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Fertilization and early development of rat oocytes induced to mature by forskolin

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Summary

Forskolin has been shown to successfully induce maturation of rat oocytes as assessed by morphological markers. The present study was designed in an attempt to elucidate whether oocytes, induced to mature by forskolin (10^{-4} M, group A) in a follicle-enclosed oocyte culture, are fertilizable and can further develop into two-cell embryos. Oocytes exposed in vitro to either luteinizing hormone (LH, 5 μg/ml, group B) or a GnRH agonist analogue (10^{-7} M, group C) as well as oocytes that underwent maturation in vivo (group D), served as positive controls. We found that similar rates of fertilization were obtained in the experimental and all of the above mentioned control groups ($A = 78.9 \pm 4.2\%$, $B = 77.9 \pm 3.1\%$, $C = 77.5 \pm 5.5\%$ and $D = 84.7 \pm 2.7\%$). Cleavage rate of fertilized eggs from group A was significantly higher than that of eggs from groups B & C, and similar to that of eggs from group D ($A = 63.1 \pm 6.7\%$, $B = 37.8 \pm 4.9\%$, $C = 50.0 \pm 4.1\%$, $D = 67.8 \pm 4.1\%$). Using functional parameters we hereby demonstrate that forskolin and LH are at least equally potent in producing fertilizable eggs that have a high potential of development into two cell embryos. These results further support the idea that cAMP is a mediator of LH action in inducing oocyte maturation.

Introduction

Meiosis of the mammalian oocyte is initiated during fetal life. It proceeds up to the diplotene stage of the first prophase and is arrested at birth. Resumption of meiosis, also known as oocyte maturation, is clearly dependent upon the preovulatory surge of luteinizing hormone (LH; Lindner et al., 1974). It has been suggested that, similar to the other ovarian responses to this hormone, maturation of the oocyte is mediated by cAMP (Dekel and Sherizly, 1983; Dekel, 1986; Holmes et al., 1986; Yoshimura et al., 1992). On the other hand, since oocyte maturation can be induced by stimulants that act via cAMP independent biochemical pathways (Dekel et al., 1983; Dekel and Sherizly, 1985; Downs, 1989; Hillensjo and LeMaire, 1980), this idea seems to deserve a further evaluation.

The mediatory role of cAMP in LH-induced oocyte maturation has been suggested on the basis of previous reports demonstrating that forskolin, a potent activator of adenylate cyclase (Seamon et al., 1981), can mimic LH action, induces maturation in rat follicle-enclosed oocytes (Dekel and Sherizly, 1983; Holmes et al., 1986; Yoshimura et al., 1992). Maturation of the oocytes in these studies was assessed by germinal vesicle breakdown (GVBD). In order to further evaluate forskolin action in this system, we have employed in the present study functional, rather than morphological tests, for assessment of oocyte maturation. Specifically, we evaluated the capacity of oocytes that underwent maturation in response to forskolin to be fertilized and tested the potential of these fertilized eggs to further develop into two-cell embryos.

Materials and methods

Animals

Wistar-derived rats were maintained in air conditioned rooms (25°C) and supplied with laboratory animal pellets, (Ambar Israel) and tap water ad libitum. Artificial illumination was provided for 14 h daily.

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Hormones and chemicals

The following agents were used for induction of oocyte maturation in vitro: luteinizing hormone, ovine (oLH NIH LH S-20), forskolin (Sigma, USA), a GnRH agonist analogue, buserelin [D-Ser(t-Bu)^6]des-Gly^{10}-GnRH-*N*-ethylamide](GnRHa, Hoechst, Germany).

For induction of oocyte maturation and ovulation in vivo, immature female rats were injected with pregnant mares' serum gonadotropin (PMSG, Intervent, Holland) followed by human chorionic gonadotropin (hCG, CG-10 Sigma).

