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Regulation of Intracellular Calcium in the Mouse Egg: Calcium Release in Response to Sperm or Inositol Trisphosphate Is Enhanced after Meiotic Maturation[1]

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ABSTRACT

Fertilization of the immature, prophase I-arrested mouse oocyte produces multiple Ca^{2+} transients similar to those of the mature, metaphase II egg; however, the first Ca^{2+} transient is much lower in amplitude and shorter in duration. In contrast to prophase I-arrested oocytes, maturing oocytes fertilized after germinal vesicle breakdown have first Ca^{2+} transients similar to those of mature fertilized eggs. Immature, prophase-arrested oocytes release less Ca^{2+} in response to injection of inositol 1,4,5-trisphosphate (IP_3) than eggs. At high concentrations, the sulfhydryl reagent, thimerosal (200 μM), causes Ca^{2+} oscillations in eggs and produces similar oscillations in oocytes. A lower concentration of thimerosal (25 μM) does not cause Ca^{2+} oscillations, but does sensitize IP_3-induced Ca^{2+} release in both eggs and oocytes, since IP_3-induced Ca^{2+} release is enhanced in the presence of 25 μM thimerosal. Incubation of oocytes in 25 μM thimerosal before injection of 2.2 μM IP_3 causes oocytes to release as much Ca^{2+} as is released in eggs injected with 2.2 μM IP_3. These results indicate that immature mouse oocytes possess intracellular stores of releasable Ca^{2+} similar in size to Ca^{2+} stores in eggs; however, these stores are less sensitive to IP_3. Development of the IP_3-induced Ca^{2+} release mechanism may be an important component of maturation; at fertilization of the egg, Ca^{2+} must be elevated to levels sufficient to activate further development and establish a block to polyspermy. Mouse oocytes appear to develop an increased sensitivity to IP_3 during the course of oocyte maturation.

INTRODUCTION

It is well established that an increase in intracellular free Ca^{2+} is the principal signal responsible for egg activation at fertilization [1–6]. The importance of Ca^{2+} is illustrated by the fact that both cortical granule exocytosis and resumption of meiosis are prevented in mouse eggs when the increase in intracellular Ca^{2+} ($[Ca^{2+}]_i$) is prevented [7]. It is less clear whether a change in $[Ca^{2+}]_i$ has a role in mammalian oocyte maturation. The mammalian oocyte is arrested at prophase of the first meiotic division and must undergo a maturation process that produces a mature oocyte arrested at second metaphase of meiosis. In this paper, we refer to immature, prophase I-arrested oocytes or maturing oocytes as "oocytes." To distinguish the fully mature oocyte from immature oocytes, we refer to metaphase II-arrested oocytes as "eggs." Fertilization of the egg normally occurs at second metaphase. Oocyte maturation is a complex process involving release from prophase arrest and breakdown of the germinal vesicle (GV), as well as cytoplasmic changes necessary for subsequent fertilization and early embryonic development [8, 9]. In vivo, mammalian oocyte maturation is triggered by a preovulatory surge of LH [10], and maturation is regulated, at least in part, by cAMP, which serves to maintain meiotic arrest ([11–14], reviewed in [8]). However, there is evidence that other intracellular signalling agents, such as protein kinase C [15, 16] and calmodulin [17], may also be important for oocyte maturation. There is also evidence that Ca^{2+}, too, may play a role in the maturation of hamster [13], porcine [18], and bovine [19] oocytes (reviewed in [20]). Investigations to ascertain the role of Ca^{2+} in oocyte maturation in the mouse have used isolated oocytes which mature spontaneously when released from the follicle in the absence of an LH surge. Some reports suggest that an increase in $[Ca^{2+}]_i$ is not required for spontaneous oocyte maturation [21, 22], while another study has indicated that an increase in Ca^{2+} is necessary for oocyte maturation [23]. In this paper, we present data to show that an increase in $[Ca^{2+}]_i$ is not required for spontaneous maturation of mouse oocytes.

Although the intracellular signals initiating oocyte maturation are not completely understood, it is clear that many changes take place during maturation which allow eggs to undergo normal fertilization, activation, and subsequent development. Particularly important are changes associated with the regulation of $[Ca^{2+}]_i$. Frog oocytes, for example, undergo a complex rearrangement of endoplasmic reticulum during maturation [24–27]; this rearrangement is thought to be associated with the development of a calcium store that releases Ca^{2+} during fertilization or artificial activation of the egg [25]. Likewise, starfish oocytes develop the capability to produce a fast polyspermy block [28] and the ability to decondense sperm nuclei and form asters during maturation [29]. The polyspermy block and sperm decondensing competence are associated with the magnitude of Ca^{2+} released at fertilization, suggesting a modification of Ca^{2+} stores or Ca^{2+} release mechanisms during starfish oocyte maturation.

Direct evidence for alteration of calcium stores or release mechanisms during maturation has been obtained for two species. Maturing starfish and hamster oocytes exhibit an increasing sensitivity to inositol 1,4,5-trisphosphate (IP_3);

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the IP_3-induced Ca^{2+} release mechanism is poorly developed in prophase I-arrested oocytes [30, 31]. IP_3-induced Ca^{2+} release has been shown to be the principal, and probably only, Ca^{2+} release mechanism responsible for Ca^{2+} transients during fertilization of hamster and mouse eggs [3, 32, 33].

Cortical granule exocytosis does not occur in prophase I-arrested, GV-intact mouse oocytes treated with the ionophore A23187 to release Ca^{2+} from intracellular stores, suggesting that the immature mouse oocyte contains an inadequate Ca^{2+} store [34, 35]. This is supported by direct measurement of Ca^{2+} release in immature oocytes during application of the Ca^{2+} ionophore, ionomycin; Ca^{2+} is elevated to about one-fourth the level seen in eggs [22]. The competence of mouse oocytes to undergo cortical granule exocytosis may be correlated with an increase in cortical membrane vesicles. These membrane vesicles are three times more abundant in the cortex of eggs than in oocytes and may represent an elaboration or modification of a Ca^{2+} store [36]. Normal fertilization responses of the mouse egg appear to require some modification of Ca^{2+} stores during oocyte maturation. We examined the magnitude of Ca^{2+} release in the immature and maturing mouse oocyte in response to injection of IP_3, which is known to elevate $[Ca^{2+}]_i$ [21, 37], and compared this response to the Ca^{2+} release initiated by sperm and calcium ionophore. Further, we compared Ca^{2+} release induced by IP_3, sperm, and ionophore in oocytes with that induced in eggs. Our data suggest that mouse oocytes develop an increased sensitivity to IP_3 during maturation.

