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Different triggers for calcium oscillations in mouse eggs involve a ryanodine-sensitive calcium store

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Relative intracellular free Ca^{2+} concentrations ($[Ca^{2+}]_i$) were monitored in mature unfertilized mouse eggs by measuring fluorescence of intracellular fluo3. A number of different agents were found to cause sustained repetitive transient $[Ca^{2+}]_i$ oscillations. These were microinjection of a cytosolic sperm factor, sustained injection of Ins-(1,4,5)P_1, or extracellular addition of the thiol reagent thimerosal. Stimulating G-protein activity by injection of guanosine 5'-[γ-thio]triphosphate plus application of carbachol also caused $[Ca^{2+}]_i$ oscillations, but less reliably than other stimuli. A role for Ca^{2+}-induced Ca^{2+} release and a ryanodine-sensitive Ca^{2+} channel in mouse eggs was suggested by the finding that microinjection, or external addition, of ryanodine also caused $[Ca^{2+}]_i$ increases. Furthermore, ryanodine, along with thimerosal, increased the sensitivity of eggs to Ca^{2+}-induced $[Ca^{2+}]_i$ oscillations. When ryanodine was added to eggs oscillating in response to the sperm factor, InsP_3 or thimerosal, it caused a decrease in amplitude of oscillations and eventually a block of $[Ca^{2+}]_i$ oscillations associated with a sustained elevation of $[Ca^{2+}]_i$. These data suggest that a ryanodine-sensitive Ca^{2+}-release mechanism exists in mouse eggs and that a ryanodine-sensitive Ca^{2+} store plays a role in generating intracellular $[Ca^{2+}]_i$ oscillations.

INTRODUCTION

During fertilization of mouse and hamster eggs *in vitro* the sperm causes a series of oscillations in cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) that last for several hours after gamete fusion (Cuthbertson & Cobbold, 1985; Miyazaki, 1991; Kline & Kline, 1992). These $[Ca^{2+}]_i$ oscillations are the trigger for mammalian egg development (Fulton & Whittingham, 1978; Ozil, 1990; Kline & Kline, 1992). It has been suggested that the sperm triggers these $[Ca^{2+}]_i$ oscillations by acting on membrane receptor and GTP-binding proteins that stimulate phosphoinositide turnover and increased InsP_3 production (Jaffe, 1990; Miyazaki, 1988). However, an alternative idea is that the sperm triggers Ca^{2+} release by fusing with the egg and introducing a soluble sperm factor (Swann & Whitaker, 1990). This idea is supported by the finding that injecting a protein factor from sperm cytosol triggers $[Ca^{2+}]_i$ oscillations in hamster eggs identical with those at fertilization (Swann, 1990). It is not known how this sperm factor causes these oscillations.

Several different mechanisms have been suggested to explain how cells generate cytosolic $[Ca^{2+}]_i$ oscillations (Berridge & Galione, 1988; Tsien & Tsien, 1990). The mechanisms are based on InsP_3-induced Ca^{2+} release from internal stores, with some models also suggesting the involvement of a Ca^{2+}-induced Ca^{2+} release (CICR) mechanism. InsP_3-induced Ca^{2+} release has been demonstrated in hamster eggs and immature mouse oocytes (Miyazaki, 1988; Peres, 1990), and injection of InsP_3 alone can trigger $[Ca^{2+}]_i$ oscillations in hamster eggs and mouse oocytes (Swann *et al.*, 1989; Peres, 1990). CICR has also been demonstrated in hamster eggs and immature mouse oocytes (Igusa & Miyazaki, 1983; Peres, 1990). Furthermore, the CICR mechanism is probably involved in the $[Ca^{2+}]_i$ oscillations at fertilization in hamster eggs, because after sperm–egg fusion, or sperm-factor injection, there is an order-of-magnitude increase in the sensitivity of the egg to CICR (Igusa & Miyazaki, 1983; Swann, 1990). A similar increase in CICR sensitivity and a series of $[Ca^{2+}]_i$ oscillations occur in hamster eggs after applying the thiol reagent thimerosal (Swann, 1991). Nevertheless, the contribution of CICR towards $[Ca^{2+}]_i$ oscillations triggered InsP_3 or other stimuli, and the basic nature of the CICR mechanism has remained unclear.

