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or Chelation of Intracellular Calcium Blocks FSH-Induced but Not Spontaneous Meiotic Resumption in Mouse Oocytes

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Mammalian oocytes are arrested at the diplotene phase of the first meiotic division until ovulation. In the mouse, germinal vesicle breakdown (GVBD) and progression to metaphase II is thought to be triggered by a positive signal originating in the follicular cells following stimulation by the luteinizing hormone (LH) surge. Isolated, fully grown oocytes can also undergo spontaneous reinitiation of meiosis *in vitro* in the absence of gonadotrophin stimulation. To investigate the mechanism of meiotic resumption, inhibitors of phosphoinositide metabolism and an intracellular calcium chelator were used during maturation *in vitro* under different conditions. In a series of experiments, isolated cumulus cell–oocyte complexes (COCs) maintained in meiotic arrest by hypoxanthine were induced to resume meiosis by treatment with follicle-stimulating hormone (FSH). Under these conditions, both LiCl and neomycin, which inhibit phosphoinositide hydrolysis, produced a dose-dependent inhibitory effect on meiotic resumption. Similar results were obtained when FSH-induced meiotic resumption was observed in the presence of the acetoxymethyl ester form of 1,2-bis(*o*-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid (BAPTA/AM), an intracellular calcium chelator. In hypoxanthine-arrested oocytes, GVBD induced by epidermal growth factor (EGF), which mimics FSH action in *in vitro* maturation, was also repressed by LiCl and neomycin. Conversely, meiotic resumption triggered by a pulse of 8-bromo-cyclic adenosine monophosphate (8-Br cAMP) was not affected by these two inhibitors. In experiments in which oocytes were cultured under conditions which permit spontaneous meiotic maturation, resumption of meiosis was not affected by either inhibition of phosphoinositide hydrolysis or chelation of intracellular calcium. Therefore, it appears that meiotic resumption induced by hormone stimulation requires activation of the phosphoinositide pathway and mobilization of intracellular calcium. In contrast, spontaneous maturation probably occurs through a different mechanism because it is not affected by inhibition of this signaling pathway.

INTRODUCTION

In mammals, meiosis of female germ cells is initiated before birth, but is arrested thereafter at the diplotene stage of the first meiotic division. During the early stages of folliculogenesis oocytes are intrinsically incompetent to resume meiosis, probably as a consequence of inadequate levels of the catalytic component of M-phase-promoting factor (MPF) and other cell cycle-regulating elements (Chesnel and Eppig, 1995). Competence to undergo meiotic maturation (i.e., reinitiation of meiosis and progression to metaphase II) is progressively acquired during later stages of follicle growth (Sorensen and Wassarman, 1976). However, the meiotic hiatus is maintained until shortly before ovulation by the follicular environment. It is not entirely clear how meiotic arrest is achieved in different mammalian species. In rodents, several lines of evidence suggest that inhibition of the meiotic cycle is principally derived by elevated intraoocyte levels of cyclic adenosine monophosphate (cAMP) (Downs, 1995). In mouse oocytes the pres-

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ence of a fully active adenylate cyclase has not been unequivocally demonstrated. However, as gap junctions ensure direct communication between the germ cell and somatic cells of the follicle to form a functional syncytium, it has been proposed that cAMP is generated in the granulosa cells and subsequently conveyed to the oocyte. Purines contained in the follicular fluid of various mammalian species have been suggested to contribute to the maintenance of meiotic arrest. In particular, hypoxanthine (HX), found in the follicular fluid of the mouse, inhibits spontaneous maturation *in vitro* at physiological concentrations, probably by causing an increase in cAMP levels as a direct result of cAMP phosphodiesterase repression. It is still not known how elevated intraoocyte cAMP concentrations prevent reinitiation of the meiotic cycle, although it is clear that activation of cAMP-dependent protein kinase and changes in the phosphorylation pattern of regulatory proteins are involved.

By the time a large antral cavity is formed, oocytes acquire full meiotic competence, undergoing spontaneous maturation *in vitro* after release from their follicles (Edwards, 1965; Pincus and Enzmann, 1935). *In vivo,* reinitiation of meiosis, signified by germinal vesicle breakdown (GVBD), is finally triggered by the luteinizing hormone (LH) surge (Channing *et al.,* 1978). The mechanism by which LH induces GVBD remains unknown. It has been suggested that in some species meiotic resumption may result from withdrawal of inhibitory action imposed by the follicular environment, resulting from breakdown in gap junction communication between different follicle compartments and interruption of the transfer of cAMP or other inhibitory molecules to the oocyte. Alternatively, reinitiation of meiosis may be mediated by a positive signal able to override the conditions which ensure continued meiotic arrest. In fact in the mouse, following chorionic gonadorophin administration, cAMP increases in cumulus–oocyte complexes (COCs) and gap junction communication between germ cells and cumulus cells is not substantially compromised at a time when oocyte have undergone GVBD (Eppig and Downs, 1988). Moreover, FSH and other ligands induce GVBD in cumulus cell-enclosed oocytes *in vitro,* under culture conditions which maintain continued meiotic arrest (Downs *et al.,* 1988), supporting the view of the existence of an overriding stimulus. Importantly, these ligands act through mediation by cumulus cells, as they fail to promote the same effect on denuded oocytes.

