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Rat oocyte maturation: role of calcium in hormone action

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Summary

We studied the role of extracellular calcium (Ca_0) in oocyte maturation and oocyte–cumulus cells interaction in rat follicles in vitro. Luteinizing hormone (LH) or a gonadotropin-releasing hormone analog (GnRHa) induced full maturation at $[Ca_0] = 1.3$ mM. At $[Ca_0] = 0.6$ mM, maturation induced by LH or GnRHa was inhibited by 65%. Chelatin of $[Ca_0]$ resulted in 45% maturation and neither hormone caused a further increase of maturation. $[Ca_0] = 20$ mM enhanced the response to suboptimal concentrations of GnRHa but inhibited that to LH. Divalent cation ionophores caused $[Ca_0]$-dependent maturation, which was fully inhibited by dibutyryl cAMP. Changes in $[Ca_0]$ also affected oocyte–cumulus interaction. At $[Ca_0] = 1.3$ mM, either LH or GnRHa caused partial dispersion of the cumulus. Chelation of $[Ca_0]$ also resulted in an almost complete dispersion of the cumulus. The ionophores, however, caused maturation with the oocyte–cumulus complex preserved intact. Our data suggest that GnRHa may induce maturation via cAMP-sensitive calcium mobilization into the oocyte–cumulus–granulosa complex.

Introduction

Resumption of meiosis (maturation) is an obligatory step in the preparation of the oocyte for fertilization. LH, the physiological signal for oocyte maturation in vivo (Lindner et al., 1974), also induces maturation of follicle-enclosed oocytes in vitro (Tsafriri et al., 1972).

It has been recently demonstrated that agonist analogs of GnRH also induce maturation of mammalian oocytes both in vivo, in hypophysectomized animals (Corbin and Bex, 1981; Ekholm et al., 1981; Dekel et al., 1983, 1985) and in vitro, in isolated follicles (Hillensjö and LeMaire, 1980; Dekel et al., 1983). The molecular mechanism of this effect of GnRH analogs is unknown. However, in the pituitary gonadotrops, GnRH induces the release of LH via the phosphoinositide–calcium–protein kinase C cascade (Harris et al., 1985; Hirota et al., 1985; Naor et al., 1985).

Calcium is an important participant in the transduction of numerous signals in various tissues and cell types (Berridge and Irvine, 1984). Hence, the role of calcium in oocyte maturation has been studied in invertebrates and amphibians (for reviews see Baulieu et al., 1978; Masui and Clarke, 1979; Morill and Kostellow, 1986) and,

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less extensively, in mammals. In mammalian oocytes, the data are often contradictory and no clear pattern emerges concerning the role of calcium in this process (Tsafriri and Bar-Ami, 1978; Leibfried and First, 1979; Paleos and Powers, 1981; Jagiello et al., 1982; Maruska et al., 1984; Bae and Channing, 1985; Rakowsky, 1986).

Most of the studies mentioned above were on spontaneous, hormone-independent maturation in vitro. We have studied the role of calcium in the induction of maturation using the rat follicle-enclosed oocyte system. In this in vitro system, meiotic arrest is fully maintained in the absence of hormonal stimulation, allowing analysis of the processes controlling hormonally induced maturation. It has been suggested that the maintenance of meiotic arrest is effected by continuous supply of cAMP via gap junctions from the surrounding cumulus and granulosa cells and that meiotic reinitiation is preceded by disruption of this intercellular communication (Dekel, 1986). Our data point to complex involvement of calcium in hormone-induced oocyte maturation and the control of the oocyte–cumulus cells complex organization.

Materials and methods

Cultures

Follicle-enclosed oocytes were isolated from pregnant mare serum gonadotropin (PMSG)-primed sexually immature Wistar rat females and cultured as previously described (Dekel et al., 1983). The desired concentrations of hormones and other agents were included in the culture medium.

Morphological examination

At the end of the indicated periods of incubation the follicles were incised and the cumulus–oocyte complexes recovered and transferred for morphological examination by Nomarski interference contrast microscopy (Dekel et al., 1983). Resumption of meiosis in the individual oocytes was indicated by germinal vesicle breakdown (GVB). Wherever desired, oocytes, with or without the adhering cumulus cells, were photographed using a 40 × magnification objective.