The existence of a CICR mechanism in nerve and muscle cells is due to a specific Ca^{2+}-activated Ca^{2+}-release channel in the sarcoplasmic or endoplasmic reticulum (Lai *et al.*, 1988; McPherson *et al.*, 1991). Such channels are specifically bound by ryanodine (Lai *et al.*, 1989). Ryanodine has dramatic affects on $[Ca^{2+}]_i$ homoeostasis in muscle cells, stimulating or inhibiting CICR depending on the conditions (Meissner, 1986; Sutko *et al.*, 1985). Ryanodine has also been shown to affect Ca^{2+} release in some secretory cell types, hepatocytes and sea-urchin eggs (Malgaroli *et al.*, 1990; Shoshan-Barmatz, 1990; Galione *et al.*, 1991; Foskett & Wong, 1991). However, despite the demonstrations of CICR, ryanodine has not previously been reported to affect Ca^{2+} release in mammalian eggs and oocytes.

In this paper I show that $[Ca^{2+}]_i$ oscillations are triggered in mouse eggs by a cytosolic sperm factor and thimerosal. Injecting InsP_3, applying carbachol and injecting guanosine 5'-[γ-thio]-triphosphate (GTP[S]) also trigger $[Ca^{2+}]_i$ oscillations. Furthermore, ryanodine is shown to cause Ca^{2+} release, alter the CICR mechanism and inhibit all types of $[Ca^{2+}]_i$ oscillations in mouse eggs. The mechanisms of Ca^{2+} release in mammalian eggs are discussed.

MATERIALS AND METHODS

Female B6CBF1 hybrid mice were superovulated by serial injections of pregnant-mare-serum gonadotropin and human choriogonadotropin (Fulton & Whittingham, 1978). Eggs were recovered from animals 12–16 h after human choriogonadotropin injection. Eggs were freed from cumulus cells by washing in hyaluronidase and the zona pellucida was removed by brief treatment with acid Tyrode solution. Eggs were maintained

Abbreviations used: $[Ca^{2+}]_i$, intracellular free Ca^{2+} concentration; CICR, Ca^{2+}-induced Ca^{2+} release; InsP_3, inositol 1,4,5-trisphosphate; GTP[S], guanosine 5'-[γ-thio]triphosphate.

in M2 medium plus 4 mg of BSA/ml (Fulton & Whittingham, 1978), and kept at 37 °C with experiments carried out within 4 h of egg collection. Injections and fluorescence measurements were on eggs placed in drops of M2 medium at 30–33 °C as described previously (Swann *et al.*, 1989). Drugs were added rapidly to medium containing the eggs, and all quoted values are the final concentration of each drug.

$[Ca^{2+}]_i$ was monitored by measuring the fluorescence of intracellular fluo3 (Minta *et al.*, 1989). Eggs were incubated in 50 μM-fluo3-AM plus 0.02% Pluronic in M2 medium for either 10–15 min. Fluorescence was measured as described previously (Swann, 1990, 1991). Fluorescence values in Figures are in arbitrary units and the $[Ca^{2+}]_i$ levels are uncalibrated. Initial studies on eggs showed a decline in baseline fluorescence of about 10%/h from fluo3-loaded eggs. The decline in fluorescence appeared to be due to dye leakage from the egg, since ratio measurements of resting $[Ca^{2+}]_i$ with indo-1 did not suggest any decrease in baseline $[Ca^{2+}]_i$ with time in unfertilized or fertilizing eggs (K. Swann, unpublished work). A constant level of fluo-3 fluorescence was achieved by inclusion of 0.25 μM-fluo-3-AM (which is not fluorescent) in the medium bathing the eggs. This allowed for prolonged recordings of relative $[Ca^{2+}]_i$ levels and did not affect egg viability, nor inhibit fertilization or early signs of egg activation such as second polar-body formation.