The LH surge is the physiological stimulus which induces GVBD. Although this hormone typically activates the cAMP pathway in target cells, it has been shown that in granulosa cells it can also promote phosphoinositide turnover and elevation of inositol trisphosphate ($InsP_3$) (Davis *et al.,* 1986; Dimino *et al.,* 1987; Sadighian *et al.,* 1989) as well as calcium mobilization (Mattioli *et al.,* 1991). It is also suspected that phosphoinositide metabolism and transient changes in intracellular free calcium are involved in the regulation of the reinitiation of meiosis in the oocyte. Studies on calcium as a possible candidate for an overriding signal that induces GVBD have not produced conclusive evidence, probably as a consequence of the use of different systems and culture conditions (Homa, 1995). Similarly, experiments on phosphoinositide metabolism during spontaneous meiotic resumption in mouse oocytes have also generated contrasting results (Bagger *et al.,* 1993; Pesty *et al.,* 1994).

In this study, the hypothesis suggested by various authors (Eppig, 1991, 1993; Homa *et al.,* 1993) that phosphoinositide metabolism and changes in intracellular calcium mediate hormone-induced and spontaneous reinitiation of meiosis in mouse oocytes was investigated. In experiments in which hormone-induced meiotic resumption was studied, fully grown germinal vesicle (GV)-stage oocytes enclosed in their cumulus cells were released into medium containing HX, to prevent spontaneous GVBD, and were exposed to FSH to promote GVBD. LH was not tested as in cumulus cells expression of the LH receptor gene is repressed by paracrine factor(s) produced by the oocyte (Eppig *et al.,* 1997). Lithium and neomycin were employed to inhibit phosphoinositide metabolism and generation of second messengers. Lithium decreases the level of cytosolic inositol (Berridge *et al.,* 1989) and inhibits enzymes that hydrolyze inositol phosphates (Berridge *et al.,* 1989; Gee *et al.,* 1988; Majerus *et al.,* 1988). Neomycin, an aminoglycoside antibiotic, binds with high affinity to phosphatidylinositol 4,5-bisphosphate (Schacht, 1978), preventing its hydrolysis (Downes and Michell, 1981; Tysnes *et al.,* 1988; Whitaker, 1989). In addition, this aminoglycoside can potentially affect calcium influx across the plasma membrane, by competing with calcium for channel binding (Dulon *et al.,* 1989; Hughes *et al.,* 1988). To specifically investigate a possible involvement of calcium, in some experiments hormone-mediated meiotic resumption was promoted in the presence of BAPTA, a potent and highly specific calcium chelator which accumulates in the intracellular environment following deesterification of its acetoxymethyl ester form (BAPTA/AM) (Tsien, 1980). Epidermal growth factor (EGF), whose action on target cells probably induces activation of the phosphoinositide pathway, has been shown to promote meiotic resumption in cumulus-enclosed oocytes arrested at the GV stage by HX (Downs *et al.,* 1988). Similarly, transient high concentrations of the cAMP analogue 8-Br cAMP can also induce GVBD under the same conditions. Therefore, in some experiments the effect of LiCl and neomycin was also tested on reinitiation of meiosis promoted by these agents. Furthermore, the effects of inhibition of phosphoinositide metabolism and changes in intracellular calcium were investigated under conditions which allow spontaneous resumption of meiosis.

MATERIALS AND METHODS

Chemicals

The culture medium αMEM obtained from Gibco (Life Technologies, Paisley, Scotland) was used for these experiments. Preg-

nant mare's serum gonadotropin (PMSG) was purchased from Intervet (Cambridge, England). FSH (Folligon) was provided by Organon UK. BAPTA/AM and pluronic were supplied by Molecular Probes (Cambridge Biosciences, Cambridge, England). All other chemicals were obtained from Sigma Chemical Co. (Dorset, England). Stock solutions of neomycin sulfate (10 mM) and LiCl (1 M) were prepared in αMEM and phosphate-buffered saline (PBS), respectively, containing bovine serum albumin (BSA, 3 mg · ml^{-1}) and stored at 4°C for up to 2 weeks. Stock solutions of FSH and EGF (50 IU · ml^{-1} and 100 ng · μl^{-1}, respectively) were prepared in PBS and stored at −20°C. Stock solution of BAPTA/AM (10 mM) was prepared in anhydrous DMSO and stored desiccated at −20°C. Stock solution of 8-Br cAMP (100 mM) was prepared in sterile water and stored at −20°C.