In-vitro maturation (IVM) of oocytes

Maturation in vitro was induced using the follicle-enclosed oocyte culture system (Dekel and Sherizly, 1983). Immature, 25-day-old rats were primed by PMSG (15 IU) administered sub cutaneously. The rats were killed 48 h after PMSG administration and the ovaries were removed and placed in Leibovitz's L-15 tissue culture medium (L-15, Gibco, USA) supplemented with antibiotics (penicillin 100 u/ml and streptomycin 50 μg/ml, Sigma, USA), and heat inactivated foetal calf serum (9%, Bio-Lab, Israel). Ovarian follicles of 0.9–1.0 mm diameter were dissected under a stereoscopic microscope, rinsed and further incubated in the above medium (20 follicles/2 ml medium) in the presence or absence of forskolin (10^{-4} M). Oocytes incubated in the presence of oLH (5 μg/ml) or GnRHa (10^{-7} M) served as positive controls. These concentrations were previously demonstrated to induce GVBD in the greatest percentage of the follicle-enclosed oocytes (Dekel and Sherizly, 1983; Dekel et al., 1985). Being incubated under these experimental conditions in hormone-free medium, the oocytes remain meiotically arrested (Dekel et al., 1983; Dekel and Sherizly, 1985).

Incubation of follicles was carried out in 25 ml flasks in an N_2/O_2 (1:1) atmosphere in an oscillating water bath at 37°C. At the end of the incubation period, eggs with associated cumuli were recovered from the follicles, treated with hyaluronidase (400 IU/ml, Sigma) to disperse the cumulus cells and allow oocyte examination, and further incubated for 3 h before being transferred to a sperm suspension for in-vitro fertilization (IVF).

Viability of cumulus-oocyte complexes

Cumulus-oocytes complexes (COC), recovered from the isolated follicles after culture with either forskolin, LH or GnRHa, were evaluated morphologically using a stereoscopic microscope, at the following three time points: (1) upon isolation of the complexes from the follicles; (2) after hyaluronidase treatment for removal of the cumulus cells; (3) after 3 h of culture in fertilization medium, prior to their transfer to the sperm suspensions.

A COC composed of a round oocyte with a clear and homogeneous ooplasm, surrounded by a homogeneous cumulus cell mass was considered as viable. Non-viable COCs were all composed of oocytes that were dark and flat, surrounded by clumps of cumulus cells.

Cumulus mucification

Cumulus mucification was analyzed morphologically using Nomarsky interference-contrast microscopy and scanning electron microscopy (SEM). Cumulus masses with an expanded appearance composed of cells that were not tightly packed were considered mucified. For further evaluation by SEM some COCs were fixed in 2.5% glutaraldehyde in phosphate buffered saline (PBS) at pH 7.4 and attached to poly-L-lysine coated coverslips (Phillips and Shalgi, 1980). Samples were then dehydrated in acetone, dried in a Sorvall critical-point drying apparatus using liquid CO_2, coated with

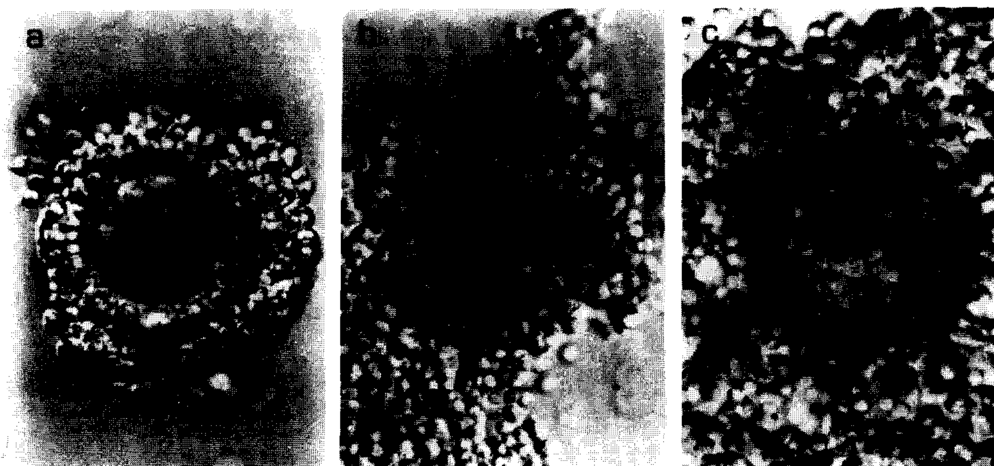

Fig. 1. Cumulus-oocyte complexes recovered from follicles that were incubated in the presence of LH: (a) at 4 h of incubation; (b) at 6 h of incubation; (c) at 16 h of incubation. (a & b & c ×200).