Microinjections were carried out as described previously for hamster eggs (Swann *et al.*, 1989; Swann, 1990, 1991). The egg volume was taken as 200 pl. All injections were made during the brief breaks in the fluorescence records, since the photomultiplier tubes were closed while eggs were observed under bright-field microscopy. With most sperm-factor and GTP[S] injections the micropipettes were removed from the egg immediately after injections were made; Ca^{2+} and $InsP_3$ injection pipettes were kept in the egg unless otherwise stated. In cases where injection damaged the egg and led to egg lysis, the data were discarded.

The cytosolic sperm extracts used were the high-molecular-mass fractions of ejaculated boar sperm or epididymal hamster sperm. They were made as described previously (Swann, 1990), except that boar sperm extracts were concentrated on C-100 ultrafiltration membranes, and hamster sperm extracts on C-10 membranes (Amicon, Stonehouse, U.K.). GTP[S] and $InsP_3$ were made up in a buffer containing 120 mM-KCl, 20 mM-Hepes and 100 μM-EGTA, pH 7.5. Thimerosal was dissolved directly into M2 medium. Ryanodine consisted of a 95–99% mixture of ryanodine and dehydroryanodine (produced by Calbiochem, obtained through Novabiochem, Nottingham, U.K.). $InsP_3$ and thimerosal were purchased from Sigma (Poole, Dorset, U.K.).

RESULTS

Different triggers for $[Ca^{2+}]_i$ oscillations in mouse eggs

Fig. 1 shows that microinjecting cytosolic sperm extracts triggers a series of intracellular Ca^{2+} oscillations in unfertilized mouse eggs, as measured by fluo-3 fluorescence. The series of $[Ca^{2+}]_i$ increases started with a prolonged $[Ca^{2+}]_i$ increase that lasted from 40–70 s to over 30 min, followed by oscillations at frequencies of one every 40 min to one every 10 min. Each increase in Ca^{2+} had a characteristic rapid upstroke and downstroke, with the initial $[Ca^{2+}]_i$ increase occasionally showing smaller oscillations on top of a large increase (Fig. 1*b*). Such $[Ca^{2+}]_i$ oscillations were seen in all of 24 eggs injected with sperm extracts, and in two cases oscillations were seen to persist for over 5 h (Fig. 1*c*). Control injections of the KCl buffer failed to cause any significant $[Ca^{2+}]_i$ increase in 11/11 eggs. These data show that a sperm factor causes $[Ca^{2+}]_i$ oscillations in mouse eggs similar to those reported in hamster eggs (Swann, 1990). As with hamster eggs, the frequency of oscillations in mouse eggs depended on the particular batch and the amount of sperm extract injected.

In addition to sperm extracts, the thiol reagent thimerosal also triggered a series of $[Ca^{2+}]_i$ oscillations. Adding final concentrations of 100 μM-thimerosal to the bathing medium containing the eggs caused regular repetitive $[Ca^{2+}]_i$ oscillations in all 18/18 eggs (Fig. 2*a*). The pattern of $[Ca^{2+}]_i$ oscillations triggered by thimerosal was more variable than that after sperm-extract injection, in that the initial $[Ca^{2+}]_i$ transients were sometimes brief spikes that later developed into the longer-lasting responses that are shown in a more typical experiment in Fig. 2(*a*). Also, unlike the sperm factor, thimerosal sometimes caused oscillations that later developed into a sustained increase in $[Ca^{2+}]_i$ indicated by a sustained high level of fluo3 fluorescence (results not shown).