Materials

The following are the agents used and their sources: GnRH analog [D-Ser(*t*-Bu)^6]des-Gly-GnRH-*N*-ethylamide (GnRHa, Hoechst, F.R.G.); ovine LH (oLH, NIH LH S-20); calcium ionophore A23187 (Sigma, U.S.A.) or ionomycin (free acid, Calbiochem, U.S.A.); ethyleneglycol bis-aminoethyl ether *N,N,N′,N′*-tetraacetic acid (EGTA, Sigma). Calcium-channel blockers: verapamil (Ikapharm, Israel), nifedipine (Taro, Israel) or diltiazem (Sigma). Calcium-channel activator BAY-K-8644 (Bayer, F.R.G.); isoquinoline sulfonamide derivative H-7 (a protein kinase inhibitor, Hidaka et al., 1984) (Seikagaku America, U.S.A.); dibutyryl 3′,5′-cyclic adenosine monophosphate (dbcAMP, Sigma).

Results

The effect of changes in [Ca_0] on follicle-enclosed oocytes

Follicle-enclosed rat oocytes cultured in medium that contains physiological [Ca_0] (1.3 mM) are maintained in meiotic arrest. In five experiments (156 oocytes), only 5% or less of the inspected oocytes exhibited GVB and virtually none were observed with the first polar body. Likewise, all the recovered oocytes had a compact enclosure of cumulus cells with a large stalk clearly visible (Fig. 1*A*). Hence, the follicle-enclosed oocyte presents a superior experimental system to investigate changes in function and oocyte–cumulus interaction induced by hormones and other agents.

Decrease of [Ca_0] affects pronounced changes in cultured follicle-enclosed oocytes. When [Ca_0] was decreased, we observed increasing dispersion of the cumulus that culminated in an almost complete loss of the cumulus in all the recovered oocytes when [Ca_0] was chelated by EGTA (Fig. 1*B*). This morphological change was accompanied by a decrease in the recovery of oocytes (40% vs. 77% in control medium) and a high proportion of GVB oocytes (45 ± 10%, Fig. 2). An increase in [Ca_0] to 20 mM, on the other hand, resulted in a modest induction of maturation (14 ± 4%, not shown).