gold in Edwards 150 Sputtercoater and examined with an ETEC autoscan microscope. Sensitivity of the cumulus mass to hyaluronidase treatment was further used for quantitation of the extent of mucification. Sensitivity was classified as: (1) none, (2) partial mucification characterized by detachment of all the cumulus cells, except of those of the corona radiata, and (3) full mucification represented by detachment of all the cumulus and corona cells resulting in a denuded oocyte.

In-vivo maturation of oocytes

Oocytes that underwent maturation in vivo served as a bridge-control. These oocytes were recovered from 27-days old immature female rats injected with 15 IU hCG 54 h following PMSG administration, and sacrificed 17–19 h later. The ovulated cumulus-enclosed oocytes were collected from the excised oviductal ampullae and transferred to a sperm suspension for IVF. This group was also used for standardization of the results according to daily variations in fertilization rate which may result from the use of different males as donors of spermatozoa.

In-vitro fertilization

Spermatozoa were collected from the uteri of mature cycling rats soon after mating and diluted in modified rat fertilization medium (RFMm) (Shalgi, 1991), to a final concentration of $7–13 \times 10^5$ spermatozoa/ml. Aliquots of sperm suspension (100 μl) were incubated in petri dishes under heavy paraffin oil (BDH-England) at 37°C for 5 h in a humidified atmosphere of 5% CO_2 in air, to allow capacitation (Shalgi et al., 1983). Eggs that underwent maturation either in vitro or in vivo were then introduced to the sperm suspensions (10–15 per 100 μl drop). Forty hours later, the eggs were examined by Nomarsky interference-contrast microscopy for fertilization and first cleavage. Rat oocytes cleave 21 h after sperm penetration (Shalgi et al., 1985). It should be pointed out that in contrast to some other species, the rat sperm tail remains clearly visible in the vitellus and/or blastomers, until the 8 cells stage (Shalgi et al., unpublished). Thus, only eggs containing a sperm tail in the vitellus, were classified as fertilized. The number of fragmented, polyspermic and parthenogenetic eggs was also recorded. Fragmented eggs contained more than two fragments with a sperm tail present. Polyspermic eggs contained more than one sperm tail in the ooplasm. Parthenogenesis was diagnosed in the presence of two blastomers but absence of a sperm tail.

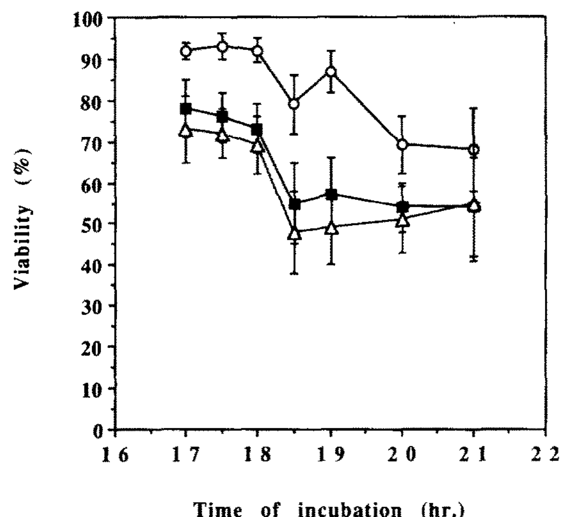

Fig. 2. Viability of cumulus-oocytes complexes (COCs) recovered from follicles incubated with LH. Isolated follicles were cultured for different time periods in the presence of LH (5 μg/ml). COCs were then recovered and their viability was evaluated using the parameters described in Materials and methods, at the following three stages: 1) Upon isolation of complexes from the follicles (– ○ –). 2) After hyaluronidase treatment for removal of the cumulus cells (– ■ –). 3) After 3 h of culture, prior to transfer to sperm suspensions (– @ –). The results are expressed as the percentage out of the total number of follicles incubated. Each data point represents the mean ± standard error of 10–12 experiments including a total of 300–600 follicles.