Oocyte Isolation

Three-week-old immature female mice (B6C3) were used for all experiments. Follicle development was stimulated by intraperitoneal injection of 5 IU PMSG. Animals were sacrificed 48 h later by cervical dislocation and their ovaries were dissected and transferred to preequilibrated culture medium under oil. Oocytes were released by puncturing large antral follicles with a fine needle. COCs with a full-sized oocyte surrounded by an uninterrupted layer of decompacted cumulus cells were selected, washed repeatedly in fresh medium, and transferred in groups of 10- to 12-, to 30- to 40-μl drops of medium under oil for subsequent culture.

Culture Conditions

BSA (3 mg · ml^{-1}), penicillin (60 μg · ml^{-1}), and streptomycin (50 μg · ml^{-1}) were added to the culture medium for all groups. Pyruvate and glutamine were not added because they were already included in the formulation of the medium. In experiments in which the effect of BAPTA/AM was examined, the agent was premixed with pluronic, to facilitate its dispersion in the medium. Media were prepared such that the final concentration of DMSO, used to dissolve BAPTA/AM, and pluronic was 0.2 and 0.01%, respectively, in test and control groups. In hormone and growth factor-induced GVBD, in addition to FSH (0.1 IU · ml^{-1}) or EGF (1 ng · ml^{-1}), media were supplemented with HX (4 mM) to prevent spontaneous resumption of meiosis. In some control experiments, NaCl (10 mM) was added to the medium to rule out the possibility of inhibitory effects, due to increased osmolarity, on FSH action and meiotic resumption. In experiments in which GVBD was induced by 8-Br cAMP, COCs were initially pulsed for 3 h with a high concentration of this agent (1 mM) in the presence of HX and subsequently transferred to medium containing HX for the remaining period of culture. In experiments in which the effect of neomycin and LiCl was assessed, oocytes were released and thoroughly washed in their respective medium before culture. For treatment with BAPTA/AM, immediately after collection oocytes were exposed to the agent for 30 min at 37°C, thoroughly washed, and cultured in BAPTA/AM-free medium. Culture was conducted for 17–18 h at 37°C in a 5% CO_2 atmosphere.

Oocyte Assessment

The endpoint of these experiments was the resumption of meiosis. Oocytes were cultured for 17–18 h, denuded of their cumulus cells by repeated pipetting, and morphologically assessed for the presence of the GV using an inverted microscope equipped with differential interference contrast. Oocytes were incubated for a period longer than that required for FSH-induced GVBD (4–5 h) because it has been reported that relatively low levels of phosphoinositide metabolism inhibitor (LiCl) produce a delay in spontaneous meiotic resumption, but are unable to maintain continued suppression (Pesty *et al.*, 1994). Oocytes showing signs of degeneration or morphological abnormality were excluded from the statistical analysis. In all experiments the proportion of oocytes with a normal appearance was at least 95%. Each experiment was repeated at least three times with at least 40 oocytes for each experiment in each group. Frequencies of GVBD in the various groups were subjected to arcsin transformation and statistically compared by ANOVA and Duncan's test. A P value less than 0.05 was considered statistically significant.

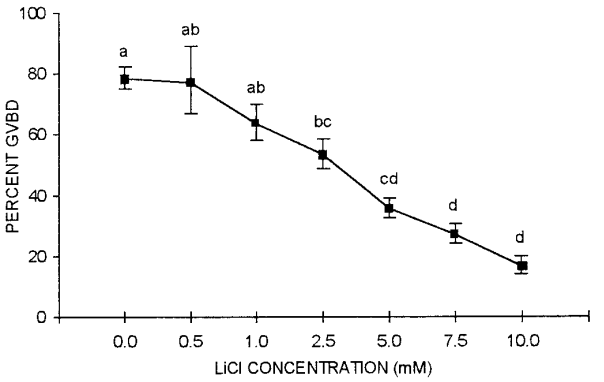

FIG. 1. Effect of LiCl on FSH-induced meiotic resumption. COCs were cultured for 17–18 h in the presence of HX, FSH, and increasing concentrations of LiCl. Data are shown as the mean percentage of GVBD ± SEM of at least three experiments. Groups presenting at least one identical letter are not significantly different.