MATERIALS AND METHODS

Media and Reagents

The media used for cell culture were MEM and in vitro fertilization medium (IVF) [38]. MEM consisted of Minimum Essential Medium with Earle's salts, L-glutamine, nonessential amino acids, 120 U/ml penicillin G (potassium salt), 50 μg/ml streptomycin sulfate, 0.24 mM sodium pyruvate, 0.1% polyvinyl alcohol (PVA), and 20 mM Hepes, pH 7.2. The composition of IVF was 99.3 mM NaCl, 2.7 mM KCl, 1.8 mM $CaCl_2$, 0.5 mM $MgCl_2$, 0.36 mM NaH_2PO_4, 25.0 mM $NaHCO_3$, 25.0 mM sodium lactate, 0.5 mM sodium pyruvate, 5.6 mM glucose, 100 U/ml penicillin G (potassium salt), 50 μg/ml streptomycin sulfate, 0.001% phenol red, and 3% BSA. Ca^{2+}/Mg^{2+}-free IVF consisted of IVF without $CaCl_2$ or $MgCl_2$, with NaCl increased to 102.8 mM; BSA was replaced by 0.1% PVA. The pH of IVF with 3% BSA was 7.4; the pH of IVF with 0.4% BSA or with PVA was adjusted by addition of HCl. Media were made with cell culture reagents and purified water. All chemicals, except where noted, were obtained from Sigma Chemical Co. (St. Louis, MO). MEM was filtered through 0.2-μm Acrodisc filters (Gelman Sciences, Ann Arbor, MI). All media were equilibrated for 1 h or more at 37°C in an atmosphere of 5% CO_2: 95% air.

Preparation of Gametes

Oocytes were obtained from 6–12-wk-old NSA (CF1) female mice (Harlan Sprague-Dawley, Indianapolis, IN), 44–48 h after i.p. injection of 10 IU eCG. Ovaries were removed and repeatedly punctured with a 26-gauge needle to release oocytes. Oocytes enclosed by cumulus cells were isolated, and cumulus cells were removed by repeated pipetting through a small-bore pipette. Fully grown, GV-intact oocytes, approximately 80 μm in diameter, were collected and thoroughly washed in MEM. When prophase-arrested oocytes were used, 0.1 mg/ml dibutyryl cAMP (dbcAMP) was included in the medium to prevent spontaneous maturation [11]. Except for the experiments presented in Figure 6, dbcAMP was not included in media for experiments using eggs.

Eggs and sperm were collected and prepared as previously described [7]. Briefly, eggs were obtained from CF1 mice after superovulation and were cultured in MEM. The cumulus cells were removed with 0.3 mg/ml hyaluronidase (Type IV-S). For experiments requiring in vitro fertilization, zonae pellucidae were removed by a brief treatment (less than 5 min) with 10 μg/ml α-chymotrypsin (Type II). Sperm were obtained from 14–20-wk-old male NSA (CF1) mice (Harlan Sprague-Dawley) and were capacitated at approximately 2–5 × 10^6 sperm/ml for 1–2 h in IVF. Oocytes, eggs, and sperm were cultured in media under light mineral oil (Fisher Scientific, Pittsburgh, PA) and incubated in a humidified atmosphere of 5% CO_2: 95% air. During injection experiments, oocytes and eggs were placed in a drop of medium under inert dimethylpolysiloxane.

To prevent movement of oocytes and eggs during sperm addition and Ca^{2+} measurements, zona pellucida-free oocytes and eggs were lightly attached to a glass-bottom dish treated with Cell-Tak (Collaborative Research, Bedford, MA). Prior to sperm addition, oocytes and eggs were in IVF without BSA; after sperm addition, the medium contained approximately 0.4% BSA. The final sperm concentration was approximately 2–5 × 10^5 sperm/ml. We confirmed that sperm enter the zona-free, immature mouse oocyte incubated in dbcAMP [39] by preloading oocytes not injected with Fura with the DNA-specific fluorochrome Hoechst 33342 and scoring for fused sperm following insemination [7, 40]. Polyspermy occurs in both zona-free eggs and oocytes. Zona-free eggs generally contain 1–3 fused sperm, while 7–10 sperm typically fuse with immature oocytes. We did not examine the degree of polyspermy in individual oocytes or eggs injected with Fura for Ca^{2+} measurements because Hoechst 33342 cannot be used with Fura.

In experiments that did not involve fertilization, zona-intact oocytes and eggs were held in place with a holding pipette to prevent movement during addition of thimerosal or ionomycin. Thimerosal, dissolved in IVF containing 0.4% BSA, was added to oocytes or eggs, which were in a drop of the same medium. Ionomycin was diluted in Ca^{2+}/Mg^{2+}-

free IVF from a stock in dimethyl sulfoxide (DMSO; Aldrich Chemical Co., Milwaukee, WI) and was added to oocytes or eggs in Ca^{2+}/Mg^{2+}-free IVF. All solutions were added directly to a drop of medium in an equal volume to ensure rapid and thorough mixing.

Intracellular microinjections were made by use of a quantitative direct pressure system [41] in which a small volume of mercury in a micropipette permits controlled injection of picoliter quantities. A beveled micropipette, backloaded with a small amount of mercury, was connected to a micrometer syringe system filled with fluorinert FC-70. Each micropipette was first front-filled with inert dimethylpolysiloxane (silicon oil, viscosity 20 centistokes), then filled with the injection solution, and finally filled with an equivalent volume of silicon oil to form a cap over the injection solution. The relative amounts of oil and injection solution were determined by linear measurement with an ocular reticle during the loading procedure. The silicon oil cap was injected into the oocyte or egg along with the injection solution, and the diameter of the oil drop was measured. The volume of the injected solution is equivalent to the volume of the injected oil cap. We observed the injection in some cases; however, we were also able to inject solutions without simultaneously viewing the eggs and interrupting the Ca^{2+} measurements. "Blind" injections are possible because the injection solution invariably enters the cell after injection of the oil cap since an aqueous solution flows readily out of the pipette once enough pressure is applied to move the silicon oil cap. The injection pipette was usually removed immediately after the injection. Intracellular concentrations were calculated from the concentration and volume of solution injected, assuming uniform distribution and an oocyte or egg volume of 205 pl. Zona pellucida-intact oocytes and eggs, in MEM, received injections of dextran-conjugated Fura-2 (Fura dextran). The final intracellular concentration of Fura dextran in these experiments was 47 μM. This concentration did not appear to cause significant Ca^{2+} buffering, since basal $[Ca^{2+}]_i$ and peak Ca^{2+} ratios were the same as in cells injected with less Fura dextran in other experiments not reported here. Injections of IP_3 were done in IVF containing 0.4% BSA. The injection buffer for IP_3 (D-myo-inositol 1,4,5-trisphosphate; Sigma) was 75 mM KCl, 20 mM Hepes, pH 7.0. Final intracellular IP_3 concentrations of 8.6 nM, 86 nM, and 2.2 μM were achieved by injecting 1.8 pl of 1-, 10-, or 250-μM stock solutions, respectively. To examine the role of Ca^{2+} in spontaneous oocyte maturation, oocytes were collected in a medium containing dbcAMP; $[Ca^{2+}]_i$ was measured as dbcAMP was flushed out of the medium, and measurements were continued for 2–3 h. To further determine whether a rise in $[Ca^{2+}]_i$ is necessary for spontaneous maturation, oocytes were loaded with the cell permeant acetoxymethyl ester of BAPTA (BAPTA AM; Molecular Probes, Eugene, OR) by incubation in 1.0–100.0 μM BAPTA AM for 30 min at 37°C in MEM + 0.1 mg/ml dbcAMP. The loading solution also contained 1.0 μl/ml of a 25% w/w solution of the dispersing agent Pluronic F-127 (Molecular Probes) in anhydrous DMSO (final DMSO ≤ 2 μl/ml). The dbcAMP was removed, and oocytes were later scored for germinal vesicle breakdown (GVBD). The free acid of BAPTA was also injected into arrested oocytes, which were scored for subsequent maturation. A stock solution of 41 mM BAPTA was prepared in water, and 8.0 pl of BAPTA was injected as described above to give an intracellular concentration of 1.6 mM.