TABLE 1

FERTILIZATION AND FIRST CLEAVAGE OF IVM OOCYTES, INCUBATED WITH LH FOR 17–18.5 h

Oocytes were isolated from the follicles after 17–18.5 h of incubation with LH, and transferred to sperm suspensions for in-vitro fertilization. Postovulatory oocytes that underwent maturation in vivo were used as a control group. After 40 h the eggs were examined by interference contrast microscopy for fertilization and first cleavage. The results represent 24 experiments.

Total no. of eggs		Fertilized eggs Total no. of eggs (%)		Two-cell embryos Fertilized eggs (%)	
Experiment	Control	Experiment	Control	Experiment	Control
269	346	36.8 ± 7.0	37.5 ± 5.1	55.0 ± 10.0	52.6 ± 7.6

Statistical analysis

An analysis of variance (ANOVA) test was used to compare the results obtained on viability of oocytes exposed to forskolin, LH and GnRHa. A Chi-squared test was used to compare fertilization and cleavage rates. Analysis was performed in the Blossom stat pack for Lotus 1-2-3.

Results

The follicle-enclosed oocyte culture system represents mature preovulatory follicles, developed in response to PMSG, that posses the capacity to respond to LH/hCG. Selection of these follicles is based on their relatively larger size as compared to the overall population of ovarian follicles.

We compared the number of follicles isolated for incubation in vitro, to that of oviductal eggs recovered

from a similar group of rats further treated with hCG for induction of ovulation. The mean number ±SD. of follicles isolated 48 h after PMSG treatment was 25 ± 5 per ovary, as compared to 25 ± 7 eggs recovered per oviduct of the parallel group 18 h after hCG administration. A ratio higher than 1 (ovarian oocytes/ ovulated oocytes) would suggest that not only the candidates for ovulation, but also other irrelevant follicles (such as cystic etc.) were included in the experiments.

In order to determine the optimal incubation time, the following parameters have been assessed in LH-stimulated cultures, terminated at different time points: (1) mucification and expansion of the cumulus; (2) viability of the oocytes; (3) fertilizability and first cleavage of the eggs.

1. *Mucification and expansion of the cumulus oophorus:* COCs that were isolated after 4 h in culture in the presence of LH were characterized by their compact cell organization (Fig. 1a). Six hours after exposure to LH, the periphery of the COCs exhibited a diffuse structure. At this time point some cumuli responded partially to hyaluronidase with the corona radiata still compact and tightly associated with the ZP (Fig. 1b). The morphological transformation in the cumulus had progressed centripetally towards the zona pellucida (ZP) but the corona radiata could not be totally dissociated by hyaluronidase even at 16 h after LH (Fig. 1c). At 17–18 h of culture, exposure to hyaluronidase resulted in total denudation of only some of the oocytes. However, longer incubation periods did not result in a