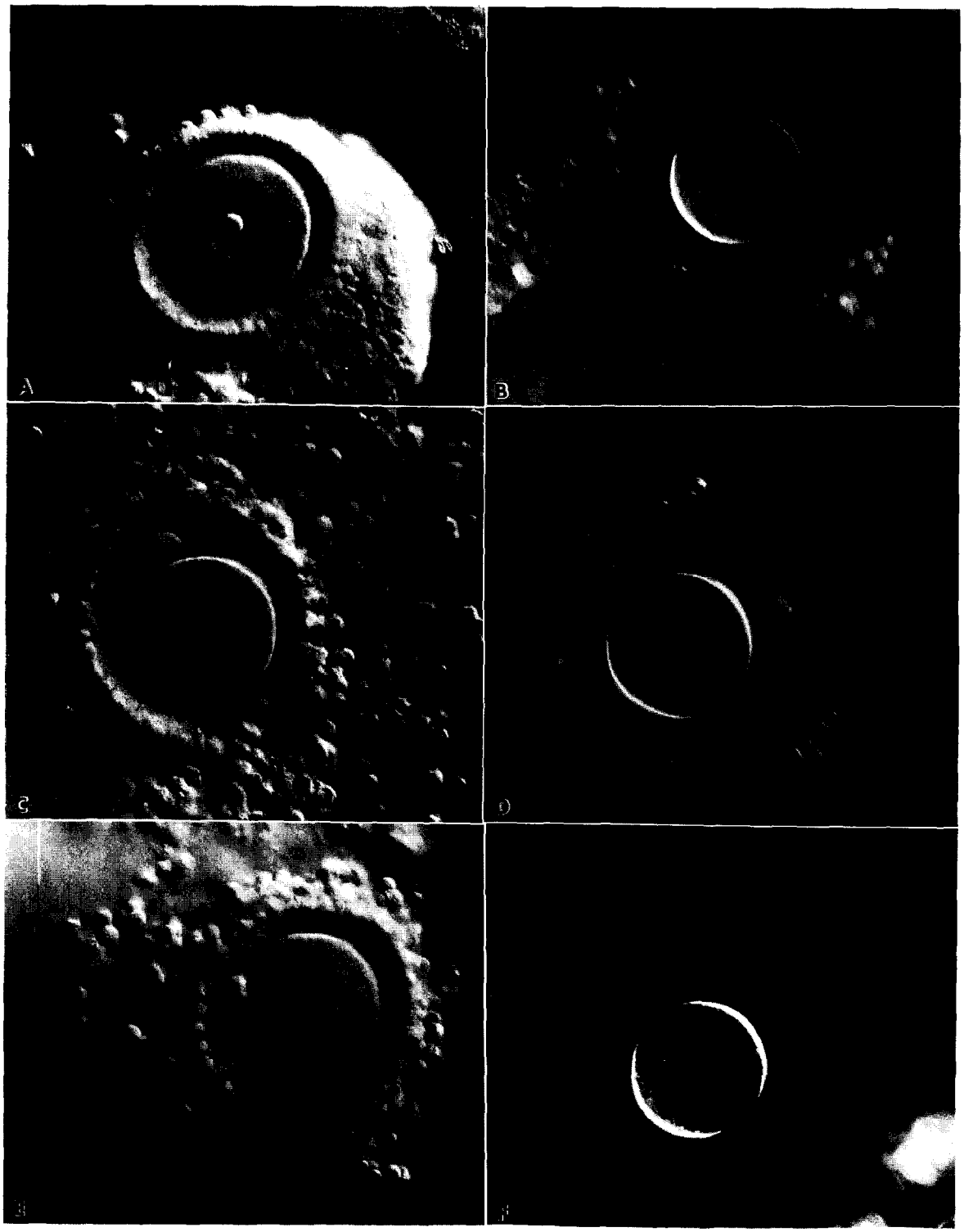


Fig. 1. Typical micrographs of oocyte–cumulus complexes recovered from follicles incubated under the following conditions: *A*: Control, no additions, [Ca_0] 1.3 mM; *B*: EGTA 2 mM; *C*: LH 1 μg/ml, [Ca_0] 1.3 mM; *D*: LH 1.0 μg/ml, EGTA 2 mM; *E*: GnRHa 0.1 μM, [Ca_0] 1.3 mM; *F*: GnRHa 0.1 μM, EGTA 2 mM.

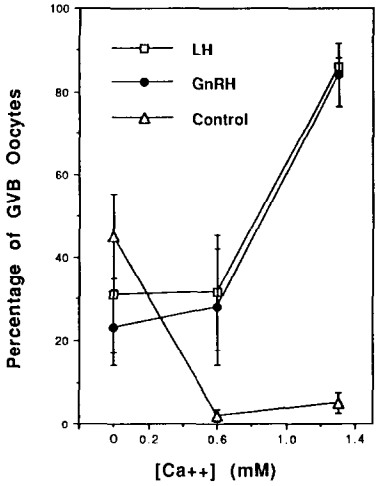


Fig. 2. The effect of lowered $[Ca_0^{2+}]$ onGnRHa- or LH-induced maturation. Isolated follicles were incubated with or without the indicated concentration of GnRHa or LH with 1.3 mM and 0.6 mM of $CaCl_2$, or with 1.3 mM $CaCl_2$ and 2 mM EGTA for 5 h. The oocytes were recovered and examined for maturation as indicated by the disappearance of GV. Between 43 and 156 oocytes were examined for each condition. Error bars indicate SEM.

Hormone-induced maturation of follicle-enclosed oocytes

Exposure of follicle-enclosed oocytes to optimal concentrations of either LH (1 μg/ml) or GnRHa (0.1 μM) resulted in a complete maturation. In three experiments, LH and GnRHa caused $86 \pm 2\%$ and $84 \pm 8\%$ GVB, respectively (Fig. 2). In many oocytes, GVB was accompanied by the extrusion of the first polar body and a partial dispersion of the cumulus in most of the recovered oocytes was observed (Fig. 1*C* and *E*). The action of either hormone required the presence of relatively high $[Ca_0]$. At $[Ca_0] = 0.6$ mM, maturation induced by either LH or GnRHa was inhibited by approximately 65% (Fig. 2). Complete removal of calcium from the medium caused partial maturation (see above) and the addition of hormones did not increase further the proportion of oocytes that exhibited GVB. Actually, the proportion of mature oocytes was slightly lower in the presence of hormones than in follicles cultured in the absence of calcium alone (Fig. 2). Either hormone improved the recovery of oocytes (from 40% to 70% and 55% for LH and GnRHa, respectively), indicating a protective effect of hormonal stimulation. Moreover, despite the partial dispersing effect of LH on cumulus in follicles incubated in 1.3 mM $[Ca_0]$, it, paradoxically, partially reversed the dispersion observed in the absence of $[Ca_0]$ in most oocytes (Fig. 1*D*). This action of LH was not shared by GnRHa (Fig. 1*F*).