RESULTS

Effect of LiCl on FSH-Induced Meiotic Resumption

Isolated fully grown oocytes normally undergo spontaneous GVBD at a high rate (see data on spontaneous meiotic resumption). In preliminary experiments, we found that a very high proportion (92.8 ± 3.2%) of COCs were conversely unable to resume meiosis spontaneously in the presence of HX for at least 24 h, consistent with previous reports (Eppig *et al.*, 1985). To mimic meiotic resumption *in vivo*, oocytes were exposed to FSH, as originally described by Downs *et al.* (1988). In these experiments LH was not utilized because in cumulus cells the receptor to this hormone is expressed at very low level (Eppig *et al.*, 1997). After 18 h of incubation, treatment with FSH promoted GVBD in 78.3% of cumulus-enclosed oocytes cultured in the presence of HX. The use of LiCl as an inhibitor of phosphoinositide metabolism in mouse oocytes has been previously reported (Pesty *et al.*, 1994). The effect of this agent on hormone-induced GVBD is summarized in Fig. 1. Culture in the presence of this agent produced an inhibition

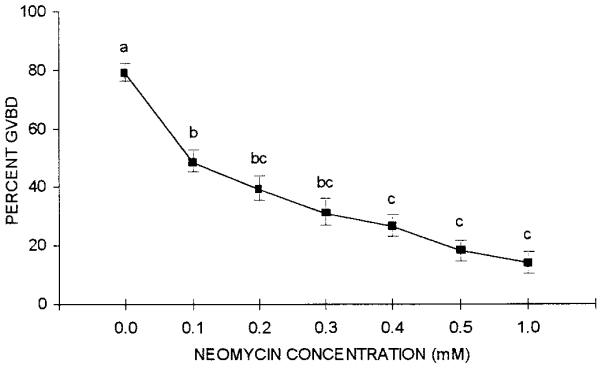

FIG. 2. Effect of neomycin on FSH-induced meiotic resumption. COCs were cultured for 17–18 h in the presence of HX, FSH, and increasing concentrations of neomycin. Data are shown as the mean percentage of GVBD ± SEM of at least three experiments. Groups presenting at least one identical letter are not significantly different.

of meiotic resumption in a dose-dependent manner. Incubation in the presence of 2.5 mM LiCl resulted in a statistically significant ($P < 0.05$) inhibition of GVBD. Approximately a twofold decrease in meiotic resumption was observed following exposure to 5 mM LiCl. Higher doses of the inhibitor, 7.5 and 10 mM, produced a more pronounced effect, GVBD occurring only in 27.3 and 16.4% of oocytes, respectively. In order to rule out any nonspecific effect of LiCl due to increased osmolarity, some control COCs were cultured in medium containing 10 mM NaCl. Under these conditions, meiotic resumption occurred in a proportion (74.6 ± 4.2%) of oocytes comparable to the control group (78.3%).

Neomycin and FSH-Induced Meiotic Resumption

The involvement of phosphoinositide metabolism in hormone-induced meiotic resumption, suggested by the experiments with LiCl, was further tested by culturing COCs in the presence of neomycin, thereby inhibiting phosphatidylinostol 4,5-bisphosphate hydrolysis. Figure 2 illustrates the effect of neomycin on GVBD. Treatment with increasing concentrations of neomycin caused a dose-dependent inhibition of meiotic resumption. A statistically significant difference in comparison to control oocytes was observed at all concentrations tested. A 50% decrease in the rate of GVBD was obtained when oocytes were exposed to 0.2 mM neomycin. At higher doses, 0.5 and 1.0 mM, only 18.3 and 14% of oocytes reinitiated meiosis, respectively.

Intracellular Calcium and FSH-Induced Meiotic Resumption

Inhibition of meiotic resumption caused by LiCl and neomycin suggests that phosphoinositide metabolism is implicated in hormone-mediated meiotic resumption. Hydrolysis of phosphatidylinositol 4,5-bisphosphate generates two distinct messengers: $InsP_3$, an intracellular Ca^{2+}-releasing agent, and diacylglycerol, a positive regulator of calcium-dependent protein kinase C (PKC). In further experiments we tested the hypothesis that intracellular calcium plays a role in hormone-induced GVBD, downstream to phosphoinositide hydrolysis. The effect of chelation of intracellular calcium with BAPTA/AM is described in Fig. 3. The proportion of oocytes in the control group, cultured in the presence of the vector, DMSO and pluronic, that underwent GVBD (79.2%) following FSH stimulation was very similar to that of the control groups of the other experiments. In a fashion similar to the experiments conducted in the presence of LiCl and neomycin, BAPTA/AM produced a dose-dependent inhibition of meiotic resumption. A statistically significant decrease in the ability of oocytes to resume meiosis ($P < 0.001$) was observed with 10 μM BAPTA/AM. Further increase in chelator concentration severely repressed reinitiation of meiosis. Chelation of intracellular calcium also appeared to reduce the proportion of oocytes which extruded the first polar body after resuming meiosis (data not shown).