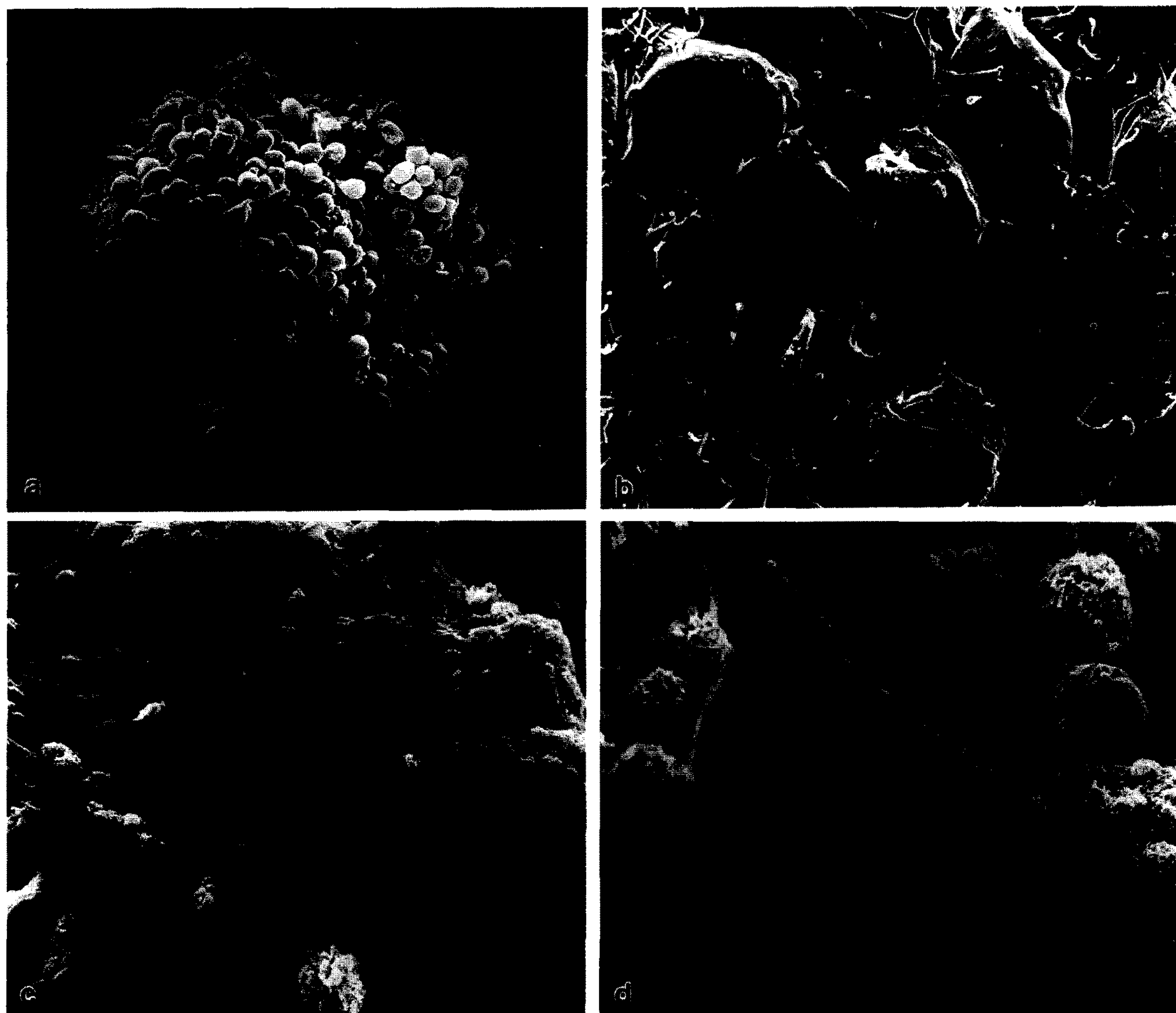

Fig. 3. Cumulus-oocyte complexes recovered from follicles incubated in the absence (a and b) or the presence (c and d) of forskolin for 18 h. (a and c, ×400; b and d, ×3000).

TABLE 2

VIABILITY OF OOCYTES THAT UNDERWENT MATURATION IN RESPONSE TO FORSKOLIN, LH AND GnRHa

Isolated follicles were incubated for 18 h with the indicated concentration of forskolin, LH or GnRHa. Cumulus-oocyte complexes were recovered and their viability evaluated, using the parameters described in Materials and methods, at the following two stages: (1) upon isolation of complexes from the follicles, and (2) after 3 h of culture in RFMm, prior to their transfer to sperm suspensions. The results are expressed as the percentage of the total number of follicles incubated. Each data point represents the mean ± standard error of 7–9 experiments including a total of 550–690 follicles.

Treatment	Follicle no. (Experiment no.)	(1) Viable COC no. Follicle no. (%)	(2) Viable oocyte no. Follicle no. (%)
Forskolin (10^{-4} M)	638 (9)	90.9 ± 2.3	78.7 ± 3.4
LH (5 μg/ml)	550	95.6 ± 1.9	83.0 ± 4.0
GnRHa (10^{-7} M)	690 (9)	93.0 ± 2.3	83.6 ± 4.0

larger fraction of COCs exhibiting total dissociation in response to hyaluronidase.