The strict requirement for relatively high $[Ca_0]$ for hormone-induced maturation implied a role of $[Ca_0]$ in this process. To further investigate this possibility, we assayed the effect of high $[Ca_0]$ on partial maturation induced by suboptimal concentrations of either hormone. Indeed, 1 nM GnRHa induced $10 \pm 1\%$ GVB at $[Ca_0] = 1.3$ mM but $59 \pm 5\%$ at $[Ca_0] = 20$ mM (Fig. 3). In contrast, elevation of $[Ca_0]$ resulted in a significant inhibition of the action of suboptimal concentration of LH (Fig. 3).

Divalent ionophore-induced maturation

The above-described effects of $[Ca_0]$ on GnRHa-induced maturation suggested that calcium entry alone may cause GVB in mammalian oocytes. To assess this possibility, we promoted calcium entry into cells of the follicle-enclosed oocyte–cumulus–granulosa complex by incubation with the divalent cation ionophores A23187 or ionomycin. Either ionophore in the presence of $[Ca_0] = 1.3$ mM induced rapid and extensive maturation. GVB was observed in 25 ±

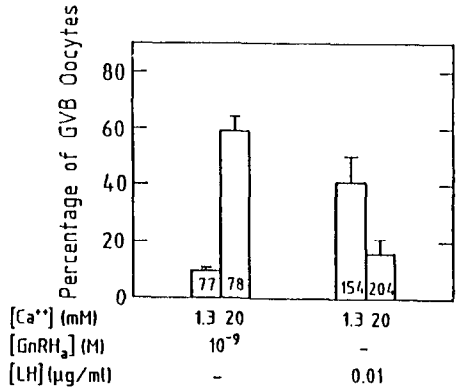
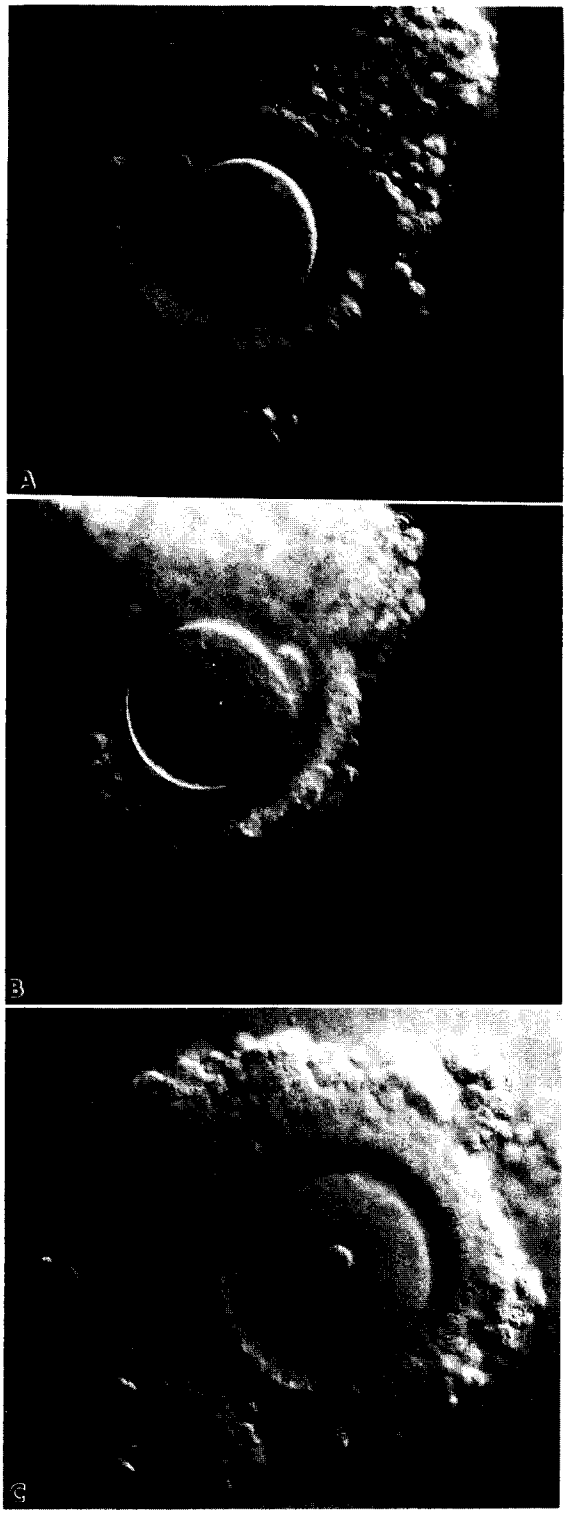