In common with hamster eggs, agents that mimic or might potentiate increased phosphoinositide turnover also caused $[Ca^{2+}]_i$ oscillations in mouse eggs. Fig. 2(*b*) shows that after inserting a micropipette containing high concentrations of $InsP_3$ there was a series of $[Ca^{2+}]_i$ oscillations in the egg that persisted in 10/10 eggs until the $InsP_3$-containing pipette was removed. No pressure or iontophoresis was needed to cause these $[Ca^{2+}]_i$ oscillations, and so it is assumed that leakage of $InsP_3$ from the pipette was sufficient to produce an effect, as described previously in hamster eggs (Swann *et al.*, 1989). External addition of the cholinergic agonist carbachol also caused a series of small $[Ca^{2+}]_i$ oscillations in 8/8 mouse eggs (Fig. 2*c*). The carbachol-induced oscillations did not persist and, although the absolute levels of $[Ca^{2+}]_i$ were not measured, the relative change in fluo3 fluorescence was less than half that typically seen after thimerosal application. This implies a rather small and transient stimulation of $InsP_3$ production by carbachol. However, if eggs were injected

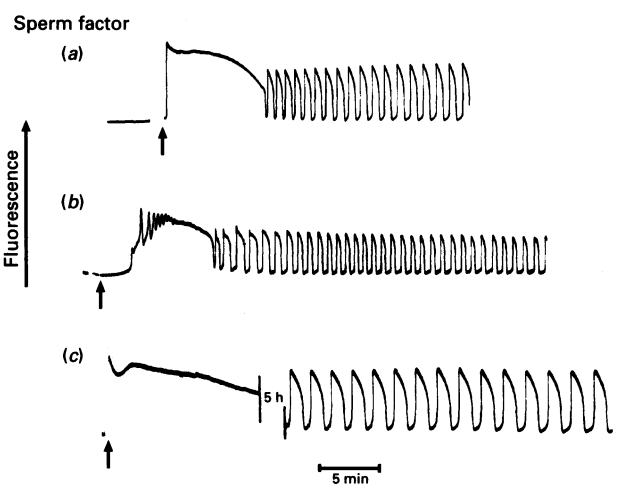

Fig. 1. Ca^{2+} oscillations, as measured by fluo3 fluorescence, in unfertilized mouse eggs injected with sperm extracts

In all three records, injection volumes of 1–2% of egg volume were made at the times indicated by the arrows. Fluorescence is in arbitrary units, although the absolute fluorescence levels and time scales are the same. In (*a*) hamster sperm extract was injected and the pipette removed immediately after injection; in (*b*) boar sperm extract was injected and the injection pipette kept inside the egg. The different types of initial $[Ca^{2+}]_i$ increase in (*a*) and (*b*) were seen with either type of sperm extract. In (*c*) a different batch of hamster sperm extract caused an initial $[Ca^{2+}]_i$ increase that lasted 30 min before oscillating, and the trace shown is for a period 5 h after injection, when oscillations still occurred.

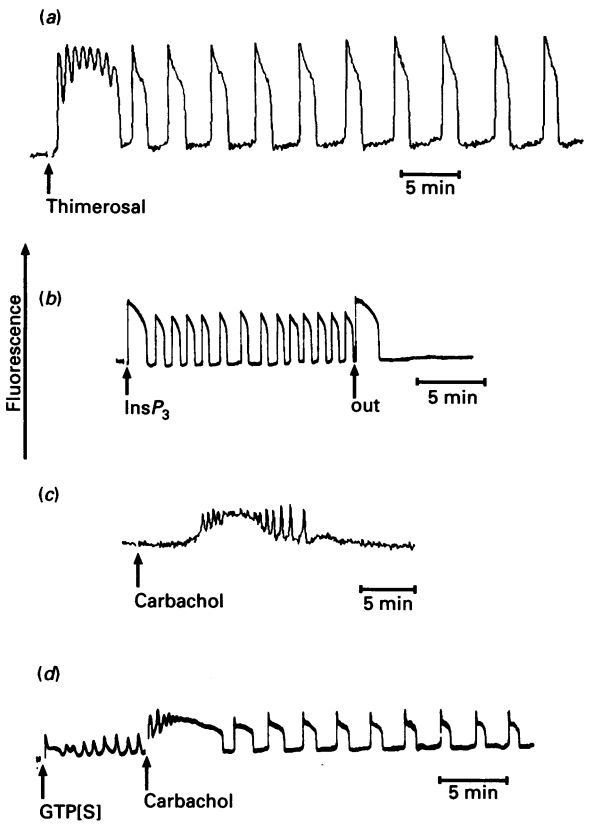

Fig. 2. $[Ca^{2+}]_i$ oscillations in unfertilized mouse eggs triggered by various agents