2. *Viability of the oocytes:* As mucification and expansion of the cumulus did not reach a maximum before 17 h of culture, viability of the oocytes around this time point has been further analyzed, employing the morphological parameters described in Materials and methods. We found that at 17–18 h of incubation a high percentage (almost 100%) of the mature oocytes were viable. On the other hand, an increase in the duration of culture beyond 18 h, was accompanied with a decrease in the fraction of viable oocytes (Fig. 2).

3. *Fertilizability and first cleavage:* For functional evaluation of the IVM culture system, fertilizability and first cleavage of 17–18.5 h cultures of follicle-enclosed oocytes exposed to LH as been assessed. Oocytes matured in vivo served as a control group. We found that the fertilization rate of oocytes incubated with LH for 17–18.5 h was not significantly different from that of the control group of postovulatory oocytes matured in vivo (Table 1). On the basis of these findings further analysis of forskolin action was carried out at 17–18.5 h in follicle-enclosed oocyte cultures.

Forskolin action

In a second series of experiments the action of forskolin on the oocyte was evaluated. COCs examined after 18 h culture in the presence of forskolin presented fully mucified cumulus masses as revealed by light microscopy as well as by SEM (fig. 3). Viability of the oocytes exposed to forskolin was compared to that of oocytes exposed to LH and GnRHa. No significant difference was found between these 3 groups, (Table 2). Furthermore, the three groups of eggs did not differ significantly in their fertilization rate (78.9 ± 4.2%, 77.9 ± 3.4% and 77.5 ± 5.5% respectively, Fig. 4). A similar rate of fertilization (78.9 ± 4.2%) was obtained in postovulatory eggs that underwent maturation in vivo. On

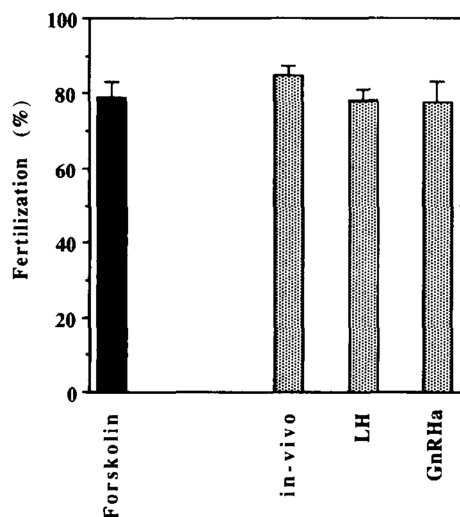

Fig. 4. Fertilization rate of oocytes matured in vitro in response to forskolin, LH and GnRHa. Oocytes that underwent maturation in vitro in response to the above agents were isolated from the follicles and transferred to sperm suspensions for in vitro fertilization. Postovulatory oocytes that underwent maturation in vivo were also analyzed. Each data point represents mean ± standard error of 9–17 experiments including 200–260 follicles.

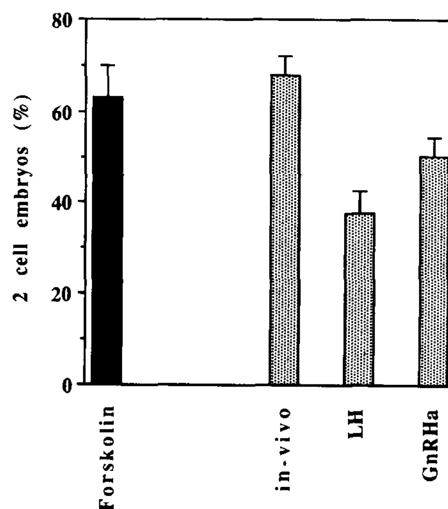

Fig. 5. Rate of first cleavage of oocytes matured in vitro in response to forskolin, LH and GnRHa. Oocytes that underwent maturation in vitro in response to the indicated agents were isolated from the follicles and transferred to sperm suspensions for in-vitro fertilization. The eggs were examined for the first cleavage as described in Materials and methods. Postovulatory oocytes that underwent maturation in vivo were also included. Each data point represents mean ± standard error of 9–17 experiments including 200–260 follicles. Significant difference ($P < 0.01$) was demonstrated between forskolin as compared to LH or GnRHa.