Fig. 3. The effect of elevated $[Ca_0^{2+}]$ on GnRHa- or LH-induced maturation. Isolated follicles were incubated in the presence of the indicated suboptimal concentrations of GnRHa or LH with the indicated concentrations of $CaCl_2$ for 20 h. The presence of GVs in the oocytes was examined. The total number of oocytes examined is indicated. Error bars indicate SEM.



5% and 50 ± 8% of the examined oocytes, for ionomycin and A23187, respectively. Prolonged incubation with the ionophores resulted in numerous oocytes that exhibited first polar body (see Fig. 4*A* and *B*). However, in contrast to oocytes induced to mature by hormones (Fig. 1*C* and *E*), the cumulus of a large proportion of oocytes incubated with the ionophores was extensive and compact, resembling that of the control oocytes maintained at 1.3 mM [Ca_0] (see Fig. 1*A*) or 20 mM [Ca_0] (Fig. 4*C*).

Similarly to the GnRHa-induced maturation, the induction of maturation by ionophores was potentiated by high [Ca_0] (Fig. 5). The ionomycin-induced maturation process was rapid and resulted in almost complete maturation within 2 h (Fig. 6).

The mechanism of GnRHa and calcium-induced maturation

The apparent [Ca_0] dependence of GnRHa-induced maturation suggested that the hormone may produce its effect by mobilization of [Ca_0] and that the ionophores mimic this response by facilitation of direct calcium entry into one, or more, cell types in the follicle. To test the possible conduit of calcium ions, we investigated conditions that affect voltage-sensitive calcium channels. Neither calcium channel antagonists (verapamil, nifedipine or diltiazem at 10 mM each), nor the calcium-channel activator of these channels, BAY-K-8644 (1 mM), antagonized potentiated or mimicked maturation (not shown). We assumed, therefore, that dihydropyridine-sensitive calcium channels are not involved in hormone-induced maturation.

To tentatively identify the possible effector system of the increased cellular calcium, we used H-7, an isoquinoline sulfonamide antagonist of protein kinases of preferred specificity towards protein kinase C. 1 mM H-7 partially inhibited ionophore-induced maturation (by 50%, not shown). To investigate whether calcium-induced

Fig. 4. Ionophore-induced maturation and polar body extrusion at [Ca_0] = 20 mM. *A*: A23187 1 μM; *B*: ionomycin 10 μM; *C*: corresponding control incubated in the absence of A23187 or ionomycin.

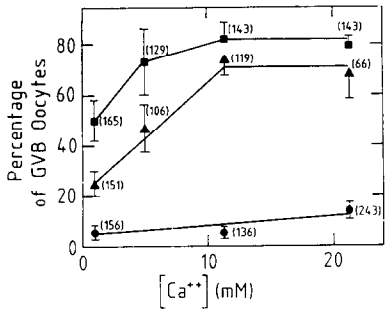


Fig. 5. Effect of $[Ca_0]$ on oocyte maturation. Isolated follicles were incubated in the presence of the indicated concentrations of $CaCl_2$ alone (●), with 1 μM A23187 (■) or 1 μM ionomycin (▲) for 20 h. Cumulus–oocyte complexes were recovered and oocytes examined for GVB. The numbers in parentheses represent the number of oocytes examined at each experimental point. Error bars indicate SE.

