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Regulation of Intracellular Calcium in the Mouse Egg: Evidence for Inositol Trisphosphate-Induced Calcium Release, but Not Calcium-Induced Calcium Release[1]

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ABSTRACT

Fertilization of the mammalian egg initiates transient and repetitive release of Ca^{2+} from intracellular stores. The mechanism by which these Ca^{2+} transients are produced is not completely known. We examined the role of two principal Ca^{2+} release mechanisms, inositol trisphosphate-induced Ca^{2+} release and Ca^{2+}-induced Ca^{2+} release, in altering intracellular Ca^{2+} in the mouse egg. Microinjection of inositol 1,4,5-trisphosphate (IP_3) transiently elevated intracellular Ca^{2+} and, at higher concentrations, produced repetitive Ca^{2+} transients. Addition of 100 μM thimerosal, a sulfhydryl reagent, caused repetitive Ca^{2+} transients. IP_3 and thimerosal responses were inhibited by prior injection of heparin, a competitive antagonist of IP_3-induced Ca^{2+} release. Addition of caffeine or injection of caffeine, ryanodine, or cyclic ADP-ribose, which are known to initiate or modulate Ca^{2+}-induced Ca^{2+} release in sea urchin eggs and other cells, produced no change in intracellular Ca^{2+}. The response to injection of Ca^{2+} was not altered by prior injection of ryanodine. The magnitude of the Ca^{2+} transients produced by injection of IP_3 was not changed by prior injection of cyclic ADP-ribose or external caffeine. We found no evidence of Ca^{2+}-induced Ca^{2+} release from ryanodine- or caffeine-sensitive stores. It is most likely that release of Ca^{2+} from intracellular stores in the mouse egg is dependent on IP_3-induced Ca^{2+} release.

INTRODUCTION

The fertilizing sperm causes elevation of intracellular free calcium ($[Ca^{2+}]_i$) in mammalian eggs by initiating the repetitive release of Ca^{2+} from intracellular stores [1–4]. The increase in $[Ca^{2+}]_i$ is necessary for activation of development, which includes the exocytosis of cortical granules and the resumption of meiosis [2]. Two principal mechanisms for the release of Ca^{2+} from intracellular stores of cells are inositol 1,4,5-trisphosphate (IP_3)-induced Ca^{2+} release mediated by an IP_3-sensitive Ca^{2+} release channel [5, 6] and Ca^{2+}-induced Ca^{2+} release [7–9] mediated by the ryanodine receptor [10–12]. IP_3 and ryanodine receptors may coexist in some cells [13, 14]; and the relative importance of IP_3-induced Ca^{2+} release and Ca^{2+}-induced Ca^{2+} release, mediated by the ryanodine receptor in the generation of Ca^{2+} transients in eggs, is now being examined. There is good evidence from studies of some species that the fertilizing sperm initiates production of IP_3 and that IP_3 releases Ca^{2+} from intracellular stores in the egg (reviewed in [15, 16]). Phosphoinositide turnover and production of IP_3 have been shown to occur at fertilization of sea urchin eggs [17, 18]. Recently, Stith and colleagues [19] reported a fivefold increase in IP_3 mass at fertilization of the frog (*Xenopus laevis*). Evidence for phosphoinositide turnover or IP_3 mass increase has not yet been obtained for mammalian eggs. However, IP_3 is known to initiate calcium release in immature hamster oocytes [20], hamster eggs [21], immature mouse oocytes [23, 24], and mature mouse eggs [25, 26]. The best evidence for the significance of IP_3-induced Ca^{2+} release in mammalian oocytes has come from experiments in hamster eggs. Miyazaki et al. have shown that the Ca^{2+} increase at fertilization of the hamster egg is blocked by prior injection of a monoclonal antibody directed against the IP_3-sensitive release channel [27].

Potentiators of calcium release from the ryanodine-sensitive release channel in muscle cells include ryanodine itself and caffeine [9, 11, 28, 29]. Ryanodine and caffeine also promote calcium release in non-muscle cells [30–33]. Evidence for an IP_3-independent, Ca^{2+}-sensitive release mechanism in eggs has come from experiments with sea urchin eggs [34–36]. Galione et al. [36] found that ryanodine and caffeine cause Ca^{2+} release from sea urchin egg homogenates. Furthermore, injection of ryanodine also releases Ca^{2+} and activates the intact sea urchin egg [35, 37–39].

These findings suggest that Ca^{2+}-induced Ca^{2+} release may contribute to the rise in calcium in the sea urchin egg. This hypothesis is supported by experiments with cyclic ADP-ribose (cADPR), a metabolite of nicotinamide adenine dinucleotide, which may modulate Ca^{2+} release in some cells [40, 41]. Cyclic ADP-ribose is as effective as IP_3 in causing Ca^{2+} release from homogenates of sea urchin eggs and, when injected into the sea urchin egg, causes a Ca^{2+} transient like that initiated by sperm [42, 43]. Cyclic ADP-ribose, produced in sea urchin eggs and other cells [44, 45], is thought to release Ca^{2+} by acting on the ryanodine-sensitive channel. In the sea urchin egg, cADPR does not appear to act on the IP_3 receptor [46]; it may act on the ryanodine receptor, since cADPR-induced Ca^{2+} release is desensitized by ryanodine and caffeine and is inhibited by procaine and ruthenium red, inhibitors of Ca^{2+}-induced Ca^{2+} release mediated by the ryanodine receptor [35, 40].

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It has been suggested that a Ca^{2+}-induced Ca^{2+} release mechanism functions in mammalian eggs. Initial reports of Ca^{2+}-induced Ca^{2+} release were based on iontophoretic injection of Ca^{2+} in hamster eggs, which released intracellular Ca^{2+} [47], or by hyperpolarization-induced Ca^{2+} influx in mouse eggs, which also triggered release of intracellular Ca^{2+} [23, 24]. In addition, Swann has suggested that the sulfhydryl reagent, thimerosal, causes calcium release in the hamster egg by sensitizing a Ca^{2+}-induced Ca^{2+} release mechanism [48]. Recently, thimerosal-induced Ca^{2+} release has been reported for immature mouse oocytes [49] and mature mouse eggs [26]. On the basis of the inability of 100 μg/ml heparin to block IP_3-induced calcium release but not thimerosal-induced Ca^{2+} transients in mouse oocytes [49], it was suggested that thimerosal may act to sensitize Ca^{2+} release from an IP_3-insensitive release channel, that is, the ryanodine-sensitive channel or a channel closely related to it [26, 49]. However, thimerosal may instead sensitize the IP_3-induced calcium release mechanism as in hepatocytes and smooth muscle cells so that Ca^{2+} is released at basal levels of IP_3 [50–52]. Furthermore, Miyazaki et al. [53] report that the monoclonal antibody to the IP_3 receptor blocked Ca^{2+} oscillations induced by thimerosal in the hamster egg, indicating that thimerosal causes Ca^{2+} oscillations by an effect dependent on the IP_3 receptor. There is now good evidence that Ca^{2+} release in the hamster egg is mediated solely by the IP_3 receptor and that any apparent Ca^{2+}-induced Ca^{2+} release is mediated by sensitization of the IP_3-induced Ca^{2+} release by Ca^{2+} [27, 53].

