medium containing only T, the P_4 content was significantly greater for each of the media containing FSH ($P<0.001$; paired t test) but was not significantly increased in medium containing LH. There was no significant difference in the stimulatory effect of impure FSH as compared with hpFSH, and supplementation of hpFSH with hpLH did not significantly enhance the secretion of P_4 above that observed in medium supplemented only with hpFSH.

To assess the role of cumulus cell P_4 secretion in the reinitiation of meiosis, intact oocytes were cultured for 12 or 24 h in unsupplemented TCM, in TCM supplemented with cyanoketone (0.7×10^{-7} M), T and FSH, or T and FSH with cyanoketone (0.7×10^{-7} M) (Fig. 6). After 12 h, maturation had been initiated in a significant proportion of those oocytes cultured in either TCM or TCM with cyanoketone, while it remained arrested in those oocytes maintained in T and FSH with or without the drug. After an additional 12 h, however, meiotic arrest was maintained in a significant proportion of oocytes cultured in medium containing T and FSH with cyanoketone, but was released in the group of oocytes cultured in the presence of T and FSH alone (Fig. 6A).

Compared with the control groups (i.e., groups cultured in TCM with or without cyanoketone), the cumulus cell exposed to T and FSH secreted significantly more E_2 during each time period (12 h, $P<0.01$; 24 h, $P<0.01$; paired t test: TCM vs. T + FSH; TCM + cyanoketone vs. T + FSH + cyanoketone). After 24 h, cyanoketone significantly inhibited E_2 secretion by the cumulus cells maintained in T and FSH compared with medium containing testosterone and FSH alone ($P<0.01$; paired t test; Fig. 6C). There were no significant differences in P_4 secretion by cumulus cells after 12 h of culture among any of the groups. After 24 h, however, cyanoketone significantly inhibited secretion of this steroid both in medium supplemented only with the drug and in medium supplemented also with T and FSH (Fig. 6D). Comparison, by linear regression analysis, of the proportions of GV stage oocytes in each of the groups maintained in medium containing T and FSH, with or without cyanoketone, with the ratio of E_2:P_4 in the appropriate spent medium (Figs. 6A and B, respectively), indicated a significant correlation between the proportion of immature oocytes and the E_2:P_4 ratio in the T and FSH system, with or without cyanoketone, at both 12 h and 24 h; the correlation coefficients were positive at 12 h for groups cultured with or without cyanoketone and at 24 h for that group maintained with cyanoketone, and negative at 24 h for the group cultured in medium containing T and FSH without the inhibitor.

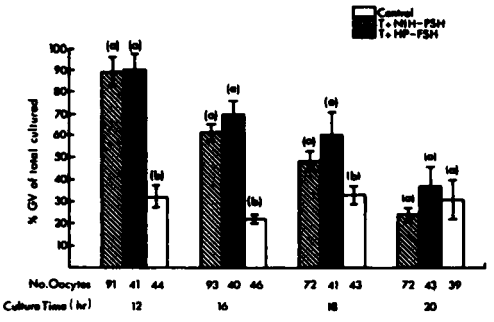

FIG. 5. Effects of impure and highly purified FSH with testosterone on the maturation of intact oocytes between 12 and 20 h of culture. Oocytes were cultured in unsupplemented medium (*open bar*), or in medium supplemented with testosterone (0.5 μM) and either NIH-FSH (10 μg/ml) (*batched bar*) or HP-FSH (0.1 μg/ml) (stippled bar). Each histogram represents the mean ± SEM of six replicate cultures. The letters in parentheses represent the subsets to which the data were assigned by Duncan's multiple range test analysis. Different letters indicate statistical significant differences at $\alpha=0.05$.

DISCUSSION

The results reported in this paper confirm and extend those of our previous investigation

TABLE 4. The effect of T + gonadotropins on the maturation of intact oocytes and the P_4 secretory capacity of cumulus cells (n=6).

Supplement	No. of oocytes	Mean % oocytes which matured: Category I[a] diakinesis–MII		Category II[b] diakinesis-MI		P_4 (pg/cumulus) (Mean ± SEM)
T[c]	85	69.9*	(64.9–74.7)	19.8†	(8.2–25.7)	298.4 ± 62.9
T + NIH-FSH-PI[d]	87	69.3*	(59.3–78.5)	70.5†††	(59.0–83.6)	1075.0 ± 85.8
T + hpFSH[e]	90	77.3*	(62.1–89.4)	78.2†††	(69.5–91.0)	1086.7 ± 93.2
T + hpLH[f]	88	73.2*	(60.4–84.2)	49.7††	(29.1–70.3)	310.3 ± 78.2
T + hpFSH + hpLH[g]	88	70.4*	(58.3–79.0)	71.6†††	(61.9–81.7)	1281.4 ± 111.3

[a]Percent of oocytes cultured.