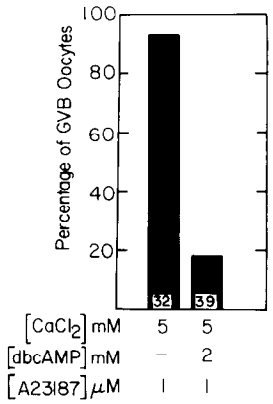


Fig. 7. Effect of dbcAMP on calcium-induced maturation. Isolated follicles were incubated for 20 h in the presence of the indicated concentration of $CaCl_2$ and 1 μM of A23187 with or without 2 mM dbcAMP. The oocytes were recovered and examined for maturation.

maturation by-passes the cAMP-sensitive step in the maturation process, we have incubated follicles with A23187 in the presence of 2 mM of the permeable derivative of cAMP, dbcAMP. This cAMP derivative fully inhibits LH- or GnRH-induced maturation (Dekel et al., 1983). The exposure to dbcAMP also fully inhibited calcium-induced maturation (Fig. 7), implying that calcium acts via a step proximal to that controlled by cAMP.

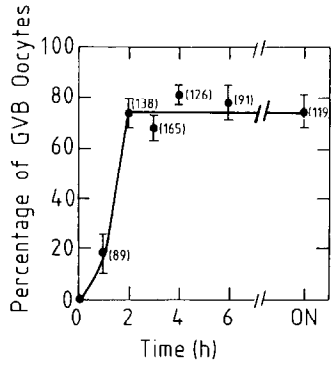


Fig. 6. Time course of $[Ca_0]$-induced maturation. Isolated follicles were incubated in the presence of 10 mM $CaCl_2$ and 10 μM ionomycin for the indicated periods of time. The values in parentheses represent the number of oocytes examined at each experimental condition. Error bars indicate SE.

Discussion

Both LH and GnRHa induce complete maturation (~ 90% GVB) of follicle-enclosed rat oocytes in the presence of physiological concentration of $[Ca_0]$ (1.3 mM). We have previously supplied evidence that LH-induced maturation is associated with elevation of cAMP in the cumulus–granulosa cells surrounding the oocyte in the follicle (Dekel and Sherizly, 1983). We have also demonstrated that the elevation of cAMP caused disruption of communication of the surrounding cells with the oocyte (Dekel et al., 1988). The continuous transfer of cAMP from cumulus–granulosa cells into the oocyte ceases and the level of this nucleotide in the oocyte decreases rapidly due to its breakdown by cAMP phosphodiesterase (Dekel, 1986). A decrease in cAMP triggers a series of as yet unidentified, biochemical events that culminate in oocyte maturation.

Unlike LH, GnRHa induces maturation without raising cAMP in the follicular complex (Dekel et al., 1985). Hence, the mechanism of the action of GnRHa appears to be different, at least initially, from that of LH. GnRH has been shown to act via phosphoinositide–calcium–protein kinase C cascade in pituitary gonadotropic cells (Harris et al., 1985; Hirota et al., 1985; Naor et

al., 1985). In order to examine the differences between LH- and GnRHa-induced maturation, we studied the role of extracellular calcium in hormone-induced maturation. Moreover, since intercellular communication plays a major role in the maintenance of meiotic arrest, we have examined the effect of calcium and hormones on the state of association of cumulus cells with the oocyte.

$[Ca_0]$ alone affects oocyte–cumulus morphology and the proportion of oocytes that undergo maturation. At $[Ca_0] = 0.6–1.3$ mM, oocytes maintain a compact envelope of cumulus cells with clearly discernible stalk. However, complete withdrawal of $[Ca_0]$ causes an extensive dispersion of the cumulus, resulting in almost cumulus-free oocytes that exhibit 45% GVB. This value may be artifactually exaggerated, becuase of the low recovery of oocytes under these conditions. Nevertheless, absence of calcium clearly causes maturation. We suggest that this effect of calcium withdrawal is a result of a complete disruption of intercellular communication.