The conditions are otherwise the same as in Fig. 1 except for the different time scales as shown. In (*a*) 100 μM-thimerosal was added to the medium bathing the egg. In (*b*) a pipette containing 1 mM-InsP_3 was inserted into the egg (at the arrow) and taken out of the egg later (at the second arrow). In (*c*) 100 μM-carbachol was added to the medium bathing an egg, and in another experiment in (*d*) an egg was first injected with a final concentration of about 50 μM-GTP[S] and then 100 μM-carbachol was added to the medium (at the times indicated by the arrows).

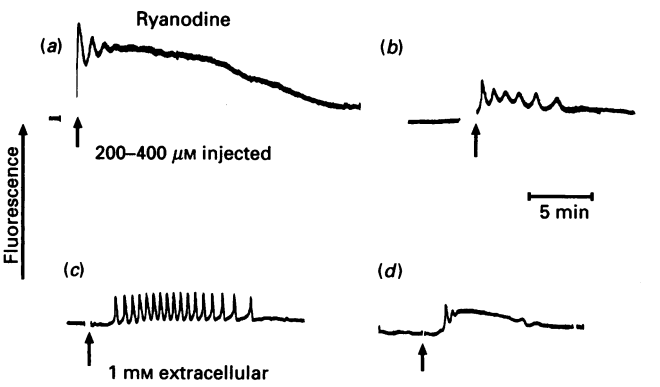

Fig. 3. Effect of ryanodine on Ca^{2+} levels in mouse eggs

In (*a*) and (*b*) eggs were injected with 2–4 pl volumes of a solution containing 20 mM-ryanodine, which caused small oscillations superimposed on more prolonged increases in $[Ca^{2+}]_i$ that varied in magnitude in six different eggs. In (*c*) and (*d*) 1 mM-ryanodine was added to the medium bathing the eggs and triggered different patterns of oscillatory $[Ca^{2+}]_i$ increases, typical of responses seen in 6/7 eggs.

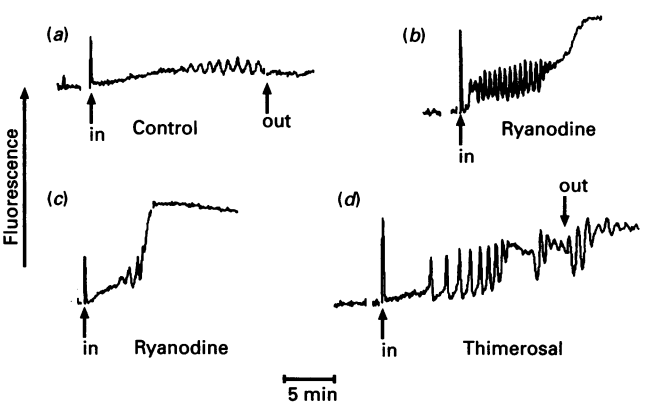

Fig. 4. Effect on $[Ca^{2+}]_i$ of inserting a Ca^{2+}-containing pipette into eggs

Ca^{2+} pipettes were inserted into the egg at the 'in' arrow and removed at the 'out' arrow. In (*a*) pipette insertion triggered an initial $[Ca^{2+}]_i$ increase and small oscillations which ceased after pipette removal. In (*b*) and (*c*) the eggs had been treated with 1 mM-ryanodine and any oscillations ceased; Ca^{2+}-pipette insertion then caused more $[Ca^{2+}]_i$ oscillations and eventually a sustained $[Ca^{2+}]_i$ increase. In (*d*) the egg was incubated in 10 μM-thimerosal for 1 h, which caused no $[Ca^{2+}]_i$ oscillations, and then inserting a Ca^{2+} pipette caused oscillations and a sustained increase in $[Ca^{2+}]_i$.