Calcium Measurements

A dual monochromator system (Photon Technology International, South Brunswick, NJ) was used for fluorescence recordings as described previously [42]. The fluorescence signal is displayed as the ratio of fluorescence for the 350-nm/385-nm excitation wavelengths after background subtraction. Emitted fluorescence was recorded at each excitation wavelength, and the ratio was calculated twice per second. $[Ca^{2+}]_i$ was estimated from the ratio equation [43]: $[Ca^{2+}]_i = K_d \times (R-R_{min})/(R_{max}-R) \times Sf_2/Sb_2$, where R is the measured ratio, R_{min} and R_{max} are the ratios obtained from standard solutions of EGTA and CaEGTA, and Sf_2/Sb_2 is the ratio of fluorescence at 385-nm excitation for Fura dextran (unbound) to Fura dextran (bound) obtained from standard solutions. The dissociation constant, K_d, was assumed to be 285 nM, a value appropriate for Fura-2 in cells at 37°C [44]. Standard solutions of EGTA and CaEGTA were used to determine R_{min}, R_{max}, and Sf_2/Sb_2 at 37°C. To account for the spectral changes of Fura fluorescence in cells and in salt solutions because of viscosity differences, 2 M sucrose was added to the standard solutions [45]. Because of the various assumptions and reliance on external calibration buffers, the calculated $[Ca^{2+}]_i$ in these cells should be considered approximations rather than absolute values, and relative ratio values are presented rather than $[Ca^{2+}]_i$, although, for reference, the estimated $[Ca^{2+}]_i$ is included in Figure 1. A fluorescence ratio of 1 corresponded to a $[Ca^{2+}]_i$ of 0.5 μM, a ratio of 2 was 1.3 μM, and a ratio of 5 was 4.1 μM. All Ca^{2+} measurements were made from oocytes or eggs held in a warming chamber with laminar flow of 5% CO_2: 95% air to maintain a temperature of 37°C and to prevent pH changes in the medium, which was kept under mineral oil or dimethylpolysiloxane. Statistical comparisons were made by using one-way analysis of variance or Student's *t*-tests (InStat software; GraphPAD, San Diego, CA).

RESULTS

The Role of Ca^{2+} in Oocyte Maturation

Before examining Ca^{2+} stores and release mechanisms in oocytes, we performed experiments to determine whether a change in $[Ca^{2+}]_i$ is required for oocyte maturation. We determined whether Ca^{2+} has a role in cell signaling during spontaneous maturation by measuring $[Ca^{2+}]_i$ during mat-

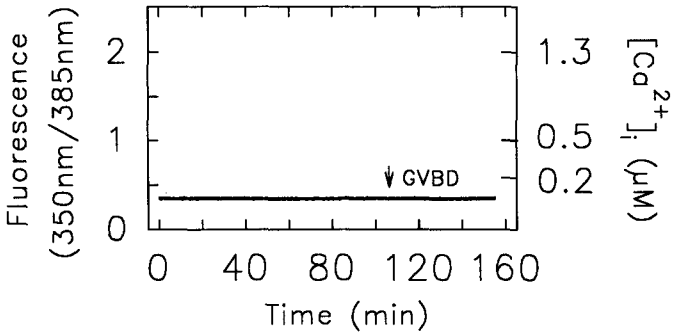

FIG. 1. There is no change in $[Ca^{2+}]_i$ during oocyte maturation. Initially, the oocyte was arrested in prophase of first meiosis by incubation in dbcAMP. $[Ca^{2+}]_i$ was monitored as the medium was replaced with one containing no dbcAMP. Time of GVBD, indicating oocyte maturation, is noted.

uration and by maturing oocytes in vitro while preventing any increase in $[Ca^{2+}]_i$. No change in $[Ca^{2+}]_i$ was observed in any oocytes undergoing spontaneous maturation, through GVBD (n = 4; Fig. 1). Furthermore, introduction of the Ca^{2+} chelator BAPTA to block any rise in $[Ca^{2+}]_i$ did not inhibit GVBD. Oocyte maturation occurred regardless of the concentration of BAPTA AM. Despite incubation in 100 μM BAPTA AM, 100% (7 of 7) of the oocytes underwent GVBD when they were removed from the medium containing dbcAMP. Eighty-two percent of oocytes incubated in 1 μM BAPTA AM underwent GVBD (n = 22), while 78% of oocytes incubated in 10 μM BAPTA AM matured (n = 23). Ninety-five percent of the oocytes not treated with BAPTA AM underwent GVBD (n = 22). As a secondary control for these experiments, 15 eggs were loaded with 1 μM BAPTA AM and were fertilized with sperm; none of these control eggs formed second polar bodies, indicating that the incubation in 1 μM BAPTA AM was sufficient to block the large Ca^{2+} increase at fertilization.

In addition to loading oocytes with BAPTA by incubation in BAPTA AM, the free acid of BAPTA was injected into arrested oocytes and subsequent maturation was examined. When 13 oocytes received injections of a final intracellular concentration of 1.6 mM BAPTA, 11 (85%) underwent GVBD. Because we never observed a change in $[Ca^{2+}]_i$ during oocyte maturation through GVBD, the Ca^{2+} changes described below can therefore be attributed to the direct release of Ca^{2+} in response to sperm, IP_3, or ionophore and not to spontaneous oscillations or changes associated with maturation.

Fertilization of Eggs and Immature Oocytes

Mature mouse eggs produce a series of Ca^{2+} transients at fertilization (Fig. 2A) that may last for several hours [42]. The zona-free, prophase I-arrested mouse oocyte, held in meiotic arrest with dbcAMP, can be fertilized; sperm-oocyte fusion and sperm entry occur, although the sperm nucleus and nuclear envelope remain intact [39]. Arrested oocytes were more polyspermic than eggs. Oocytes typically contained 7–10 fused sperm, while zona-free eggs contained 1–3 fused sperm. Fertilized prophase I-arrested oocytes produced Ca^{2+} transients (Fig. 2B). However, the first Ca^{2+} transient in oocytes was considerably different from that produced by eggs; both the size and duration of the first peak were less in the immature oocytes than in eggs (Table 1). Conversion of the average Fura dextran ratios to estimated $[Ca^{2+}]_i$ revealed that peak Ca^{2+} release in eggs was three times that of immature oocytes. The duration of the first transient was approximately two and a half times longer in eggs than in oocytes. The difference in Ca^{2+} release between oocytes and eggs does not appear to have been due to the degree of polyspermy, since oocytes contained more fused sperm than eggs, yet released less Ca^{2+}. Secondary

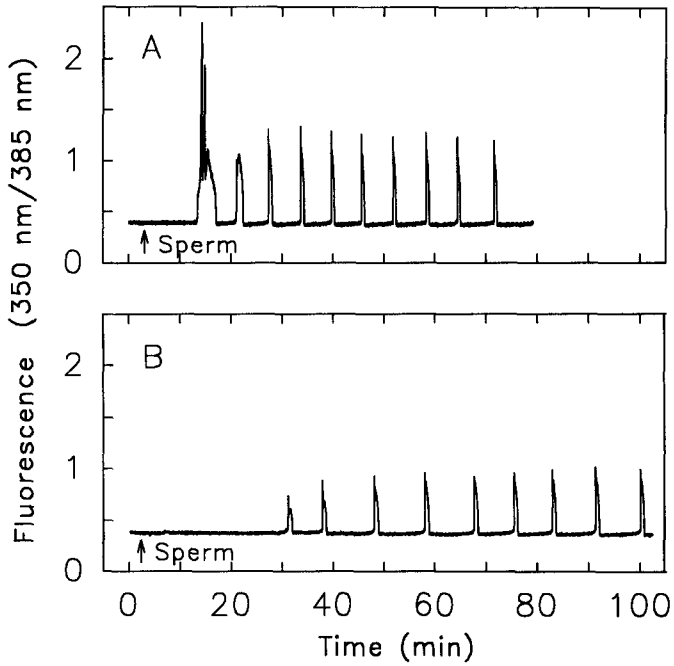

FIG. 2. Comparison of repetitive Ca^{2+} transients in a mature egg (A) and a prophase I-arrested oocyte (B) in response to sperm. Sperm were added to egg and oocyte at times indicated by arrows.