At $[Ca_0] = 1.3$ mM, either LH or GnRH caused extensive maturation accompanied by a significant dispersion of the cumulus. Even a modest decrease of $[Ca_0]$, to 0.6 mM, extensively inhibited LH- and GnRHa-induced maturation. This implies that maturation induced by either hormone includes a step that required high $[Ca_0]$. More surprising was the finding that LH (though not GnRHa) partially prevented cumulus dispersion caused by chelation of $[Ca_0]$. Moreover, either LH or GnRHa increased the recovery of oocytes under these conditions. We have no explanation for these actions of the hormones. However, neither hormone induces maturation in excess of that caused by withdrawal of $[Ca_0]$ alone. This may indicate that the action of LH or GnRHa is mediated by the surrounding cells in the follicle. Actually, the presence of LH or GnRHa resulted in a lower proportion of GVB than in follicle cultures in the absence of $[Ca_0]$ alone and may have reflected the increased recovery of oocytes in the presence of the hormones.

Elevation of $[Ca_0]$ to 20 mM resulted in inhibition of maturation induced by suboptimal concentrations of LH. Since the action of LH involves elevation of cAMP, this could be due to stimulation of cAMP phosphodiesterase and/or inhibition of adenylate cyclase (Klee et al., 1980; Eckstein et al., 1986). The same concentration of $[Ca_0]$ caused, however, a major potentiation in the action of suboptimal concentration of GnRHa.

Divalent cation ionophores caused rapid and extensive maturation, including the extrusion of the first polar body, and their action was potentiated by increasing $[Ca_0]$. It is noteworthy that, unlike the hormones, the maturation induced by calcium via the ionophores proceeds without a significant dispersion of the cumulus. We assume that either the dispersion of cumulus is not absolutely required for maturation or that enhancement of calcium entry bypasses this step in hormone action.

In conclusion, relatively high $[Ca_0]$ is required for hormone-induced maturation. Chelation of $[Ca_0]$, however, may produce partial maturation as a result of cumulus dispersion. Very high $[Ca_0]$ induces maturation in a small proportion of oocytes and potentiates maturation induced by either ionophores or GnRHa. These complex effects of either depletion or enhanced entry of calcium may have caused the conflicting results obtained by other researchers who studied oocyte maturation. For example, Tsafriri and Bar-Ami (1978) reported that A23187 caused maturation of follicle-enclosed oocytes both in the presence as well as in the absence of $[Ca_0]$. There are also contradictory reports on the effects of calcium depletion on spontaneous maturation of either isolated or cumulus-enclosed oocytes (Leibfried and First, 1979; Paleos and Powers, 1981; Jagiello et al., 1982; Maruska et al., 1984; Bae and Channing, 1985; Rakowsky, 1986).

Our results imply that GnRHa induces maturation by enhancing calcium entry into the cells of the follicle. The target cell population remains unknown, as GnRH receptors have been found both on granulosa (Clayton et al., 1979; Jones et al., 1980) and the oocyte itself (Dekel et al., 1988). If GnRHa indeed causes calcium entry into the relevant compartment of the follicle, our results appear to eliminate voltage-sensitive channels and point to receptor-operated channels as a reasonable possibility. The effector enzyme system that is responsible for calcium- or GnRHa-induced maturation has not been conclusively identified. An inhibitor of protein kinases that is moderately

selective towards protein kinase C partially blocks the action of GnRHa (Dekel et al., 1989) and that of the ionophores. This hypothesis is considerably strengthened by our finding that activation of protein kinase C alone induces maturation of rat oocytes (Aberdam and Dekel, 1985). Recently, Davis et al. (1986) suggested that the action of LH in the ovary may also be mediated by protein kinase C. This may explain the apparent requirement for high [Ca_0] for LH-induced maturation reported here. Hence, although suggestive, protein kinase C remains a likely suspect, at best.

Despite the apparent differences in the action of LH and GnRHa, do they represent two discrete pathways of induction of maturation? Recently, Gelerstein et al. (1988) suggested that maturation of *Xenopus* oocytes may proceed via cAMP-dependent as well as cAMP-independent pathways. Elevation of oocyte cAMP (via the permeable analog dbcAMP) blocks both GnRHa- and calcium-induced maturation. Hence, GnRHa may initially induce maturation by increased calcium entry and activation of protein kinase C, but it shares a common step with LH-induced maturation that is cAMP-sensitive and distal to the step mediated by calcium.

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