Spontaneous Meiotic Resumption

Fully grown oocytes released from large antral follicles spontaneously reinitiate the meiotic cycle. *A priori*, it might be argued that, under the conditions used in these experiments, interference with phosphoinositide metabolism and intracellular calcium negatively affects the oocyte cell cycle irrespective of hormone stimulation. To test this possibility, COCs were cultured under conditions which permit spontaneous meiotic resumption; i.e., in the absence of HX and FSH, but in the presence of levels of LiCl,

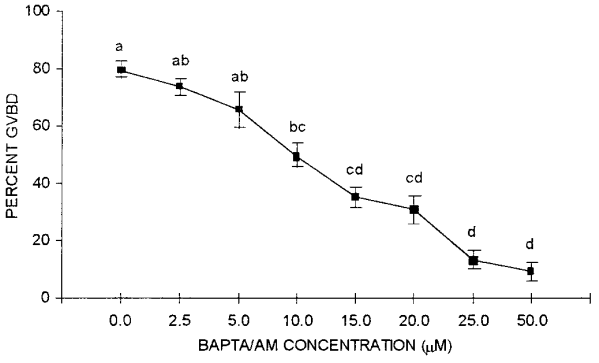

FIG. 3. Effect of treatment with BAPTA/AM on FSH-induced meiotic resumption. COCs were incubated with increasing concentrations of the calcium chelator for 30 min immediately after isolation and cultured for 17–18 h in the presence of HX and FSH. Data are shown as the mean percentage of GVBD ± SEM of at least three experiments. Groups presenting at least one identical letter are not significantly different.

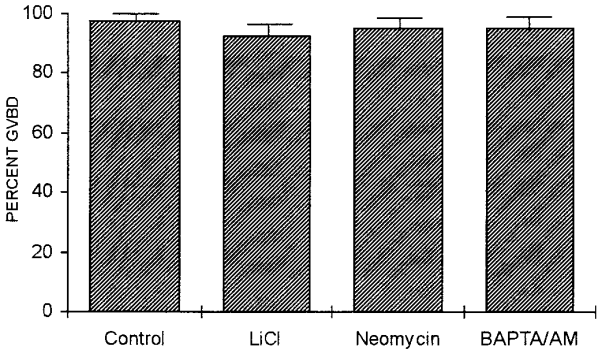

FIG. 4. Effect of treatment with LiCl (10 mM), neomycin (1 mM), or BAPTA/AM (25 μM) on spontaneous meiotic resumption. COCs were exposed to LiCl or neomycin throughout the culture period (17–18 h). COCs were loaded with BAPTA/AM for 30 min immediately after isolation, prior to culture for 17–18 h. Data are shown as the mean percentage of GVBD ± SEM of at least three experiments. No statistically significant difference was found between test groups and the control group.

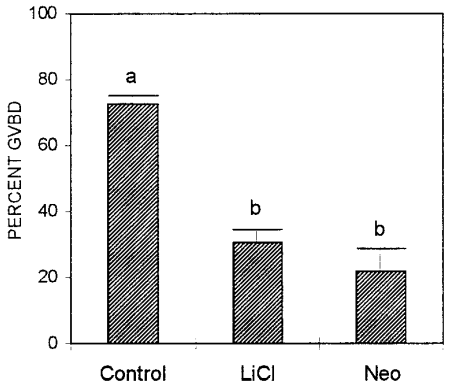

FIG. 5. Effect of treatment with LiCl (5 mM) and neomycin (0.3 mM) on EGF-induced meiotic resumption. COCs were cultured for 17–18 h with the growth factor (1 ng · ml^{-1}) in the continued presence of HX, without inhibitor (control) or with LiCl or neomycin. Data are shown as the mean percentage of GVBD ± SEM of at least three experiments. Groups presenting different letters are significantly different.

neomycin, or BAPTA/AM which severely repressed FSH-induced GVBD. As shown in Fig. 4, in comparison to the control group none of these agents produced a decrease in the proportion of oocytes that resumed meiosis, suggesting that their action is specific to FSH-stimulated GVBD.