[b]Percent of oocytes in Category I.

[c]0.5 μM testosterone added to each medium in 1 μl ethanol/ml.

[d]10 μg NIH-FSH-PI/ml.

[e]0.1 μg hpFSH/ml.

[f]30.0 ng hpLH/ml.

[g]0.1 μg hpFSH/ml + 30 ng hpLH/ml.

*,†,††,†††Figures in parentheses are the 95% confidence intervals for the means. Groups with different symbols within each category are significantly different, α=0.05.

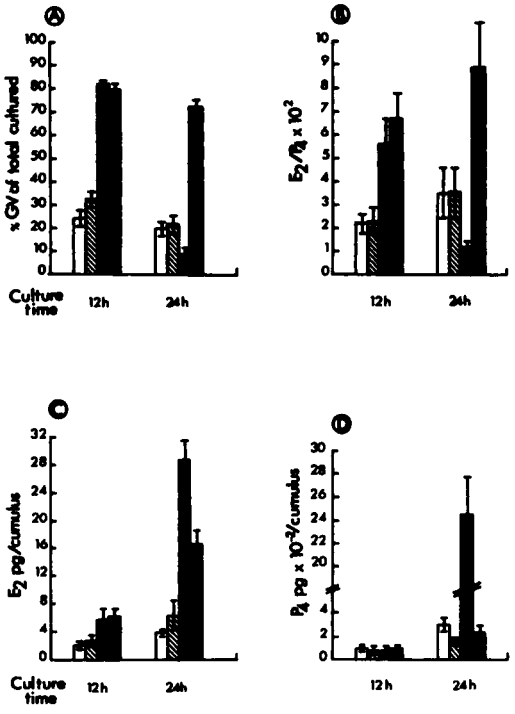

FIG. 6. Effect of cyanoketone on meiotic maturation and cumulus cell steroidogenesis during 24-h culture. Oocytes were cultured in control, unsupplemented medium *(open bar)*, or in medium supplemented with cyanoketone (0.7×10^{-7} M) *(batched bar)*, FSH (10 μg/ml) and testosterone (0.5 μM) *(stippled bar)* or FSH (10 μg/ml), testosterone (0.5 μM) and cyanoketone (0.7×10^{-7} M) *(black bar)*. Each histogram represents the mean ± SEM of six replicate cultures.

in which the effect of T and dbcAMP on meiotic maturation was examined (Rice and McGaughey, 1981). The present results show that FSH and dbcAMP each exerted significant arresting actions on meiosis of intact oocytes, and that although these cyclic AMP-dependent arrests were insignificantly different from one another, they were quite differently modulated by androgen; the arresting action of dbcAMP was significantly enhanced by T, while that of FSH was significantly depressed by both DHT and T. Furthermore, the modulations exerted by the nonaromatizable androgen, DHT, on either FSH- or dbcAMP-induced meiotic arrest, were not as marked as those exerted by T. Although substitution of androgen with E_2 did not duplicate these androgenic effects, analyses of the E_2 and P_4 secretions by the cumulus cells suggest that the expression of androgenic modulation may be regulated by aromatization. The findings are discussed with reference to previous reports of the effects on maturation of androgens and cyclic AMP inducers, and are considered in light of the two currently popular hypotheses regarding the mechanisms which control meiotic maturation (Dekel and Beers, 1978, 1980; Moor et al., 1981).

In this study, neither T nor DHT significantly suppressed the proportion of maturing oocytes compared with that of oocytes cultured in control medium. These data are consistent with those of Smith and Tenney (1980) and Schaerf et al. (1982), but do not support our previous observation (Rice and McGaughey, 1981) that T exerted a slight, albeit significant, suppressive action. The reason for this discrepancy is not clear but may reflect a variation in the populations of oocytes used.