TABLE 1. Duration and peak Fura dextran ratio of the first sperm-induced Ca^{2+} transient in oocytes and eggs.

Experiment	Mean duration[a] (min)	Mean peak Fura dextran ratio (F_{350}/F_{385})[b]
Oocytes	1.4 ± 0.4 (9)*	0.74 ± 0.18 (9)*
Eggs	3.5 ± 1.0 (9)	1.50 ± 0.48 (9)

[a]The duration of the first Ca^{2+} transient is taken beginning on the rising phase at one third of the peak height above baseline and ending at the corresponding point on the falling phase. This represents the broadest part of the Ca^{2+} transient and omits the small and variable, slow rise in Ca^{2+} that precedes the rapidly rising, regenerative increase. Values represent mean and standard deviation. Number of experiments is in parentheses.
[b]Mean ± SD. The mean ratio for basal Ca^{2+} in these experiments was 0.37 ± 0.02 (n = 18).
*Mean duration and peak Fura dextran ratio of immature oocytes, arrested with dbcAMP, are significantly different from means of mature eggs (Student's *t*-test).

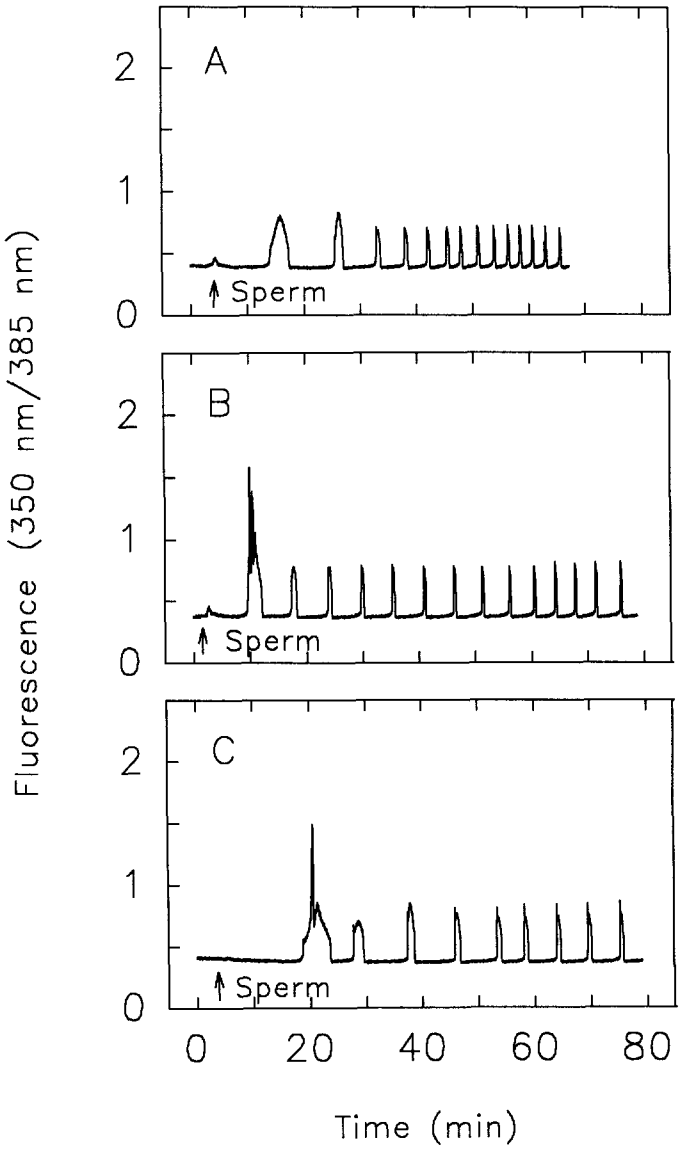

FIG. 3. Ca^{2+} transients in fertilized oocytes at different stages of maturation. Arrows indicate sperm addition. A) GV-intact, prophase I-arrested oocyte 3 h after removal of dbcAMP. B) Oocyte after GVBD (5 h after removal of dbcAMP). C) Oocyte approximately 5 h after GVBD (7 h after removal of dbcAMP).

Ca^{2+} transients in immature oocytes and eggs were similar in magnitude.

To determine whether the store of Ca^{2+} is altered or increased during oocyte maturation, oocytes were fertilized at different times during maturation and the magnitude of the first Ca^{2+} transient was examined. Oocytes were allowed to mature after removal of dbcAMP or allowed to mature spontaneously after release from the ovary. Oocytes fertilized within 2–3 h after release from meiotic arrest produced a series of Ca^{2+} transients similar to those of arrested oocytes (Fig. 3A). The peak magnitude of the first Ca^{2+} transient of oocytes fertilized within 3 h after removal of dbcAMP was not significantly different from that of arrested oocytes (Table 2), and the first Ca^{2+} transient was generally very similar in magnitude to the secondary transients. Oocytes fertilized 3–4 h after release from meiotic arrest had first Ca^{2+} transients similar to those of eggs, as

TABLE 2. Peak Fura dextran ratio of the first sperm-induced Ca^{2+} transient in oocytes inseminated at different times after the onset of maturation.

Time after onset of maturation[a]	Mean peak Fura dextran ratio (F_{350}/F_{385})[b]
—[c]	0.74 ± 0.18 (9)*
1–2 h	0.65 ± 0.10 (5)*
2–3 h	0.83 ± 0.30 (7)*
3–4 h	1.11 ± 0.38 (5)
4–5 h	1.41 ± 0.40 (7)
5–6 h	1.27 + 0.43 (6)
6–7 h	1.38 ± 0.64 (4)
7–8 h	1.64 ± 0.16 (3)
>12 h[c]	1.50 ± 0.48 (9)

[a]Prophase I-arrested oocytes were inseminated in IVF containing dbcAMP. Maturing oocytes were inseminated after removal from the ovary or from medium containing dbcAMP. The data are grouped according to the time at which the first sperm-induced Ca^{2+} transient occurred after the onset of maturation.

[b]Values indicate mean and standard deviation. The number of experiments is shown in parentheses. The average ratio for basal Ca^{2+} in these experiments was 0.37 ± 0.02 (n = 55).

[c]Data taken from Table 1 (oocytes arrested at prophase of first meiosis with dbcAMP and in vivo matured eggs arrested at second meiotic metaphase).

*Mean peak ratios are significantly different, as determined using one-way analysis of variance, from the mean of the > 12 h group (mature eggs). The rest of the means for each group are not significantly different from the mean for mature eggs.