Effects of LiCl and Neomycin on EGF-Induced Meiotic Resumption

It has been shown that EGF is able to reproduce the same effect of FSH on mouse oocytes, inducing meiotic resumption and overriding the inhibitory effect of HX through a mechanism mediated by the action of cumulus cells (Downs *et al.*, 1988). While it is generally thought that FSH affects the function of target cells mainly via a cAMP-mediated process, in contrast, response to EGF is more likely to involve a transduction mechanism based on phosphoinositide metabolism. For this reason, LiCl and neomycin were tested for their ability to repress meiotic resumption induced by EGF in the continued presence of HX (Fig. 5). In the control group, cumulus-enclosed oocytes treated with the growth factor resumed meiosis in a proportion (72.6%) comparable to the frequency of GVBD induced by FSH. More importantly, both LiCl and neomycin caused a pronounced decrease in the ability of oocytes to respond to EGF at doses producing half-maximal inhibition in the FSH experiments, supporting the view of an involvement of phosphoinositide metabolism in ligand-induced meiotic resumption.

Effects of LiCl and Neomycin on 8-Br cAMP-Induced Meiotic Resumption

Similarly to FSH and EGF, administration of high doses of cAMP analogues in a pulsatile fashion has been shown to promote GVBD in HX-arrested COCs, but not in denuded oocytes, leading to the original hypothesis that positive stimulation of meiotic resumption is based on a cAMP-mediated mechanism (Downs *et al.*, 1988; Downs and Hunzicker-Dunn, 1995). We assessed the effect of LiCl and neomycin on 8-Br cAMP-induced meiotic resumption (Fig. 6). COCs were exposed to a 3-h pulse of a high concentration of cAMP analogue (1 mM) and subsequently transferred to analogue-free medium, in the continued presence

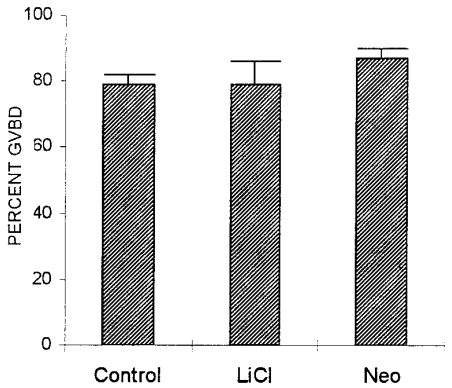

FIG. 6. Effect of treatment with LiCl (5 mM) and neomycin (0.3 mM) on 8-Br cAMP-induced meiotic resumption. COCs were pulsed with a high concentration (1 mM) of the analogue for 3 h and then transferred to analogue-free medium. Culture was conducted in total for 17–18 h in the continued presence of HX, without inhibitor (control) or with LiCl or neomycin. Data are shown as the mean percentage of GVBD ± SEM of at least three experiments. No statistically significant difference was found between test groups and the control group.

of HX. In the control group, 8-Br cAMP promoted GVBD at a high rate (79.3%). But, in contrast with the FSH and EGF experiments, LiCl and neomycin failed to inhibit meiotic resumption. This result suggests that positive induction of meiotic resumption may occur through different mechanisms. Importantly, with particular relevance to this study, it also implies that LiCl and neomycin do not produce aspecific cytotoxic effects which might in principle compromise functions of cumulus cells or the oocyte essential to positive induction of GVBD. In the mouse in particular, the integrity of gap junction communication between cumulus cells and the oocyte has been shown to be essential to FSH-mediated meiotic resumption. (Fagbohun and Downs, 1991).

DISCUSSION

In mammals the role of phosphoinositide metabolism and calcium in oocyte meiotic resumption has not been established. A number of studies have focused on the function of intracellular messengers using systems in which oocytes were induced to mature spontaneously. Spontaneous maturation fundamentally involves removal of the inhibitory influence imposed by the follicular environment. However, this model does not reproduce accurately the normal mechanism operating *in vivo,* and therefore it does not appear to be the most appropriate approach. In contrast, systems in which meiotic maturation is induced by gonadotrophins or other agents under conditions which prevent spontaneous maturation offer a more physiological model in which reinitiation of meiosis, as it presumably occurs *in vivo,* is activated by a signal which requires the interaction between the oocyte and follicle cells.