FSH and dbcAMP independently exerted similar and significant arresting actions on maturation, the degree of these actions being consistent with that previously reported for dbcAMP (Rice and McGaughey, 1981). It is noteworthy, however, that in another study (Hillensjö and Channing, 1980), highly purified FSH (0.1 μg/ml) did not significantly arrest the maturation of intact porcine oocytes cultured for 44–48 h. It is not known whether the inconsistency between these results and those reported here can be accounted for by differences in biological activities of the two FSH preparations, to variation in the sensitivities of the various batches of cumulus cells, or whether, in the previous study, the gonadotropin exerted a transient arrest which was undetectable after 48 h of culture.

The arresting action of FSH with T was demonstrated to be specific to FSH and not due to LH contamination (1% contamination by assay) of the impure gonadotropin. Such specificity was reflected in the insignificant difference between the effect of impure and highly purified FSH on the proportion of oocytes exhibiting meiotic delay at 24 h, by lack of such an effect with highly purified LH at a concentration equivalent to that present in the impure FSH preparation, and by the absence of LH stimulation of P_4 secretion comparable to that induced by either of the FSH preparations. Similar FSH specificity on the physiological status of cumulus cells has been reported with regard to mucification and

expansion of mouse cumuli oophori and the synthesis of hyaluronic acid by these cells (Eppig, 1979a,b).

It is well established that, depending upon the stage of follicular development, membrana (Lindsey and Channing, 1979) and cumulus (Bae and Channing, 1981; Channing et al., 1981) granulosa cells vary markedly in their relative abilities to respond to FSH and LH; the ability to respond to LH, as reflected by accumulation of cyclic AMP (Lee, 1978) or increased P_4 secretion (Thanki and Channing, 1978), increases with follicular maturation. Porcine cumulus cells from medium-sized follicles, however, have been shown to possess LH receptors (Channing et al., 1981), and both rat (Magnusson et al., 1979; Hillensjö et al., 1981) and pig (Hillensjö and Channing, 1980; Channing et al., 1981) cumulus cells have been demonstrated to respond to highly purified LH or human chorionic gonadotropin with a stimulation in P_4 secretion. In no case, however, has such a gonadotropic response been observed in cumulus cells either obtained from follicles prior to the LH/FSH surge or cultured for less than 48 h. In the present study, it would seem that the number of LH receptors was insufficient to elicit, in response to 30 ng/ml LH, the arrest in meiotic maturation and the increase in P_4 secretion induced by FSH. As suggested by Erickson et al. (1974), it is possible that LH receptors are unmasked by longer exposure to gonadotropin in vitro, a suggestion supported by the observation that the potential of both membrana and cumulus granulosa to secrete P_4 increased with increasing exposure to highly purified LH (Bae and Channing, 1981).

Compared with controls cultured in unsupplemented medium, the maturation of denuded oocytes was not significantly effected by culture in medium containing FSH, T or FSH with T (Table 2). Moreover, although there was no significant difference between the meiotic arrest of intact and denuded oocytes maintained in medium containing T and dbcAMP, the arrest of the denuded oocytes was found to be due to a direct action of the nucleotide derivative on the oolemma (Rice and McGaughey, 1981). Taken together, these data indicate that the surrounding cumulus cells mediate in the androgenic modulation of cyclic AMP-dependent meiotic arrest.

The results of the present study do not reveal the mechanism(s) by which this androgenic modulation is mediated. There are at least two possible explanations, however, to account for the observations obtained. Androgen may cause shunting of cyclic AMP into the aromatase pathway, thereby reducing the availability of nucleotide within the cumulus cell for transfer to the oocyte to maintain meiotic arrest. Alternatively, and/or additionally, androgens may destabilize the oocyte-cumulus complex, resulting in disruption of functional coupling between the heterologous cells. It is possible that this destabilization is regulated by the content of E_2 and P_4 within the cumulus cells such that above the critical E_2:P_4 ratio, these steroids overcome androgenic-induced destabilization, resulting in maintenance of functional intercellular coupling.