The objective of the present study was to determine whether release of Ca^{2+} from intracellular stores in the mouse egg also occurs primarily because of IP_3-induced Ca^{2+} release or whether the mouse egg also contains a ryanodine or ryanodine-like receptor that would permit the participation of a Ca^{2+}-induced Ca^{2+} release mechanism in altering $[Ca^{2+}]_i$. We examined the ability of Ca^{2+}, IP_3, the sulfhydryl reagent thimerosal, ryanodine, caffeine, and cADPR to release Ca^{2+} or potentiate Ca^{2+} release from intracellular stores in the mature mouse egg. Part of this work has been presented in abstract form [54].

MATERIALS AND METHODS

Media and Reagents

The media used for cell culture were M16, M2, and in vitro fertilization medium (IVF) [2, 55]. For Ca^{2+}-free IVF, $CaCl_2$ was omitted, NaCl was increased to 102 mM, and BSA was replaced by 0.1% polyvinyl alcohol. Fura-2, fura-2 AM, fluo-3 AM, Pluronic F-127, and BAPTA (1,2-bis(*o*-aminophenoxy)ethane-*N,N,N′,N′*-tetraacetic acid) were obtained from Molecular Probes (Eugene, OR). Anhydrous dimethylsulfoxide (DMSO) was from Aldrich Chemical Co. (Milwaukee, WI). Ryanodine was from Calbiochem (La Jolla, CA). IP_3 (D-myo-inositol 1,4,5-trisphosphate), thimerosal, heparin (M_r 4000–6000), de-*N*-sulfated heparin, and all other reagents, except as noted, were purchased from Sigma Chemical Co. (St. Louis, MO). Cyclic ADP-ribose was a gift from Dr. H.C. Lee, University of Minnesota.

Preparation of Gametes

Eggs and sperm were collected and prepared as previously described [2]. Briefly, eggs were obtained from 8- to 12-wk-old NSA (CF1) female mice (Harlan Sprague-Dawley, Indianapolis, IN) following superovulation and cultured in M16 medium. The cumulus mass was removed with 0.3 mg/ml hyaluronidase (type IV-S). For in vitro fertilization and fluo-3 records with external caffeine, the zonae pellucidae were removed by a brief treatment (approximately 1 min) with 10 μg/ml α-chymotrypsin (type II). Sperm were obtained from 14- to 20-wk-old male ND4 Swiss Webster or NSA (CF1) mice (Harlan Sprague-Dawley) and were capacitated at 2–5 × 10^6 sperm/ml for 1–2 h in IVF. Eggs and sperm were cultured under light mineral oil (Fisher Scientific, Pittsburgh, PA) and incubated in a humidified atmosphere of 5% CO_2 and 95% air. To prevent movement of the egg during sperm addition and Ca^{2+} measurements, zona pellucida-free eggs were lightly attached to a glass-bottom dish treated with Cell-Tak (Collaborative Research, Bedford, MA). Before sperm were added, eggs were in IVF without BSA; after addition of sperm, the medium contained 0.4% BSA. The final sperm concentration was 2–5 × 10^5/ml. All Ca^{2+} measurements were made using 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. Drug solutions were added directly to the drop in an equal volume of medium to ensure rapid and thorough mixing. Intracellular microinjections were made by means of a quantitative direct pressure system [56]. A beveled micropipette was connected to a micrometer syringe system filled with fluorinert FC-70. The micropipette was first filled with inert dimethylpolysiloxane (viscosity 20 centistokes); it was next filled with the injection solution and then an equivalent volume of dimethylpolysiloxane to form a cap over the injection solution. During the intracellular injection, the dimethylpolysiloxane cap was injected along with the injection solution and the diameter of the dimethylpolysiloxane drop was measured in the egg. The volume of the injected cap is equivalent to the volume of injection solution. We were able to inject solutions without simultaneously viewing the eggs and interrupting the Ca^{2+} measurements. In these "blind" injections, the injection solution invariably entered the egg after injection of the oil cap. The injection pipette was usually removed just after the injection. Intracellular concentrations were calculated from the concentration and volume of solution injected, assuming uniform distribution and an egg volume of 205 pl. Zona pellucida-intact eggs were injected in IVF containing 0.4% BSA or in Ca^{2+}-free IVF containing 0.1% polyvinyl alcohol.

Injection buffers were 75 mM KCl, 20 mM HEPES (pH 7.0) for IP_3; 10 mM HEPES (pH 7.0) for heparin, de-*N*-sulfated heparin, cADPR, and caffeine. Stocks of 7–300 mg/ml heparin and 125 mg/ml de-*N*-sulfated heparin were used for injection. Cyclic ADP-ribose was prepared at a stock concentration of 1.0–100 μM. A concentrated stock of 412 mM caffeine was prepared by heating the solution until it was dissolved and then maintaining the solution at 37°C throughout the experiments.

We used two batches of ryanodine, which according to the vendor's data contained > 30% ryanodine and > 80% dehydroryanodine + ryanodine by HPLC (item #559275; Calbiochem). One batch was prepared at a stock concentration of 37 mM in 10 mM HEPES, 100 μM EGTA (pH 7.0). EGTA was included to guard against contaminating Ca^{2+}. The other batch was prepared at the same concentration in 10 mM HEPES, pH 7.0 containing no EGTA; no difference in results from these two preparations was detected. A third preparation of "pure" ryanodine was used; according to the vendor's analysis, this contained > 99% ryanodine and essentially none of the less active dehydroryanodine (item #559276, Calbiochem). Pure ryanodine was prepared at a stock concentration of 37 mM in 10 mM HEPES, pH 7.0.

For injection of Ca^{2+}, calcium-BAPTA buffers with a range of free Ca^{2+} were prepared. The concentration of free Ca^{2+} in these buffers is given by the ratio of Ca^{2+}-bound BAPTA (CaBAPTA) to free BAPTA and the K_d of BAPTA; that is, $Ca^{2+} = K_d$ [CaBAPTA]/[BAPTA]. Calcium-BAPTA buffers were made by mixing one volume of 0.4 M BAPTA (Molecular Probes) with 1/10 volume of 0.33 to 3.3 M $CaCl_2$, giving Ca-BAPTA:BAPTA ratios of 1:10 to 10:1 and free Ca^{2+} from 0.1 × K_d to 10 × K_d. These stocks were diluted 1:10 before use, maintaining the free Ca^{2+} but reducing the amount of BAPTA and CaBAPTA injected. The approximate K_d for BAPTA is taken as 0.25 μM for low ionic strength at 36°C (from the data of Harrison and Bers [57]).