The data presented in this work imply that hormone-induced meiotic resumption in mouse oocytes may be mediated by phosphoinositide metabolism and intracellular calcium. In a first series of experiments, we observed that LiCl, which affects the activity of the *myo*-inositol-1-phosphatase and other enzymes which hydrolyze inositol phosphates, suppresses the ability of fully grown oocytes to undergo FSH-promoted GVBD in the continued presence of HX. Increasing concentrations of LiCl produced a dose-dependent suppression of meiotic resumption, with maximal inhibition at 10 mM levels. The experiments conducted in the presence of neomycin, which binds to phosphatidylinositol 4,5-bisphosphate thereby preventing its hydrolysis, further support the view that FSH-induced GVBD requires activation of the phosphoinositide pathway. However, it cannot be ruled out that neomycin may repress the reinitiation of meiosis via a different mechanism, by preventing the influx of calcium through the plasma membrane. Inhibition by neomycin was dose-dependent and statistically significant at all the concentrations tested. Maximal inhibition was observed at 1 mM levels, at which the great majority of oocytes were arrested at the GV stage. Interestingly, the highest concentrations of LiCl and neomycin resulted in a very pronounced level of inhibition (16.4 and 14% rates of GVBD, respectively). Therefore, on the basis of these results, it would appear that FSH action is mainly, if not entirely, mediated by the phosphoinositide pathway.

LiCl and neomycin were also tested for their ability to affect meiotic resumption mediated by EGF in HX-arrested cumulus-enclosed oocytes. This growth factor does not play a physiological role in promoting the reinitiation of meiosis. Nevertheless it has been found to act in a fashion similar to FSH in the induction of GVBD *in vitro* and it is believed to trigger phosphoinositide metabolism in target cells. The two inhibitors clearly showed the ability to repress EGF-mediated meiotic resumption, confirming the FSH experiments.

In mouse COCs it has been found that GVBD can be positively promoted by stimulation of cumulus cells with pulses of cAMP analogues (Downs *et al.,* 1988; Downs and Hunzicker-Dunn, 1995). In our experiments LiCl and neomycin failed to repress 8-Br cAMP-mediated stimulation of meiotic resumption in HX-arrested oocytes. This suggests that phosphoinositide metabolism is not involved downstream of the stimulation of cumulus cells with high cAMP levels and that different stimuli which trigger the reinitiation of the meiotic cycle may activate alternative pathways. Furthermore, this experiment also rules out the possibility that the two agents can cause inhibition of stimulus-mediated GVBD by producing cytotoxic effects which are not compatible with normal cumulus cell or oocyte function but vice versa indicates that the inhibitory effect is specific to FSH and EGF action.

Concentrations of LiCl and neomycin generating maximal inhibition were also tested in experiments in which oocytes were cultured under conditions which permit spontaneous maturation. In this case, the two test groups underwent GVBD at a very high rate, similar to the control group, suggesting that spontaneous maturation occurs irrespective of inhibition of phosphoinositide metabolism, at least under the conditions used in these experiments. In contrast with our experiments, other studies (Pesty *et al.,* 1994) suggest that LiCl affects mouse spontaneous oocyte maturation. In those experiments, a high concentration of the inhibitor (30 mM) produced a suppression of GVBD, whereas a lower concentration, 10 mM (the highest dose tested in our work), produced only a delay in GVBD which was not apparent after 8 h of culture. It is worth noting that different conditions were applied in those experiments, such as culture in the presence of FCS and in the absence of surrounding cumulus cells, which are known to affect profoundly the process of meiotic maturation.

An important question is whether in the system we used phosphoinositide metabolism is induced in follicle cells or the oocyte during hormone-induced meiotic resumption. It has been shown that LH can promote elevation of $InsP_3$ (Davis *et al.,* 1986; Dimino *et al.,* 1987; Sadighian *et al.,* 1989) in granulosa cells. This is also supported by the

finding that stimulation of the cloned murine LH receptor leads to stimulation of adenylylcyclase as well as formation of inositol phosphates (Gudermann *et al.,* 1992). Based on this evidence, it is tempting to suggest that in our system FSH may induce activation of the phosphoinositide pathway and possibly changes in intracellular calcium in cumulus cells. This is further supported by the finding that EGF, which most likely activates phosphoinositide turnover in target cells and is sensitive to LiCl and neomycin inhibition, requires the mediation of the cumulus cells to promote GVBD (Downs *et al.,* 1988). However, the action of $InsP_3$ may not necessarily be confined to cumulus cells. In the mouse, the integrity of cumulus cell–oocyte gap junctions has been shown to be essential to FSH-stimulated GVBD (Fagbohun and Downs, 1991). This suggests that cumulus cell–oocyte gap junctions may provide a route through which a positive stimulus, such as $InsP_3$, calcium or other small molecules, may be conveyed into the oocyte, thereby inducing the resumption of meiosis (Eppig, 1991, 1993; Homa *et al.,* 1993).