The first explanation is compatible with the hypothesis advanced by Moor et al. (1981) which suggests that it is a qualitative or quantitative change in the signal communicated from the cumulus cells to the oocyte, but not a termination of the cellular coupling between the heterologous cells, which results in release from meiotic arrest. Furthermore, much of the data presented in this paper are consistent with this proposition: T significantly decreased the proportion of oocytes maintained in meiotic arrest by FSH; dose-dependent increases and decreases were established between the proportions of cultured oocytes which matured in the presence of increasing concentrations of T and FSH, respectively (Fig. 2); significantly fewer FSH-arrested oocytes were released from meiotic arrest by the nonaromatizable androgen, DHT, than by T; and the extent of E_2 secretion has recently been found to be correspondingly less in the presence of DHT (Racowsky, in preparation). Nevertheless, some of the other results reported here suggest that androgens may destabilize the coupling between the oocyte and cumulus cells. Such a possibility is consistent with the hypothesis advanced by Dekel and Beers (1978, 1980) which proposes that maintenance of the minimum level of cyclic AMP required for meiotic arrest is dependent upon functional coupling between the oocyte and its adherent cumulus cells. Compatible with this hypothesis is the observation that both DHT and T markedly enhanced the proportion of intact oocytes that remained arrested in medium containing dbcAMP. Under such conditions, disruption of functional coupling would render the intact oocyte "denuded," in a physiological sense,

thereby increasing accessibility of the derivatized nucleotide to the oolemma.

The observations that dose-dependent increases and decreases were established between the proportions of cultured oocytes which matured in the presence of increasing concentrations of T and FSH, respectively, (Fig. 2) are compatible with both of the above suggested mechanisms for androgenic modulations of cyclic AMP time-dependent meiotic arrest.

Substitution of T with E_2 did not duplicate the arresting actions of either dbcAMP or FSH with the androgen. A role for cumulus cell E_2 is implicated in the observed androgenic modulations, however, by the observations that the arresting action of DHT with either dbcAMP or FSH was less marked than that induced by T, and that E_2 secretion has been found to be correspondingly lower in the presence of the nonaromatizable androgen (Racowsky, in preparation). In addition, a positive correlation was established between the proportion of GV stage oocytes cultured in medium containing T and FSH and the E_2:P_4 ratio in the medium, and the results of the experiments with cyanoketone revealed a similar correlation between these two parameters. While this drug is an inhibitor of 3β-hydroxysteroid dehydrogenase (Goldman et al., 1965), it has been shown marginally to suppress the 17β-dehydrogenase system. The data support this effect of the drug since the secretions of both E_2 and P_4 were significantly depressed by the drug. The suppression of E_2 secretion was presumably a reflection of both the preferential utilization by cumulus cells of androstenedione as the aromatase substrate (Schaerf et al., 1982), and the extremely low cross-reactivity of the E_2 antibody with estrone (2.6%). Since the relative inhibition of P_4 secretion was so substantially greater than that of E_2, however, the ratio of secreted E_2:P_4 was maintained at a high level beyond 12 h of culture. This ratio was significantly and positively correlated with the proportion of immature oocytes after both 12 h and 24 h of culture.

The observations of several other studies suggest that the integrity of functional intercellular coupling is under steroidal control: E_2 regulates the appearance and maintenance of gap junctions between cells of the membrana granulosa (Merk et al., 1972), myometrium (Bergman, 1968; Merk et al., 1980) and an adenocarcinoma (Dahl and Bergman, 1978), and smooth muscle when P_4 levels are very low (Garfield et al., 1977). In contrast, P_4 retards the formation of large gap junctions in gravid rat uteri (Garfield et al., 1977). Although there is no evidence to support the concept of disruption of functional coupling by androgens, a negative correlation has been reported between the number of granulosa cells within the human follicle and the ratio of androgen to estrogen in antral fluid (McNatty et al., 1979).

Taken together, these observations raise the possibility that androgens may disrupt functional coupling between the oocyte and adherent cumulus cells and that the estrogenic: progestogenic status of the cumulus cell may regulate this androgenic modulation of cyclic AMP-dependent meiotic arrest. Such a possibility, however, is based upon the assumption that the secretion of cumulus cell steroids is an accurate reflection of the steroidal content of these cells. Clearly, further work is needed to investigate this assumption. In addition, carefully designed experiments are required not only to establish the relative concentrations of cyclic AMP within the cumulus cell and oocyte prior to, and at the onset of maturation, but also to determine the precise relationship that exists between disruption of functional coupling between the oocyte and its adherent cumulus cells and the reinitiation of meiosis. Since release from meiotic arrest in medium supplemented with T and FSH occurs automatically against a background of E_2:P_4 ratios which change in a manner similar to those known to accompany maturation in vivo (reviewed by Gérard et al., 1979), this system may be appropriate for these investigations.

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