Fura-2 and Fluo-3 Loading

Eggs were loaded with fura-2 by incubation in 0.33 μM fura-2 AM (from a 330 μM stock in anhydrous DMSO) at 37°C for 30 min. The loading medium (M2) contained 1 μl/ml of a 25% (w/w) solution of the dispersing agent Pluronic F-127 in anhydrous DMSO and 2.5 mM probenecid to inhibit intracellular compartmentalization and excretion of the indicator [58]. Eggs loaded with fura-2 AM had fluorescence similar in intensity to that of eggs injected with the free acid of fura-2 at a final concentration in the egg of 50 μM. Fluo-3 was introduced by a 30-min incubation in 1 μM fluo-3 AM in M2 containing 1 μl/ml Pluronic and 2.5 mM probenecid. Measurements with fura-2 and fluo-3 were qualitatively similar in all cases. All $[Ca^{2+}]_i$ measurements were made in the presence of 2.5 mM probenecid.

Calcium Measurements

A Deltascan system (Photon Technology International, South Brunswick, NJ) was used for fluorescence recordings as described previously [25]. For fluo-3 measurements, an excitation wavelength of 490 nm was used; the emitted fluorescence was obtained through a dichroic beamsplitter (510 nm) and emission filter (530 nm) and collected by a photon-counting photomultiplier. For fura-2 measurements, dual wavelength excitation was used; 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 calculated twice per second. $[Ca^{2+}]_i$ was estimated from the ratio equation [59]: $[Ca^{2+}]_i = K_d \times (R - V \times R_{min})/(V \times R_{max} - R) \times Sf_2/Sb_2$, where R is the measured ratio, V is the viscosity correction factor, 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-2 (unbound) to fura-2 (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 [60]. 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-2 fluorescence in cells and in salt solutions due to viscosity differences, 2 M sucrose was added to the standard solutions and the viscosity correction factor for cells was estimated [61]. 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 ratio values rather than $[Ca^{2+}]_i$ values are presented. A fluorescence ratio of 2.4 corresponded to a $[Ca^{2+}]_i$ of 1.0 μM, and a ratio of 4.0 was 2.0 μM. Statistical comparisons were made by use of one-way ANOVA (InStat software; GraphPAD, San Diego, CA).

RESULTS

A long series of transient elevations of $[Ca^{2+}]_i$ occur after fertilization of the mouse egg (Fig. 1). Such transients can

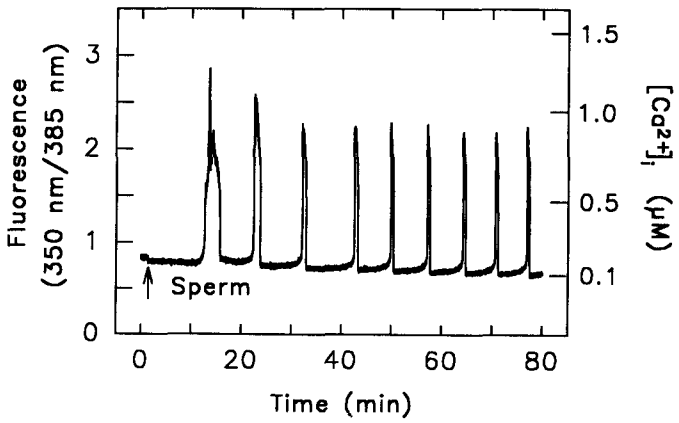

FIG. 1. Calcium transients after fertilization of the egg by the sperm. At the arrow, sperm were added to the medium containing the egg. The egg was fertilized in normal fertilization medium (IVF). In this figure, the fura-2 fluorescence ratio (350 nm/385 nm; left ordinate) and the estimated $[Ca^{2+}]_i$ from the ratio equation (right ordinate) are both indicated (see *Materials and Methods*).

TABLE 1. Effects of caffeine, ryanodine, and cyclic ADP-ribose injection on $[Ca^{2+}]_i$.

Injection	Concentration*	Number of eggs with a change in $[Ca^{2+}]_i$†	Number of eggs examined	
			Fura-2	Fluo-3
Caffeine	10, 18, 24 (mM)	0		3
	12 (mM)	0	3	
Cyclic ADP-ribose	0.009, 0.086, 0.86, 2.4, 3.5 (μM)	0		5
	3.5 (μM)	0	13	
Ryanodine (mixture)	0.004–6 (μM)	0		4
	18–970 (μM)	0		6
	16 (μM)	0	3	
	160 (μM)	0	20	
	320 (μM)	0	3	
Pure Ryanodine	16 (μM)	0	2	
	160 (μM)	0	3	
	320 (μM)	0	3	
	875 (μM)	1	3	
Ryanodine and 8–9 mM caffeine	0.009, 0.095, 0.95, 9.5, 119 (μM)	0		5

*Final concentration in the egg. The ryanodine mixture contained >30% ryanodine and >80% ryanodine + dehydroryanodine. Pure ryanodine contained >99% ryanodine (see *Materials and Methods*). The following injection volumes and stock concentrations were used: caffeine, 4.9–12.1 pl of a 412 mM stock; cyclic ADP-ribose, 1.8–7.2 pl of a 1.0–100 μM stock; ryanodine (mixture), 0.9–14.0 pl of a 0.5–50 mM stock; pure ryanodine, 1.8–4.9 pl of a 1.85–37 mM stock; ryanodine (mixture) and caffeine, 3.9–4.9 pl of stocks containing 0.5–5.0 mM ryanodine and 371–412 mM caffeine (8–9 mM final caffeine concentration).

†An increase in the 350:385 ratio for fura-2 of more than 0.1; in most recordings there was no detectable increase.