We also decided to test the hypothesis that calcium is involved in meiotic resumption since, in principle, the original signal derived by phosphoinositide hydrolysis can be potentially transmitted through changes in intracellular levels of calcium. Experiments aiming to investigate the role of PKC, which is also activated by phosphoinositide hydrolysis, were not performed because this pathway has been shown to inhibit, rather than promote, meiotic resumption (Bornslaeger *et al.,* 1986; Carroll and Swann, 1992). There is evidence that calcium mobilization can occur in granulosa cells in response to LH (Gudermann *et al.,* 1992; Mattioli *et al.,* 1991, 1998). This indicates that FSH may trigger this signal in cumulus cells during meiotic resumption. However, similar to the case of phosphoinositide metabolism, calcium may also play a role within the germ cell. In mouse oocytes, cytosolic calcium oscillations occur during spontaneous meiotic resumption, before the GV breaks down (Carroll and Swann, 1992). These calcium transients appear to be generated by increased production of $InsP_3$, since by injecting exogenous $InsP_3$ new oscillations can be generated in oocytes which have ceased to exhibit spontaneous oscillations. This view is also supported by the finding that heparin, an $InsP_3$ receptor antagonist, blocks spontaneous oscillations (Carroll and Swann, 1992). Interestingly, in the mouse the mechanism of spontaneous calcium oscillations is developed during oocyte growth and is not observed in immature oocytes which are unable to undergo spontaneous maturation (Carroll *et al.,* 1994).

More recently, it has been found that injection of $InsP_3$ promotes a longer sequence of oscillations in 15-day-old mouse oocytes than in 12-day-old oocytes. In addition, the same treatment increases the rate of GVBD in 15-day-old oocytes, but it does not reproduce the same effect in 12-day-old oocytes (Lefevre *et al.,* 1997). Therefore, based on this evidence, it appears that the ability to generate spontaneous cytosolic calcium oscillations and the acquisition of meiotic competence in mouse oocytes is correlated with the development of the $InsP_3$ pathway. But despite this evidence the involvement of calcium in meiotic resumption is still controversial. BAPTA/AM inhibits GVBD in cow (Homa, 1991) and pig (Kaufman and Homa, 1993) oocytes. Conversely, in other experiments reinitiation of meiosis in mouse oocytes was not affected by chelation of intracellular calcium (Carroll and Swann, 1992) or depletion of extracellular calcium (Tombes *et al.,* 1992). In our experiments, loading oocytes with the intracellular calcium chelator BAPTA/AM produced suppression of FSH-induced GVBD in a dose-dependent fashion. In contrast, spontaneous maturation did not appear to be influenced by chelation of intracellular calcium because oocytes cultured in medium not supplemented with HX underwent GVBD at a very high rate. This discrepancy appears to confirm that spontaneous maturation is probably not an appropriate system for studying the regulation of the meiotic cycle.

In conclusion, our data suggest that in mouse cumulus-enclosed oocytes FSH-induced meiotic resumption requires activation of phosphoinositide metabolism and release of intracellular free calcium. Both somatic cells and the oocyte are potentially sensitive to these messengers, but the experimental design of this study did not allow us to localize precisely the site where this regulation occurs. Our data are not in contrast with the hypothesis that, following positive stimulation by gonadotrophin, $InsP_3$ and possibly calcium may be generated in cumulus cells and subsequently transferred to the oocyte via intercellular communication. In this respect, it is important to note that it has been recently shown that in sheep COCs, LH triggers rises in intracellular calcium which initially develop in the somatic cells and then, provided that gap junction communication is maintained, spread into the oocyte (Mattioli *et al.,* 1998). Nevertheless, other mechanisms may account for the generation of $InsP_3$ and subsequent calcium release in the oocyte. Stimulation of cumulus/granulosa cells may lead to release of factors acting on the oocyte plasmalemma. In this respect, it has been shown that the mouse oocyte can respond to external stimuli, since carbachol, a ligand of a cholinergic muscarinic receptor, stimulates calcium oscillations (Carroll and Swann, 1992). However, the integrity of gap junctions appear to be essential to the induction of GVBD promoted by a positive stimulus (Fagbohun and Downs, 1991), at least in the mouse.

Irrespective of the possible mechanism of production, calcium signaling probably plays a central role in meiotic resumption directly within the oocyte, in view of its function as a universal regulator of the cell cycle (Whitaker and Patel, 1990). Future studies will have the priority, using alternative systems, to focus on the mechanism of action of LH, the physiological trigger of meiotic resumption, as well as the identification of the site where the $InsP_3$/calcium pathway is activated.

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