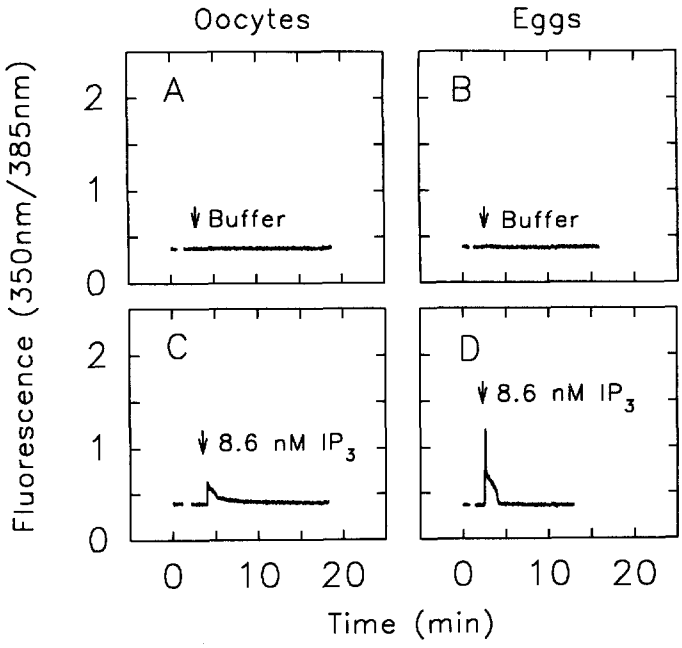

FIG. 4. Ca^{2+} release in prophase I-arrested oocytes and mature eggs in response to injection of IP_3. Control injections of oocyte (A) and egg (B) with IP_3 buffer only. Oocyte (C) and egg (D) injected with 8.6 nM IP_3. All injection times are indicated by arrows. Break in record is time interval during which injection pipette was inserted. Injections were done in IVF containing 0.4% BSA.

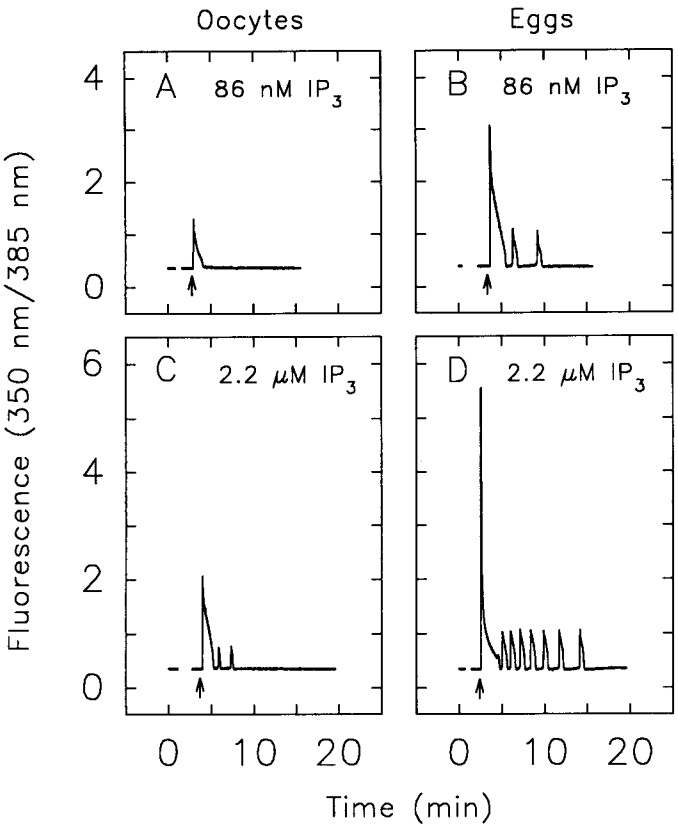

FIG. 5. Ca^{2+} release in prophase 1-arrested oocytes and mature eggs in response to IP_3 injection. A and C) Oocytes injected with 86 nM and 2.2 μM IP_3, respectively. B and D) Eggs injected with 86 nM and 2.2 μM IP_3, respectively. Arrows indicate time of injections. Breaks in records indicate time period in which injection pipette was inserted. Injections were done in IVF containing 0.4% BSA.

did oocytes fertilized at later times (Fig. 3, B and C). The peak Ca^{2+} increase in these groups did not differ in magnitude from that of eggs (Table 2). In addition, during these later time periods, the first transient was generally higher in amplitude than the secondary transients, as seen in eggs.

Ca^{2+} Release Due to Injection of IP_3

Since the initial Ca^{2+} release at fertilization in oocytes was substantially less than in eggs, we examined Ca^{2+} release mechanisms more directly by injecting IP_3. IP_3 was injected into arrested mouse oocytes to determine whether oocytes and eggs release different quantities of Ca^{2+} after injection of the same amount of IP_3. Injection of IP_3 into oocytes produced a dose-dependent release of Ca^{2+} (Table 3; Figs. 4 and 5). Injection of a small quantity of IP_3 (8.6 nM final intracellular concentration) caused Ca^{2+} release in oocytes that was not significantly different from the amount released by eggs. However, injection of 86 nM and 2.2 μM IP_3 resulted in significantly less Ca^{2+} release in oocytes; eggs released nearly three times more Ca^{2+} than did oocytes.

In some cells, cAMP may act directly to reduce the effectiveness of IP_3 in causing Ca^{2+} release by initiating phosphorylation of the IP_3 receptor by cAMP-dependent protein kinase [46–49]. To determine whether diminished Ca^{2+} release in oocytes arrested with dbcAMP might be due to an effect of dbcAMP on IP_3-induced Ca^{2+} release, we injected 2.2 μM IP_3 into immature oocytes and mature eggs in the presence or absence of 0.1 mg/ml dbcAMP. Incubation in

TABLE 3. Comparison of IP_3-induced Ca^{2+} release in oocytes and eggs.

Treatment*	Mean peak Fura dextran ratio (F_{350}/F_{385}) after IP_3 Injection† Oocytes	Eggs
8.6 nM IP_3	0.77 ± 0.30 (8)[a]	1.04 ± 0.50 (8)[a]
86 nM IP_3	1.51 ± 0.51 (9)[a,b]	3.32 ± 0.42 (9)[b‡]
2.2 μm IP_3	1.92 ± 0.49 (11)[a,b]	4.43 ± 0.80 (8)[c‡]
8.6 nM IP_3 + T	1.81 ± 0.22 (10)[a]	2.38 ± 0.38 (10)[d‡]
2.2 μM IP_3 + T	4.01 ± 2.33 (11)[c]	5.20 ± 1.41 (9)[c]

*Final concentration of IP_3 after injection of the prophase I-arrested oocyte or mature egg. "+ T" indicates experiments in which oocytes and eggs were incubated in 25 μM thimerosal and then injected with IP_3.

† Values are mean and standard deviation. The number of experiments is indicated in parentheses. The average ratio for basal Ca^{2+} was 0.38 ± 0.03 (n = 93). Different superscripts within columns denote significant differences ($p < 0.05$), as determined using one-way analysis of variance and Student Neuman Keuls post-hoc tests.

‡ Denote significant differences between oocytes and eggs, as determined by Student's t-tests, for the same treatment.