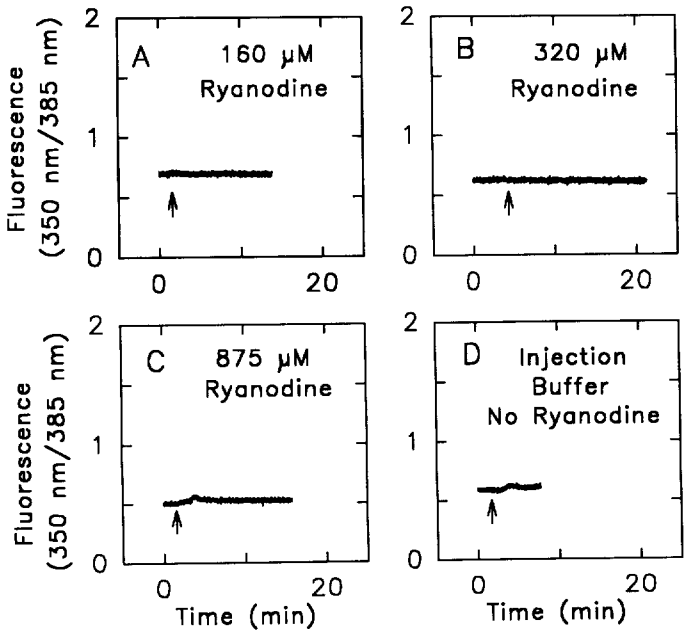

FIG. 2. Ca^{2+} transients are not produced by injection of ryanodine. A-C) Representative recordings showing injections of ryanodine at the final concentration indicated in the figure. Injection consisted of 1.8 to 4.9 pl of 18.5 to 37.0 mM stock solutions of pure ryanodine (> 99% ryanodine) in 10 mM Hepes buffer. D) Control injection of 1.8 pl 10 mM Hepes buffer without ryanodine. All experiments were done in IVF.

continue for as long as 4 h after fertilization [25]. The repetitive transients are associated with the periodic release of Ca^{2+} from intracellular stores, and they depend on Ca^{2+} influx across the plasma membrane and the refilling of intracellular stores [25]. We injected caffeine, ryanodine, and cADPR and applied external caffeine to examine the possibility that a Ca^{2+}-induced Ca^{2+} release mechanism mediated by the ryanodine receptor is present in the mouse egg. None of these substances caused an increase in intracellular Ca^{2+}. Of the agents we examined, only thimerosal and injection of IP_3, Ca^{2+}, or Ca^{2+}-BAPTA buffers caused an increase in $[Ca^{2+}]_i$.

Injection of 10–24 mM caffeine or 0.009–3.5 μM cADPR caused no change in intracellular Ca^{2+}. Injection of 0.004–970 μM ryanodine mixture (> 30% ryanodine, > 80% ryanodine + dehydroryanodine) did not elevate $[Ca^{2+}]_i$, nor did injection of 0.009–119 μM ryanodine (mixture) together with 8–9 mM caffeine (Table 1). To be certain of the ryanodine results, we also injected a highly purified ryanodine preparation. Injection of 16–875 μM pure ryanodine (> 99% ryanodine) did not significantly increase $[Ca^{2+}]_i$ (Table 1; Fig. 2). Addition of 10 mM caffeine to the medium caused no change in $[Ca^{2+}]_i$ (n = 12; data not shown).

Injection of $CaCl_2$ is known to initiate development of mouse eggs [62]. Injection of $CaCl_2$ equivalent to a final

TABLE 2. Summary of Ca^{2+} transients caused by injection of Ca^{2+} buffers with and without prior injection of ryanodine.

Ryanodine concentration[a] (μM)	Ratio CaBAPTA:BAPTA	Free Ca^{2+} in injection solution[b] (μM)	Average fura-2 peak ratio[c] (number of eggs)
—	1:10	0.025	0.62 ± 0.04 (3)
160	1:10	0.025	0.73 ± 0.08 (3)
—	1:4	0.063	0.67 ± 0.12 (4)
160	1:4	0.063	0.72 ± 0.02 (3)
—	1:2	0.125	0.90 ± 0.12 (3)
160	1:2	0.125	0.89 ± 0.08 (3)
—	1:1	0.25	0.94 ± 0.06 (3)
16	1:1	0.25	0.96 ± 0.05 (3)
160	1:1	0.25	0.93 ± 0.08 (3)
320	1:1	0.25	0.81 ± 0.09 (3)
—	2:1	0.5	1.34 ± 0.18 (4)
—	5:1	1.25	1.50 ± 0.24 (3)
—	10:1	2.5	2.00 ± 0.09 (3)

[a]Final intracellular concentration of ryanodine (mixture containing >30% ryanodine and >80% ryanodine + dehydroryanodine).
[b]Free Ca^{2+} in the injection solution, estimated from the equation $Ca^{2+} = K_d$[CaBAPTA]/[BAPTA], where K_d = 0.25 μM (see *Materials and Methods*).
[c]Average fura-2 peak ratio of the Ca^{2+} transient caused by Ca^{2+} buffer injection. In all cases, when compared to control injections at the same CaBAPTA:BAPTA ratio, prior injection of ryanodine did not significantly alter the peak ratio.

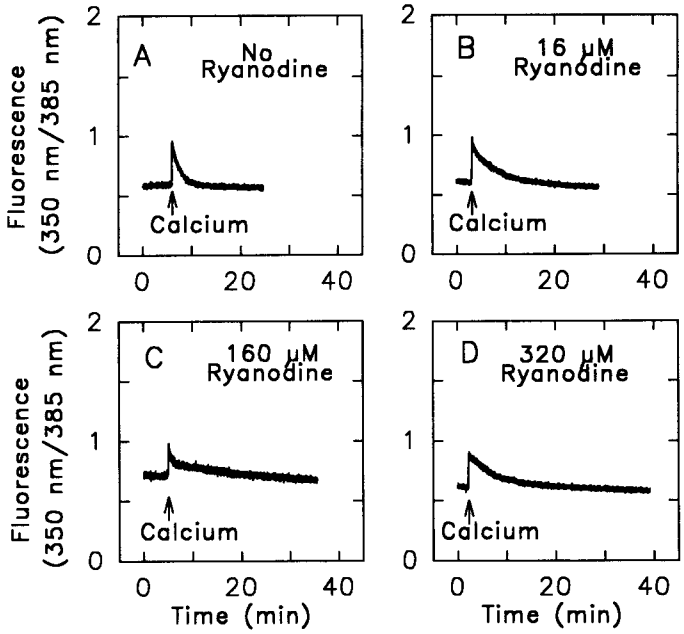

FIG. 3. Prior injection of ryanodine does not alter Ca^{2+} transients produced by a secondary injection of Ca^{2+}-containing BAPTA buffers. A) Control egg injected with 1.8 pl of Ca^{2+}-BAPTA having a 1:1 ratio of CaBAPTA:BAPTA and free Ca^{2+} of 0.25 μM. B-D) Prior to the recordings shown, eggs were injected with 0.9 to 1.8 pl of 1.85 to 37 mM ryanodine (mixture containing > 30% ryanodine and > 80% ryanodine + dehydroryanodine), giving the final concentrations indicated in the figure. The eggs were then injected with 1.8 pl of Ca^{2+}-BAPTA having a 1:1 ratio of CaBAPTA:BAPTA and a free Ca^{2+} of 0.25 μM. All experiments were done in IVF.

concentration in the egg of 39 to 426 μM resulted in a rapid, transient increase in $[Ca^{2+}]_i$ (n = 11) and, in all cases, only a single Ca^{2+} transient occurred; repetitive transients were not initiated by $CaCl_2$ injection (data not shown).