TABLE 3

RATE OF EGGS EXHIBITING ABNORMAL DEVELOPMENT

Oocytes that underwent maturation in vitro under the influence of the indicated agents, were isolated from the follicles and transferred to sperm suspensions for in-vitro fertilization. A group of oocytes that underwent maturation in vivo was also included. The results for polyspermy and fragmentation are expressed as the percentage out of the total number of fertilized eggs. Parthenogenesis is expressed as a fraction of the total number of eggs transferred to IVF. Each data point represents the mean ± standard error.

Treatment	Total egg no. (exp. no.)	Polyspermic Fertilized (%)	Fragmented Fertilized (%)	Partheno-genetic Total (%)
Forskolin (10^{-4} M)	253 (9)	10.2 ± 3.9	12.3 ± 5.0	4.1 ± 0.9
In vivo	407 (18)	7.4 ± 1.9	14.4 ± 2.6	2.9 ± 0.9
LH (5 μg/ml)	228 (17)	16.4 ± 4.0	19.2 ± 6.1	4.9 ± 1.4
GnRHa (10^{-7} M)	195 (11)	24.2 ± 5.9	10.1 ± 2.7	6.2 ± 2.1

the other hand, while fertilized eggs that underwent maturation in response to LH and GnRHa were similarly potent to develop into two-cell embryos (37.8 ± 4.0% and 50.3 ± 4.1% respectively), the developmental potential of the forskolin group was significantly higher (63.1 ± 6.7%, $P < 0.01$, Fig. 5) and equal to that of eggs matured in vivo (67.8 ± 4.1%). A small fraction of eggs diagnosed as fertilized were penetrated by more than one spermatozoon. No significant differences in the rate of polyspermic eggs were observed among all groups examined (Table 3). The extent of fragmentation and parthenogenesis was very low (10–20%, 3–6% respectively), with no significant differences between the groups examined (Table 3).

Discussion

Our present study strongly supports the idea that LH-induced oocyte maturation is mediated by cAMP. This conclusion is based on our findings that forskolin, a potent activator of adenylate cyclase promotes the production of mature fertilizable oocytes, that have the capacity to develop into two-cell embryos.

The ability of forskolin to induce oocyte maturation has been demonstrated previously in studies that utilized morphological markers such as GVBD (Dekel and Sherizly, 1983; Holmes et al., 1986; Yoshimura et al., 1992). Fertilizability of such oocytes, and the potential of the fertilized eggs to further develop into two-cell embryos, are functional parameters that have been employed in the present study for evaluation of the quality of oocyte maturation.

Fertilizability and developmental capacity of embryos derived from the IVM/IVF system has been studied in several species (rat, Daniel et al., 1989; mouse, Eppig and Schroeder, 1989; bovine, Fukuda et al., 1990). These studies so far employed isolated oocytes that matured in vitro spontaneously. Unlike spontaneous maturation, the follicle-enclosed oocyte system does require a positive trigger for reinitiation of meiosis. This in-vitro system provides therefore, an excellent experimental tool for clarification of mechanisms involved in oocyte maturation, induced by different stimulants such as forskolin. Furthermore, unlike hormones that can be examined for their effect in vivo in the intact animal, agents like forskolin, do not affect exclusively ovarian components, eliciting an entire repertoire of physiological responses. These agents therefore should be examined in vitro.

Previous attempts to use follicle-enclosed oocytes that underwent maturation in vitro in response to either LH or GnRHa for IVF experiments, yielded a very low rate of first cleavage (Dekel and Shalgi, 1987). Our initial studies aimed therefore at reexamination of the follicle-enclosed oocyte IVM culture conditions. We found that sperm penetration was most effective and that the cleavage rate was maximal when follicle-enclosed oocytes were incubated with LH, for 17–18 h. The results obtained using this modified experimental protocol represent a significant improvement in the rate of cleavage as compared to our previous study in which longer periods of incubation have been employed (Dekel and Shalgi, 1987).