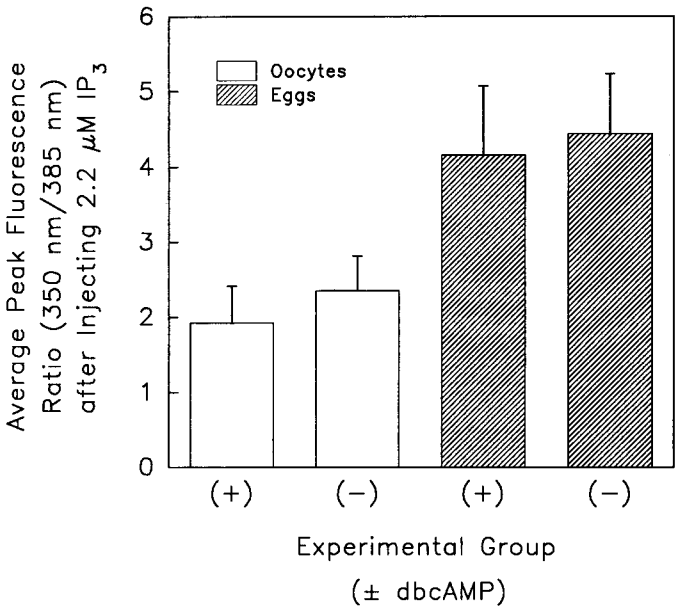

FIG. 6. Incubation in dbcAMP does not significantly alter Ca^{2+} release in GV-intact oocytes or mature eggs injected with IP_3. Average peak fluorescence ratios in oocytes and eggs injected with 2.2 μM IP_3 in presence (+) or absence (−) of 0.1 mg/ml dbcAMP in IVF containing 0.4% BSA. 11 oocytes received injections of IP_3 in medium containing dbcAMP, and 8 GV-intact oocytes received injections of IP_3 within 1 h after removal from the medium containing dbcAMP. Six eggs were examined in presence of, and 8 in absence of, dbcAMP. Whereas incubation in dbcAMP did not significantly alter Ca^{2+} release in oocytes or eggs, calcium release in eggs with or without dbcAMP was significantly greater than in oocytes with or without dbcAMP (Students's *t*-test). Error bars indicate standard deviation. Data showing oocytes that received injections in presence of dbcAMP and eggs that received injections in absence of dbcAMP are taken from Table 3.

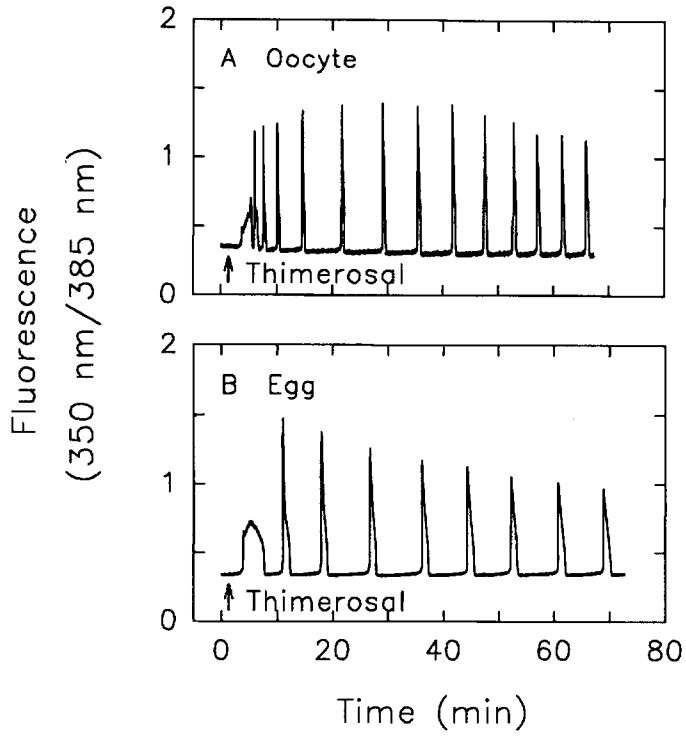

FIG. 7. Thimerosal-induced Ca^{2+} transients in prophase I-arrested oocyte (A) and mature egg (B). Thimerosal (200 μm) was added, at time indicated by arrow, to oocyte or egg, which was in IVF containing 0.4% BSA.

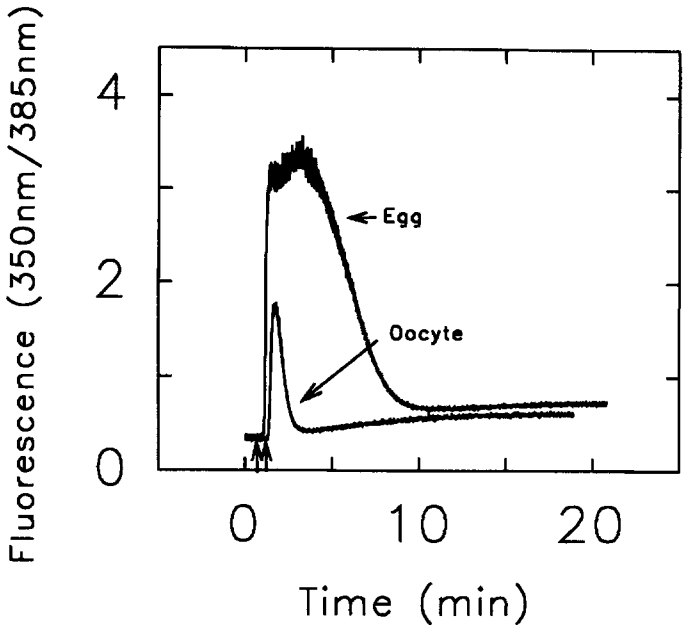

FIG. 8. Intracellular Ca^{2+} release in prophase I-arrested oocyte and mature egg after addition of 5 μM ionomycin. Arrows indicate when ionomycin was added to oocyte or egg. Medium used for this experiment was Ca^{2+}/Mg^{2+}-free IVF.

dbcAMP did not reduce the magnitude of Ca^{2+} release in eggs receiving injections of 2.2 μM IP_3, nor did incubation in dbcAMP significantly alter the average peak ratio in oocytes that had received injections of 2.2 μM IP_3 (Fig. 6). Therefore, the lower amount of Ca^{2+} released by arrested oocytes in response to IP_3 injection was probably not a consequence of including dbcAMP in the oocyte medium, since dbcAMP did not significantly diminish IP_3-induced Ca^{2+} release in either oocytes or eggs. In all other experiments using eggs, dbcAMP was not included in media.

Effect of Thimerosal on Ca^{2+} Release

The sulfhydryl reagent, thimerosal, elicits a series of Ca^{2+} transients in eggs of a number of mammalian species [4, 33, 50–52]. Application of 200 μM thimerosal elevated Ca^{2+} in 9 of 10 immature oocytes arrested with dbcAMP. Repetitive Ca^{2+} transients were initiated by 200 μM thimerosal in most cases (Fig. 7A); however, one oocyte had only one Ca^{2+} transient, while another had two. Application of 200 μM thimerosal produced repetitive Ca^{2+} oscillations in all eggs examined in these experiments (n = 10; Fig. 7B). The Ca^{2+} transients induced by thimerosal in eggs and oocytes were similar in magnitude and duration, although they varied in latency. Generally, a longer latent period was observed in oocytes. In both oocytes and eggs, the first transient, as well as one or two secondary transients, was usually smaller in amplitude than subsequent transients (Fig. 7).