We also examined the possibility that ryanodine, while not causing Ca^{2+} release directly, might potentiate or alter Ca^{2+} release from intracellular stores induced by injection of Ca^{2+}. Because of the concern that direct injection of $CaCl_2$ might result in vesiculation of the injected volume, we injected buffered solutions of BAPTA containing free Ca^{2+}. Injection of Ca^{2+}-BAPTA buffers having a ratio of CaBAPTA:BAPTA of 1:10 to 10:1, giving 0.025 to 2.5 μM Ca^{2+} in the injection solution, caused intracellular Ca^{2+} to increase from baseline to peak ratios of 0.60 to 2.00 (Table 2; Fig. 3A). Repetitive Ca^{2+} transients were not initiated by injection of any of the Ca^{2+} buffers. The change in $[Ca^{2+}]_i$ on injection of Ca^{2+}-BAPTA buffers was not altered by prior injection of 16 to 320 μM ryanodine (Table 2; Fig. 3, B-D).

IP_3 injections of 8.6 to 86 nM caused a single Ca^{2+} transient (Table 3; Fig. 4, A and D). Injections of ≥ 430 nM IP_3 caused repetitive transients in media with or without external Ca^{2+} (Table 3; Fig. 4F). ADP-ribose and caffeine are thought to potentiate Ca^{2+}-induced Ca^{2+} release from a ryanodine-sensitive store. To test whether this might occur in the mouse egg, we injected cADPR or caffeine and then injected IP_3. The total Ca^{2+} increase after IP_3 injection should have been greater if Ca^{2+} release from the IP_3-sensitive store induced further Ca^{2+} release from a Ca^{2+}-induced Ca^{2+} release system sensitized by cADPR or caffeine. Ca^{2+} transients induced by injection of 8.6 or 86 nM IP_3 were not altered after prior injection of 3.5 μM cADPR or inclusion of 10 mM caffeine in the medium (Table 4; Fig. 4, B and C).

Injection of a final concentration of 4.5 mg/ml heparin, a competitive inhibitor of IP_3-induced Ca^{2+} release [63, 64], almost completely inhibited the Ca^{2+} increase induced by a secondary injection of 86 nM IP_3 (Fig. 4E; Table 4). Injection of 4.5 mg/ml heparin did not completely block the Ca^{2+} increase caused by the much larger injection of 432 nM IP_3, but heparin injection did prevent the occurrence of repetitive transients in 3 of 4 eggs. The magnitude and number of repetitive transients caused by injection of 432 nM IP_3 was unchanged in 4 of 4 eggs after prior injection of 4.4 mg/ml de-*N*-sulfated heparin, which does not inhibit IP_3-induced Ca^{2+} release (data not shown).

Addition of 100 μM thimerosal caused repetitive Ca^{2+} transients in IVF containing Ca^{2+} and in Ca^{2+}-free IVF (Table 5; Fig. 5, A and B). The effects of this sulfhydryl reagent should be reversed by addition of dithiothreitol (DTT). The Ca^{2+} transients induced by thimerosal were prevented when thimerosal was added with 5 mM DTT (Table 5; Fig. 6D). In 4 of 10 eggs examined in IVF after the addition of 100 μM thimerosal, the Ca^{2+} level remained elevated after five

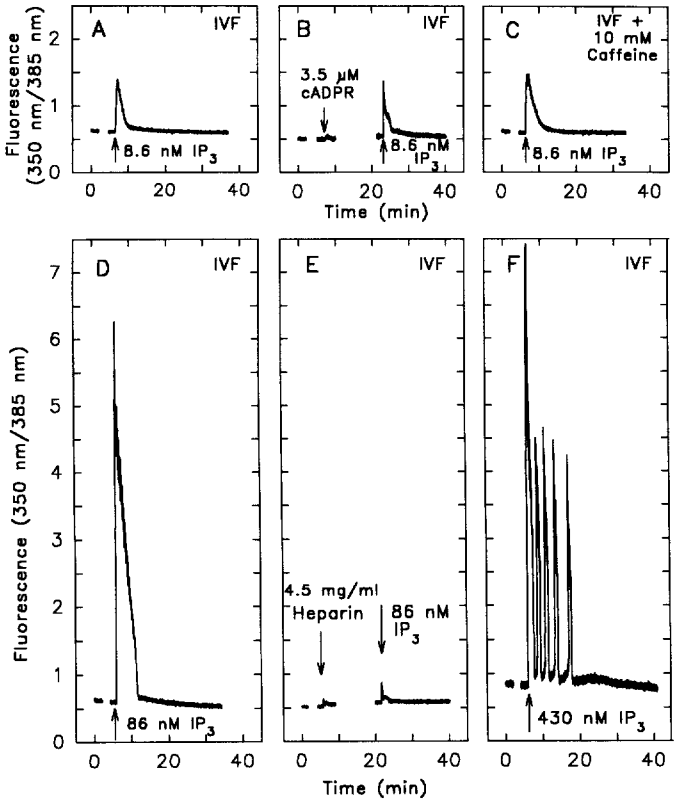

FIG. 4. IP_3 causes Ca^{2+} transients that are unaffected by cADPR or 10 mM caffeine but are inhibited by heparin. The culture medium was the normal fertilization medium (IVF). A) Ca^{2+} transient in response to injection of 1.8×10^{-18} mol IP_3 (final concentration in the egg of 8.6 nM). B) At the first arrow, 3.5 μM cADPR was injected. IP_3 (8.6 nM) was injected at the second arrow. C) An injection of IP_3 (8.6 nM) into an egg in IVF medium containing 10 mM caffeine. D) Injection of 86 nM IP_3. E) Heparin (final concentration in the egg of 4.5 mg/ml) was injected at the first arrow. IP_3 (86 nm) was injected at the second arrow. F) Multiple Ca^{2+} transients caused by injection of 430 nM IP_3.

to twelve transients and did not return to the basal level, usually stabilizing at a level greater than or equal to that reached during each of the previous transients.

We injected eggs with heparin before adding thimerosal to determine whether thimerosal acts on an IP_3-sensitive Ca^{2+} pool in the egg. The effect of heparin injection was quantified by counting the number of Ca^{2+} transients during the first 40 min of recordings that lasted 40–80 min (Table 5). Thimerosal-induced Ca^{2+} transients were not affected by control injections of 4.4 mg/ml de-*N*-sulfated heparin, which does not block the IP_3 receptor (Table 5; Fig. 6A). Prior injection of 0.1 or 1.0 mg/ml heparin did not significantly alter the number of early Ca^{2+} transients caused by addition of 100 μM thimerosal although, as shown in Figure 6B, the transients sometimes ended earlier than in control eggs. Thimerosal-induced Ca^{2+} transients were reduced in number or inhibited by 2.0 or 4.5 mg/ml heparin (Table 5; Fig. 6C).