Using this IVM culture system we hereby demonstrate that forskolin and LH are equally potent in producing a mature fertilizable egg, suggesting that cAMP is a mediator of LH-induced oocyte maturation. Furthermore, the rate of first cleavage obtained in those oocytes exposed to forskolin is even higher than that obtained in oocytes exposed to LH. These results may represent the differences in the mechanism of signal transduction of forskolin as compared to that of the hormone. Forskolin directly activates the catalytic sub unit of adenylate cyclase, while LH should initially bind to the G-protein linked receptor that in turn leads to the activation of the catalytic subunit of the enzyme. In this case, any regulatory mechanism imposed by the hormone-receptor interaction is bypassed by forskolin, resulting in a higher intensity of response.

Maturation of the oocyte is accompanied by expansion and mucification of the cumulus oophorus. These maturational changes are prerequisite for fertilization (Bar-Ami et al., 1989; de Loos et al., 1989). Facilitates the transport of the ovum along the oviduct and allowing penetration of the spermatozoon between the cumulus cells to finally reach the oocyte (Larsen and Wert, 1988; Cuasnicu and Bedford, 1991). Forskolin induces mucification very effectively, and COCs isolated from follicles exposed to forskolin appeared morphologically similar to those that underwent matura-

tion in vivo (Dekel et al., 1979). It seems however, that the kinetics of cumulus expansion examined in vitro in the present study was somewhat slower than that observed in vivo (Dekel et al., 1979). Furthermore, unlike the response in vivo, the exposure to LH in vitro induced mucification at the periphery of the cumulus mass with almost no effect at the corona radiata.

In addition to LH and forskolin, that act by activation of adenylate cyclase, oocyte maturation can be induced in vitro by other stimulants, such as GnRHa and EGF that act via alternative biochemical pathways, independent of cAMP (GnRH-Hillensjo and LeMaire, 1980; Ekholm et al., 1981; Dekel et al., 1983; Erickson et al., 1983; EGF-Dekel and Sherizly, 1985; Fagbohun and Downs, 1990; Ben-Yosef et al., 1992). In the pituitary gonadotrophs, GnRH activates the calcium dependent protein kinase C (PKC), (Harris et al., 1985; Hirota et al., 1985; Naor et al., 1989). Activation of PKC has been reported to mediate GnRH action also in the ovary (Naor and Yavin, 1982). Our experiments included therefore another group of control oocytes induced to mature by GnRHa. This additional positive control allows comparison of the quality of oocytes induced to mature via a cAMP dependent mechanism to that of oocytes induced to mature by activation of PKC. Fertilizability and early embryonic development of oocytes matured in vitro in response to GnRHa was not significantly different from that of oocytes stimulated by LH. These results are in accordance with our earlier findings that rat oocytes matured in vivo by LH and GnRHa showed a similar rate of successful fertilization and equal potential of development into two-cell embryos, implantation in the uterus and subsequent development into live embryos (Shalgi and Dekel, 1990). Taking into account that a GnRH-like protein was found in ovaries of domestic animals as well as the rat (Aten et al., 1986, 1987), and that specific receptors for GnRH were identified in rat ovaries including the oocyte itself (Clayton et al., 1979; Dekel et al., 1988; Hazum and Nimrod, 1982), our findings could possibly suggest that a GnRH like agent may act as a paracrine regulator for oocyte maturation. Alternatively, the recent evidence for dual coupling of the murine LH receptor to adenylate cyclase as well as to phosphoinositide breakdown and calcium mobilization (Gudermann et al., 1992), may suggest the LH employs both these biochemical pathways for the induction of oocyte maturation.

Our present study demonstrates that cAMP elevation by itself is sufficient for the production of a fertilizable egg. On the other hand, it does not deny possibility that calcium dependent PKC can also be involved in the induction of oocyte maturation. The intriguing possibility that LH stimulates these two intracellular signal transduction mechanisms is subjected to further investigation in our laboratory.

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