Effect of Thimerosal and IP_3 on Ca^{2+} Release

Thimerosal, at a final extracellular concentration of 25 μM, did not produce Ca^{2+} transients in oocytes or eggs. We incubated oocytes and eggs in 25 μM thimerosal and then injected 8.6 nM or 2.2 μM IP_3. The IP_3-induced Ca^{2+} release in eggs and oocytes was compared (Table 3). Eggs incubated in thimerosal and then receiving injections of 8.6 nM IP_3 released a significantly larger amount of Ca^{2+} than those not treated with thimerosal. Incubation of eggs in 25 μM thimerosal did not significantly enhance the Ca^{2+} release caused by injection of 2.2 μM IP3.

Like eggs, immature oocytes treated with 25 μM thimerosal had, on the average, higher peak Ca^{2+} levels after they had received injections of 8.6 nM IP_3, although in these experiments the increase was not statistically significant (Table 3). Unlike eggs, however, oocytes released significantly more Ca^{2+} after injections of 2.2 μM IP_3 in the presence of 25 μM thimerosal. Oocytes incubated in thimerosal and then receiving injections of 2.2 μM IP_3 released nearly the same amount of Ca^{2+} as eggs receiving injections of 2.2 μM IP_3, whether or not the eggs were preincubated in thimerosal.

Effect of Ionomycin on Ca^{2+} Release in Oocytes and Eggs

Ionomycin (5 μM) was added to oocytes or eggs in Ca^{2+}/Mg^{2+}-free IVF. Ionomycin caused considerably less Ca^{2+} release in oocytes than in eggs (Fig. 8). Both the magnitude and duration of Ca^{2+} release was significantly less in oocytes than in eggs. The average fluorescence ratio at the peak of the ionomycin-induced Ca^{2+} increase in immature

oocytes was 1.40 ± 0.33 (SD, n = 7), corresponding to a peak $[Ca^{2+}]_i$ of 0.8 μM. In comparison, peak Ca^{2+} reached 2.7 μM in eggs treated with ionophore (fluorescence ratio 3.54 ± 0.38, n = 5), a level similar to the peak $[Ca^{2+}]_i$ in eggs that received injections of 2.2-μM IP_3 (see Table 3). The average duration of the Ca^{2+} change in oocytes was approximately 1.2 min, while the average duration in eggs was approximately 4.9 min.

DISCUSSION

The results of our initial experiments confirm previous findings that an increase in $[Ca^{2+}]_i$ is not required for spontaneous oocyte maturation in mice [21, 22]. No change in $[Ca^{2+}]_i$ was seen when $[Ca^{2+}]_i$ was monitored from the start of maturation through GVBD. Spontaneous Ca^{2+} oscillations occur in some hamster oocytes [31] and have been reported to occur in one mouse strain [21]. However, in mice, Ca^{2+} oscillations must not be required for spontaneous oocyte maturation. We, like Tombes et al. [22], did not observe spontaneous Ca^{2+} oscillations in arrested or maturing oocytes. Furthermore, blocking any possible rise in $[Ca^{2+}]_i$ by incubation in BAPTA AM or injection of BAPTA did not prevent GVBD, even when very high concentrations of BAPTA AM and BAPTA were used. Tombes et al. [22] also reported that incubation in BAPTA AM did not prevent GVBD in mouse oocytes, and Carroll and Swann [21] found that inhibition of Ca^{2+} oscillations with BAPTA did not inhibit maturation in mouse oocytes displaying such oscillations. Therefore, an increase in $[Ca^{2+}]_i$ is not associated with spontaneous oocyte maturation. Although a Ca^{2+} rise is not necessary for spontaneous maturation of mouse oocytes after release from the ovary, it is possible that Ca^{2+} plays a part in LH-induced maturation in vivo. Further study is required to examine Ca^{2+}-dependent events in oocytes or granulosa cells that might mediate oocyte maturation in vivo.

The immature mouse oocyte, like the egg, is capable of producing repetitive Ca^{2+} transients when fertilized. The release of Ca^{2+} at fertilization of oocytes and eggs differs: the first Ca^{2+} transient in the egg is much larger. Peak Ca^{2+} release in eggs is approximately three times that of immature oocytes, and the duration of the first transient is more than twice as long in eggs than in oocytes. The first Ca^{2+} transient in oocytes fertilized after GVBD is similar to that exhibited by mature fertilized eggs. In these experiments, 88% (n = 51) of arrested oocytes underwent GVBD within 2 h after removal of dbcAMP. Approximately 3–4 h after the onset of maturation, the magnitude of the first transient is similar to that of eggs. Thus, maturation-induced changes in Ca^{2+} release at fertilization appear within 1–2 h of GVBD.

The Ca^{2+} transients at fertilization are produced by Ca^{2+} release from an IP_3-sensitive Ca^{2+} store. To better understand Ca^{2+} release mechanisms in the oocyte and egg, IP_3 was injected. Injection of IP_3 caused a dose-dependent increase in $[Ca^{2+}]_i$ in oocytes. The amount of Ca^{2+} released in immature oocytes was considerably less than the amount released in eggs. After injection of 86 nM and 2.2 μM IP_3, eggs released nearly three times more Ca^{2+} than oocytes. This suggests that either the mouse oocyte contains a smaller store of releasable Ca^{2+} than the egg, or, if the Ca^{2+} store is the same size, the Ca^{2+} store in the oocyte is less sensitive to IP_3 than that found in the egg.

There is evidence that the sulfhydryl reagent, thimerosal, sensitizes IP_3-induced Ca^{2+} release by increasing the affinity of the IP_3 receptor for IP_3, and, in some cases, causing Ca^{2+} release at basal levels of IP_3 [33, 51, 53–57]. Thimerosal causes Ca^{2+} oscillations in both mouse oocytes [21] and eggs [33], as well as in hamster eggs [50]; in mouse eggs, these oscillations are independent of external Ca^{2+} [33]. We found that 25 μM thimerosal, while not sufficient to produce Ca^{2+} oscillations in oocytes or eggs, did sensitize Ca^{2+} release. Both oocytes and eggs treated with 25 μM thimerosal released more Ca^{2+} after injection of 8.6 nM IP_3 than did untreated cells. Treatment of eggs with 25 μM thimerosal caused a larger Ca^{2+} change following injection of 8.6 nM IP_3. However, treatment of eggs with 25 nM thimerosal did not enhance Ca^{2+} release when 2.2 μM IP_3 was injected. This indicates that, on injection of 2.2 μM IP_3, eggs liberate most of the releasable stored Ca^{2+}. In contrast, oocytes incubated in thimerosal released significantly more Ca^{2+} on injection of 2.2 μM IP_3 than did untreated oocytes that received injections of 2.2-μM IP_3. Moreover, when thimerosal was present, injection of 2.2 μM IP_3 released as much Ca^{2+} in oocytes as in eggs. This suggests that the mouse oocyte contains a large Ca^{2+} store similar in size to that of the egg. The oocyte is less responsive to IP_3, and enhanced IP_3 sensitivity arises during oocyte maturation.