DISCUSSION

Response to Ryanodine, Caffeine, and cADPR

Injection of ryanodine, caffeine, or cADPR, which mediate or modulate Ca^{2+}-induced Ca^{2+} release, caused no change in $[Ca^{2+}]_i$; nor did these substances alter subsequent Ca^{2+} increases after injection of Ca^{2+} or IP_3. Swann reported that caffeine did not alter intracellular Ca^{2+} in the mouse egg [26]. However, the results we have obtained differ from some of the results previously reported for Ca^{2+} regulation in the mouse egg. Swann reported that injection of 200 to 400 μM ryanodine produced Ca^{2+} increases in B6CBF1 hybrid mice [26]. We observed no change in Ca^{2+} in many experiments using three different ryanodine preparations. The dissimilar results may be attributable to differences in the two studies between mouse strains, ryanodine preparations, or experimental approaches and methods. However, the results we have obtained in studies of the mouse egg are very similar to those acquired from examination of Ca^{2+} transients produced in the hamster egg. Miyazaki and coworkers found no evidence for ryanodine receptor-mediated Ca^{2+}-induced Ca^{2+} release in the hamster egg. In the hamster, injection of 1–5 mM caffeine or 0.5–25 μM ryanodine produced no change in intracellular Ca^{2+}. The ryanodine receptor was not detected in hamster eggs by immunoblot analysis with an antibody to the rabbit ryanodine receptor [27].

TABLE 3. Summary of Ca^{2+} transients caused by injection of IP_3.

Medium	IP_3 concentration[a] (nM)	Number of eggs with a change in $[Ca^{2+}]_i$[b]	Number of Ca^{2+} transients (range)
IVF	8.6	3/3	1
IVF	17	1/1	1
IVF	86	3/3	1
IVF	430	4/4	5–12
Ca^{2+}-free IVF	430	3/3	2–3
Ca^{2+}-free IVF + 5 mM BAPTA	430	3/3	2–4
IVF	864	1/1	2
IVF	1180	2/2	6–9

[a]Final concentration in the egg. The concentration of IP_3 in the pipette was 1.0–50.0 μM, and 1.8–4.9 pl was injected.
[b]All measurements were made with fura-2.

TABLE 4. Effects of cyclic ADP-ribose, caffeine, and heparin on Ca^{2+} transients produced by IP_3 injection.

Experiment[b]	Average peak ratio (F_{350}:F_{385}) after IP_3 injection[a]	
	8.6 nM IP_3	86 nM IP_3
Control	1.33 ± 0.30 (3)	6.34 ± 0.13 (3)
After injection of 3.5 μM cyclic ADP-ribose	1.56 ± 0.22 (3)	5.91 ± 1.29 (4)
In 10 mM external caffeine	1.45 ± 0.01 (3)	5.72 ± 0.72 (3)
After injection of 4.5 mg/ml heparin	—	0.85 ± 0.14 (3)[c]

[a]The average fluorescence ratio of eggs prior to injection (indicating resting $[Ca^{2+}]_i$) in this series of experiments was 0.56 ± 0.06 (n = 22). The number of experiments is indicated in parentheses.

[b]Concentrations are final concentrations in the egg. Cyclic ADP-ribose (7.2 pl of a 100 μM stock) and heparin (3.0 pl of a 300 mg/ml stock) were injected before the IP_3 injection. Caffeine was added to the medium before the IP_3 injection; 1.8 pl of a 1.0 μM or 10.0 μM stock of IP_3 was injected.

[c]Significantly different from the average peak in control eggs ($p < 0.001$). Results of all other experiments were not significantly different from control results.

Response to Injection of IP_3 or Addition of Thimerosal

Ca^{2+} transients were produced by injection of IP_3 or application of thimerosal. Injection of small amounts of IP_3 produced a single Ca^{2+} transient, while larger injections caused repetitive Ca^{2+} transients. Thimerosal produced Ca^{2+} transients that were similar, but not identical, to those occurring at fertilization. We found that both the IP_3-induced Ca^{2+} transients and the repetitive transients induced by thimerosal were blocked by heparin, indicating that thimerosal is acting on the IP_3-induced Ca^{2+} release mechanism in the mouse egg.

The thimerosal-induced Ca^{2+} transients we observed are very similar to those reported in mouse eggs [26] and to those that occur in immature mouse oocytes ([49]; our unpublished results). On the basis of injection of 0.1 mg/ml heparin into immature oocytes, Carroll and Swann [49] concluded that Ca^{2+} oscillations produced by thimerosal were not inhibited by heparin and that thimerosal therefore sensitized an IP_3-independent Ca^{2+}-induced Ca^{2+} release system. However, we show that 0.1 mg/ml heparin is insufficient and that a higher concentration of heparin—2.0 to 4.5 mg/ml—is necessary to block the thimerosal-induced Ca^{2+} transients. Control injections of 4.4 mg/ml de-*N*-sulfated heparin, which does not block IP_3-induced Ca^{2+} release, had no effect on the thimerosal-induced transients. The intracellular concentration of heparin needed to block the thimerosal effect is higher than the 1 mg/ml or less that is usually suggested for inhibition of IP_3-induced Ca^{2+} release in permeabilized cell preparations [50, 63, 64]. Higher heparin concentrations may be required in the intact cell [65, 66]. Furthermore, if thimerosal does indeed sensitize IP_3-induced Ca^{2+} release [51, 52], higher concentrations of heparin may be necessary to inhibit the sensitized system. We recognize that heparin may act on molecules other than the IP_3 receptor, but our results do agree with studies of the hamster egg. The antibody to the IP_3 receptor blocks thimerosal-induced Ca^{2+} oscillations in hamster eggs [53]. Thus, there is evidence with respect to both the mouse and the hamster egg that thimerosal-induced Ca^{2+} transients are mediated by the IP_3 receptor.

The initiation of Ca^{2+} release from intracellular stores in mouse, hamster, and other cells by thimerosal is similar to the release of Ca^{2+} from the sarcoplasmic reticulum triggered by sulfhydryl group oxidation. Salama and colleagues have shown that Ca^{2+} release from the sarcoplasmic reticulum is triggered by application of Cu^{2+}/cysteine, reactive disulfides, or heavy metals [67]. This response, however, may be due to an effect on a 106-kDa protein that may be the ryanodine receptor [68]. Inhibition of thimerosal-induced Ca^{2+} release by heparin and the antibody to the IP_3 receptor indicates that thimerosal modifies the IP_3 receptor in mouse and hamster eggs.

Mechanisms of Ca^{2+} Release

The primary mechanism for release of Ca^{2+} from intracellular stores in the mammalian egg is dependent on the release of Ca^{2+} from IP_3-sensitive stores, mediated by the IP_3 receptor. This conclusion is supported by the results presented in the present paper and is most explicitly demonstrated by Miyazaki and his colleagues, who were able to block all Ca^{2+} oscillations in the hamster egg—whether induced by sperm, IP_3, or thimerosal—by a monoclonal antibody to the IP_3 receptor [27, 53]. Furthermore, Miyazaki and coworkers [27] have examined the mechanism behind the apparent Ca^{2+}-induced Ca^{2+} release seen when Ca^{2+} is injected into hamster eggs [47] or immature mouse oocytes [23]. Ca^{2+}-induced Ca^{2+} release in the hamster egg, which is seen as a nonlinear increase in $[Ca^{2+}]_i$ during injection of small amounts of Ca^{2+} by iontophoresis, is also inhibited by the antibody to the IP_3 receptor. Thus, Ca^{2+}-induced Ca^{2+} release in the hamster egg is actually Ca^{2+}-sensitized, IP_3-induced Ca^{2+} release (see below). A similar mechanism may account for the apparent Ca^{2+}-induced Ca^{2+} release from the mouse oocyte.

It is not known how the fertilizing sperm triggers the first Ca^{2+} transient in the mammalian egg. Based on our conclusions from examination of Ca^{2+} stores in the mouse egg and observations made from studies of the hamster egg, it appears that the Ca^{2+} transients result primarily from IP_3-induced Ca^{2+} release from intracellular stores in the egg.

TABLE 5. Effects of thimerosal on $[Ca^{2+}]_i$.

Thimerosal concentration (μM)	Medium and experiment[a]	Number of eggs with Ca^{2+} transients[b]	Average number of Ca^{2+} transients[c]
1	IVF	0/2	0
10	IVF	1/4[d]	0.25 ± 0.5
100	IVF	10/10	5.3 ± 1.6
100	Ca^{2+}-free IVF	5/5	7.2 ± 2.9[e]
100	Ca^{2+}-free IVF + 5.0 mM BAPTA	4/4	9.2 ± 1.9
100	IVF + 5.0 mM DTT	0/3	0
100	IVF after 4.4 mg/ml de-*N*-sulfated heparin	4/4	5.2 ± 2.1
100	IVF after injection of 0.1 mg/ml heparin	3/3	4.3 ± 1.0
100	IVF after injection of 1.0 mg/ml heparin	3/3	3.3 ± 1.2
100	IVF after injection of 2.0 mg/ml heparin	1/3[f]	0.7 ± 1.2
100	IVF after injection of 4.5 mg/ml heparin	1/5[g]	0.2 ± 0.05

[a]Eggs were incubated in IVF or Ca^{2+}-free IVF with or without 5.0 mM BAPTA; 1–100 μM thimerosal was added. DTT (5.0 mM) was added with 100 μM thimerosal to eggs in IVF, where indicated. Before addition of 100 μM thimerosal in the heparin experiments, the eggs were incubated in IVF and injected with heparin to give the indicated final concentration in the eggs.
[b]Data from fura-2 and fluo-3 experiments are combined.
[c]Average number of Ca^{2+} transients for all eggs during the 40-min recording. Multiple transients occurred in all eggs except where noted. The average includes eggs in which no transients occurred.
[d]A single transient occurred in one egg; no change in $[Ca^{2+}]$, was detected in the other three eggs.
[e]Multiple transients occurred in all eggs. The recording was less than 40 min for three eggs, and the average number of transients is given for the remaining two.
[f]Two transients occurred in 40 min in one egg; no change in $[Ca^{2+}]$, was detected in the other two eggs.
[g]One transient occurred in 40 min in one egg; no change in $[Ca^{2+}]$, was detected in the other four eggs.

The observations are consistent with the hypothesis that the sperm elevate intracellular Ca^{2+} in the egg by elevating intracellular IP_3, perhaps through receptor-mediated activation of a guanine nucleotide-binding protein (G-protein) in the egg. Injection of guanosine-5′-*O*-(3-thiotriphosphate) (GTP-γ-S), which activates G-proteins, initiates repetitive Ca^{2+} transients in hamster [21] and mouse eggs [54]. Application of an acetylcholine agonist after introduction of the muscarinic acetylcholine receptor also elevates $[Ca^{2+}]_i$ in the mouse egg (Kline and Kline, unpublished), further suggesting that the mouse egg contains a G-protein, capable of interacting with phospholipase C and transducing the signal to elevate $[Ca^{2+}]_i$.

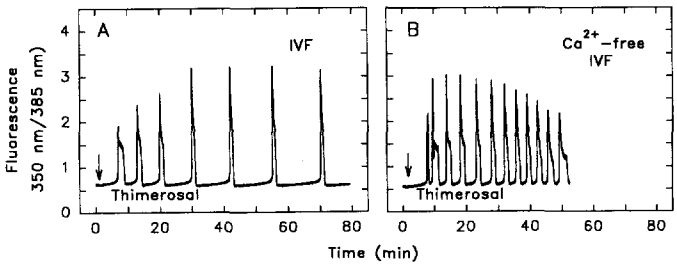

FIG. 5. Thimerosal caused repetitive Ca^{2+} transients in media with or without Ca^{2+}. A) Thimerosal (100 μM) was added to the egg (arrow) in normal fertilization medium (IVF) that contained 1.7 mM Ca^{2+}. B) Thimerosal (100 μM) added to an egg in Ca^{2+}-free IVF.

Alternatively, sperm may cause elevation of $[Ca^{2+}]_i$ in the egg by some mechanism not involving receptor-mediated activation of a G-protein. It has been suggested that a sperm-derived factor, capable of causing Ca^{2+} release in the egg, might be introduced into the egg after fusion of the egg and sperm membranes [64, 70]. It has also been proposed that sperm initiate Ca^{2+} release in the egg by introducing an additional Ca^{2+} conductance (open Ca^{2+} channels) into the egg membrane after sperm-egg fusion [71, 72]. The entry of Ca^{2+} might then induce Ca^{2+} release in the egg, although on the basis of the results presented here, Ca^{2+} release from stores in the egg would not be mediated by elevation of cytosolic Ca^{2+} and activation of a ryanodine-