A higher concentration of thimerosal (200 μM) causes Ca^{2+} transients in both oocytes and eggs of the mouse. Thimerosal probably sensitizes IP_3-induced Ca^{2+} release in mouse oocytes as it does in other cells. In hamster eggs, a function-blocking antibody that recognizes an epitope near the Ca^{2+} channel region at the carboxyl terminus of the IP_3 receptor blocks Ca^{2+} transients in response to thimerosal, as well as to IP_3 and sperm [3, 51]. Carroll and Swann [21] reported that 100 μg/ml heparin, a competitive inhibitor of IP_3 binding to the IP_3 receptor, failed to block thimerosal-induced Ca^{2+} oscillations in the mouse oocyte; however, they did not test higher concentrations. It required 1–4 mg/ml heparin to block thimerosal-induced Ca^{2+} release in mature rabbit and mouse eggs [4, 33]. IP_3-induced Ca^{2+} release is the principal, and perhaps only, Ca^{2+} release mechanism utilized at fertilization of hamster and mouse eggs [3, 32, 33]. The ryanodine receptor is not detected by immunoblot analysis of proteins from the hamster egg [32]. Although evidence for Ca^{2+} release mediated by the ryanodine receptor has been reported for eggs of one strain of mouse [58], the eggs of the NSA (CF1) strain used in these experiments do not appear to contain a ry-

anodine-sensitive Ca^{2+} store [33]. Although we cannot rule out the possibility that thimerosal sensitizes an IP_3-insensitive Ca^{2+} store in mouse oocytes, the evidence from studies of mouse and hamster eggs and the data presented in this paper suggest that the IP_3 receptor in oocytes is sensitized by thimerosal, and that thimerosal has effects in oocytes similar to those in eggs.

Hamster [31] and starfish [30] oocytes also release less Ca^{2+} in response to fertilization and to injected IP_3. As in the mouse, the lower amount of Ca^{2+} released in oocytes of these species is due to a reduced IP_3 sensitivity in the oocyte rather than to smaller Ca^{2+} stores. When hamster oocytes received injections of high concentrations of IP_3, Ca^{2+} release was increased to about 80% of that in eggs [31].

Previous work by Tombes and co-workers [22] suggested that the immature mouse oocyte does not contain as much stored Ca^{2+} as the egg and that the Ca^{2+} reserve enlarges during oocyte maturation. Their conclusion was based on results showing that oocytes released only about one fourth of the Ca^{2+} that eggs did in response to the Ca^{2+} ionophore ionomycin. We also found that oocytes released significantly less Ca^{2+} than did eggs upon application of 5 μM ionomycin. In oocytes, peak Ca^{2+} transients caused by application of 5 μM ionomycin or injection of 2.2 μM IP_3 were similar; however, oocytes preincubated with 25 μM thimerosal and then injected with 2.2 μM IP_3 released significantly more Ca^{2+} than oocytes treated with ionomycin.

There are several possible reasons why the ionomycin experiments might not reveal the full size of the Ca^{2+} stores in the oocyte. Because the Ca^{2+} ionophores ionomycin and A23187 are effective in a Ca^{2+}-free medium, the rise in Ca^{2+} is thought to be due to partitioning of the lipid-soluble ionophore in membranes of intracellular organelles as well as in the plasma membrane. It is conceivable that ionomycin partitions differently in the oocyte than in the egg. The magnitude of Ca^{2+} release initiated by ionomycin depends on direct release of Ca^{2+} caused by insertion of the ionophore and any additional Ca^{2+} release through stimulation of Ca^{2+}-induced Ca^{2+} release, which is mediated by the IP_3 receptor [32, 33]. Ducibella et al. [36] reported that oocytes contain substantially fewer membrane-enclosed cortical vesicles than eggs. Therefore, Ca^{2+} stores may be less accessible to ionophore in the oocyte, causing a smaller initial Ca^{2+} increase in oocytes. We also demonstrate in this paper that the oocyte is less sensitive to IP_3 than the egg. Therefore, the reduced Ca^{2+} release in oocytes treated with ionophore may also be due, in part, to the reduced sensitivity of the IP_3-induced Ca^{2+} release mechanism in the oocyte.

The data presented in this paper indicate that the mouse oocyte contains a substantial Ca^{2+} store that is less sensitive to IP_3 than that contained in the egg. Development of the IP_3-induced Ca^{2+} release mechanism during oocyte maturation may result from a change in IP_3 receptor sensitivity, receptor number, or the organization of Ca^{2+} stores. Since the effectiveness of IP_3 in releasing Ca^{2+} is reduced by IP_3 receptor phosphorylation in rat brain microsomes [46], it is possible that the maturational changes we observe are associated with a net dephosphorylation of IP_3 receptors. The IP_3 receptor is phosphorylated by cAMP-dependent protein kinase A [46, 48], protein kinase C, and calcium calmodulin-dependent protein kinases ([59]; reviewed in [49]). Although we found that treatment with dbcAMP did not substantially alter IP_3-induced Ca^{2+} release in the oocyte or egg, it is possible that the IP_3 receptor in the oocyte may be phosphorylated by some kinase.

Since cAMP is responsible for maintaining meiotic arrest, cAMP-dependent phosphorylation of the oocyte IP_3 receptor could be important. However, the function of phosphorylation events in $[Ca^{2+}]_i$ regulation may be more complex, since cAMP-dependent protein kinase phosphorylation can enhance the amount of Ca^{2+} accumulated in the endoplasmic reticulum and consequently enhance Ca^{2+} release despite reduced IP_3 receptor sensitivity [46]. Indeed, in permeabilized hepatocytes, IP_3-induced Ca^{2+} release is enhanced by treatment with 8-bromo-cAMP or the catalytic subunit of cAMP-dependent protein kinase [60]. Further study will be necessary to delineate a role for IP_3 receptor phosphorylation in Ca^{2+} release in oocytes and eggs. In addition, the distribution of IP_3-sensitive stores and the IP_3 receptor will need to be examined. The potential role of membrane bound vesicles, described by Ducibella and his colleagues [36], that appear in greater numbers in the mouse egg cortex after maturation must also be examined.

The rise in Ca^{2+} at fertilization is necessary to initiate resumption of meiosis in the metaphase II-arrested egg. Furthermore, the rise in Ca^{2+} is required for cortical granule exocytosis, which is responsible for the alteration of the zona pellucida that prevents polyspermy [61]. In addition to experiments in which exocytosis is inhibited by preventing the rise in Ca^{2+} [7], the Ca^{2+} dependence of cortical granule exocytosis is illustrated in studies of the hamster egg. Exocytosis is closely linked to the first Ca^{2+} transient: 75% of the cortical granules undergo exocytosis within 13 sec of the first Ca^{2+} transient [62]. Fertilized immature, zona pellucida-intact oocytes are often polyspermic, which may be due to the inability of these oocytes to undergo cortical granule exocytosis [35]. Injection of 1 μM IP_3 fails to stimulate exocytosis in GV-intact mouse oocytes [63]. Exocytosis does occur in response to treatment with 12-*O*-tetradecanoyl phorbol 13-acetate (TPA), indicating that the exocytic machinery is fully competent [63]. The inability of IP_3 to cause exocytosis in the immature oocyte may be due to the diminished IP_3 sensitivity of the oocyte Ca^{2+} release mechanism or due to the spatial organization of Ca^{2+} stores. Complete development of the IP_3-sensitive Ca^{2+} release mechanism in the oocyte is clearly important for activation of the egg at fertilization; mouse oocytes develop an increased sensitivity to IP_3 during oocyte maturation.

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