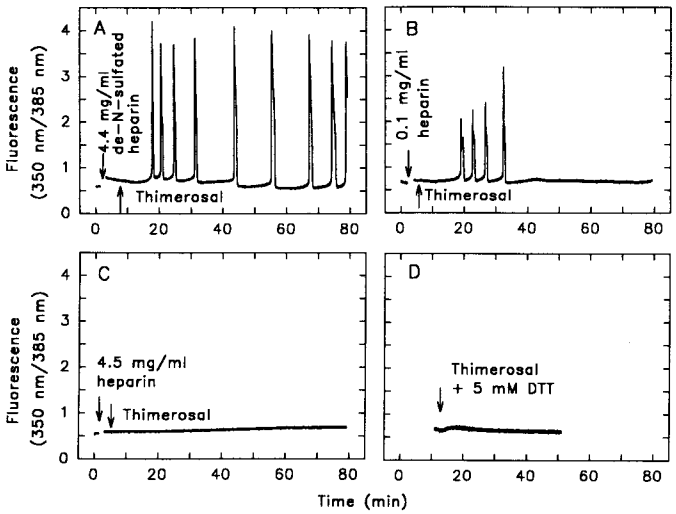

FIG. 6. Thimerosal-induced Ca^{2+} transients are inhibited by heparin or DTT. All experiments were performed in IVF containing 1.7 mM Ca^{2+}. A) Control injection of 7.3 pl of stock solution of de-*N*-sulfated heparin (125 mg/ml), giving a final concentration of 4.4 mg/ml in the egg. Thimerosal (100 μM) was added at the second arrow. B) Injection of 3.1 pl of 6.7 mg/ml heparin stock solution giving a final concentration of 0.1 mg/ml. Thimerosal (100 μM) was added at the second arrow. C) Injection of 3.1 pl of 300 mg/ml heparin stock giving a final concentration of 4.5 mg/ml. Thimerosal (100 μM) was added at the second arrow. D) Addition of 100 μM thimerosal combined with 5 mM DTT at the arrow.

like receptor but rather by a form of Ca^{2+}-sensitized IP_3-induced Ca^{2+} release. Regardless of how the initial Ca^{2+} transient is initiated by sperm, either through receptor-mediated activation of a G-protein or by some other mechanism, release of intracellular Ca^{2+} is likely to occur from IP_3-sensitive stores.

Repetitive Ca^{2+} Transients in Mammalian Eggs

The repetitive Ca^{2+} transients that follow the first sperm-induced Ca^{2+} transient are characteristic of mouse and hamster eggs; they depend on continued influx of extracellular Ca^{2+} [25, 47]. We have proposed [25] that the repetitive Ca^{2+} transients may occur because of the periodic overfilling and subsequent release of Ca^{2+} from intracellular stores due to increased Ca^{2+} influx after fertilization. The increase in Ca^{2+} influx following fertilization may be associated with the depletion of an intracellular Ca^{2+} store during the first sperm-induced Ca^{2+} transient; treating the egg with thapsigargin, an inhibitor of the Ca-ATPase, depletes intracellular Ca^{2+} stores and activates a divalent cation influx pathway [25]. Refilling the stores is necessary because the repetitive transients are inhibited both by lowering extracellular Ca^{2+} to reduce Ca^{2+} influx and by treatment with thapsigargin to inhibit the endoplasmic reticulum Ca-ATPase [25]. Irvine [73] proposed that Ca^{2+} release through the IP_3 receptor might be modulated by the concentration of Ca^{2+} in the lumen of the Ca^{2+} store. This has been supported by experiments in some [51, 52, 74], but not all, cells [75, 76]. The IP_3-sensitive store in hepatocytes releases Ca^{2+} spontaneously when overloaded with Ca^{2+}, presumably because the sensitivity to endogenous IP_3 is increased [50–52].

In the mouse egg, the increased Ca^{2+} influx following fertilization may serve to overfill the intracellular store and trigger repetitive Ca^{2+} transients. The Ca^{2+} transients in mouse eggs consist of a slow pacemaker-like rise in Ca^{2+} followed by a rapidly rising phase indicating some form of positive feedback. The positive feedback is most likely to be due to the known biphasic sensitivity of the IP_3 receptor to cytosolic Ca^{2+} [77–79]. IP_3-induced Ca^{2+} release in smooth muscle cells is enhanced by increasing Ca^{2+} up to about 300 nM [77], while higher Ca^{2+} inhibits IP_3-induced Ca^{2+} release. Similarly, the opening of the cerebellar IP_3 receptor ion channel exhibits a bell-shaped Ca^{2+} sensitivity; Ca^{2+} up to 200 nM increases the probability of channel opening, while higher concentrations reduce it [79]. Consequently, the kinetics of Ca^{2+} release and the frequency of Ca^{2+} transients in the mouse egg, as in other cells, may be regulated by IP_3 concentration and by both luminal and cytosolic Ca^{2+}.

The initiation of Ca^{2+} transients in the mouse egg by thimerosal is consistent with the suggestion that thimerosal may increase the sensitivity of the IP_3 receptor to low levels of endogenous IP_3 [50–52]. The relatively slow metabolism of IP_3 may account for the repetitive Ca^{2+} transients observed when 430 μM IP_3 was injected into the egg. While Ca^{2+} influx is clearly the driving force for repetitive Ca^{2+} transients in the fertilized egg [25], Ca^{2+} influx is not necessary for Ca^{2+} transients induced by thimerosal or by large concentrations of IP_3, since repetitive transients occurred in a Ca^{2+}-free medium. In these cases, if refilling the Ca^{2+} store is necessary for Ca^{2+} transients, Ca^{2+} must be provided from the cytosol. It is important to note, therefore, that while thimerosal may sensitize IP_3-induced Ca^{2+} release in the mouse egg, it does not completely mimic the fertilization response. The pattern and frequency of Ca^{2+} transients induced in the mouse egg by IP_3, thimerosal, and sperm are somewhat different (compare Figs. 1, 3, and 4) because of the nature of the stimulus.

The results presented here for the mouse and findings for the hamster egg (reviewed in [80]) are in strong contrast to those obtained from studies of echinoderm eggs. Ryanodine, caffeine, and other agents that promote release of intracellular Ca^{2+} from muscle cells provoke release of Ca^{2+} from intracellular stores and cause exocytosis of cortical granules in sea urchin eggs [35, 37–39]. In addition, an antibody against the ryanodine receptor of skeletal muscle sarcoplasmic reticulum binds to the cortical endoplasmic reticulum in sea urchin eggs [81]. These observations, together with the suggestion that cADPR may be an important modulator or regulator of $[Ca^{2+}]_i$ [35], indicate that Ca^{2+}-induced Ca^{2+} release might be an important component of the Ca^{2+} increase at fertilization in the sea urchin egg. Evidence suggests that Ca^{2+} release during fertilization in the sea urchin egg involves both IP_3-induced Ca^{2+} release and

Ca^{2+} release mediated by cADPR- and ryanodine-sensitive mechanisms [36, 82]. In *Xenopus* oocytes, apparent Ca^{2+}-induced Ca^{2+} release is mediated by the IP_3 receptor and there is no evidence for ryanodine receptor-mediated Ca^{2+}-induced Ca^{2+} release [65, 82, 83]. Accordingly, Ca^{2+} release in the *Xenopus* oocytes may be similar to that in mammalian eggs in which Ca^{2+} release appears to be mediated by an IP_3-sensitive mechanism.

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