with GTP[S] to stimulate G-proteins, a small series of $[Ca^{2+}]_i$ oscillations were potentiated by addition of 100 μM-carbachol, and oscillations similar to those induced by InsP_3 injection were seen (Fig. 2*d*). These data suggest that G-protein-controlled InsP_3 production can lead to repetitive $[Ca^{2+}]_i$ release in mouse eggs. However, it was found that even the $[Ca^{2+}]_i$ oscillations triggered by GTP[S] plus carbachol were not readily induced in all eggs; 4/8 eggs showed no response with GTP[S] injection alone and only two $[Ca^{2+}]_i$ transients in 15 min after further addition of carbachol. Only sustained InsP_3 injection was as reliable at causing prolonged $[Ca^{2+}]_i$ oscillations as thimerosal and the sperm factor.

Ryanodine causes $[Ca^{2+}]_i$ increases in mouse eggs

The above data support the idea that an InsP_3-gated Ca^{2+}-release channel exists in mammalian eggs (Miyazaki, 1991). A different type of Ca^{2+}-release channel has been identified that is specifically stimulated by ryanodine (Lai *et al.*, 1989). When 200–400 μM-ryanodine (final concns.) was microinjected into mouse eggs, it triggered a $[Ca^{2+}]_i$ increase in all cases (Figs. 3*a* and 3*b*), although the size and type of response to ryanodine differed between eggs. Since extracellular application of 200 μM-ryanodine did not trigger $[Ca^{2+}]_i$ oscillations, the plasma membrane appears to present a barrier to ryanodine uptake into the egg cytoplasm. Higher concentrations of ryanodine (1 mM), however, were effective at triggering $[Ca^{2+}]_i$ oscillations when applied to the medium bathing the eggs (Figs. 3*c* and 3*d*). The typical response to 1 mM extracellular ryanodine was a series of $[Ca^{2+}]_i$ oscillations that did not persist.

CICR in mouse eggs

In sarcoplasmic reticulum, ryanodine can potentiate CICR (Meissner, 1986). To see if this was also true of mouse eggs, CICR was assessed in them by inserting pipettes containing 0.5 M-$CaCl_2$. Fig. 4(*a*) shows that in control eggs inserting the Ca^{2+} pipette caused a brief $[Ca^{2+}]_i$ increase. This caused no further $[Ca^{2+}]_i$ change in five eggs and small oscillations after 10 min in three other eggs. These data suggest that there is a degree of CICR demonstrable in some unfertilized mouse eggs, as previously found in hamster and immature mouse oocytes (Igusa & Miyazaki, 1983; Peres, 1990). However, after eggs had

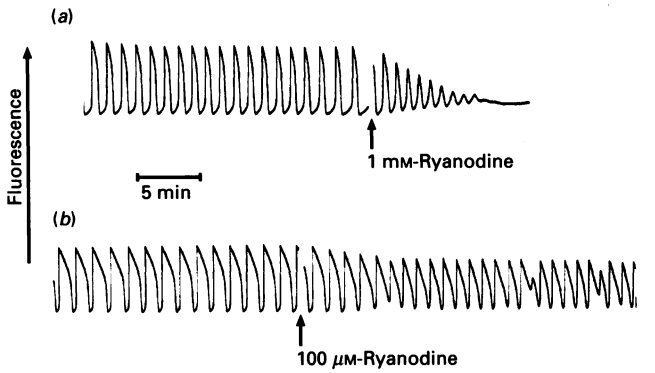

Fig. 5. Ryanodine-induced modulation and inhibition of $[Ca^{2+}]_i$ oscillations triggered by the sperm factor

In both cases eggs had been injected with 1–2 % of injection volumes of hamster sperm extract and undergoing $[Ca^{2+}]_i$ oscillations for about 1 h, at which point in (*a*) 1 mM- and in (*b*) 100 μM-ryanodine was added to the medium bathing the eggs. Each record is representative of at least three experiments.

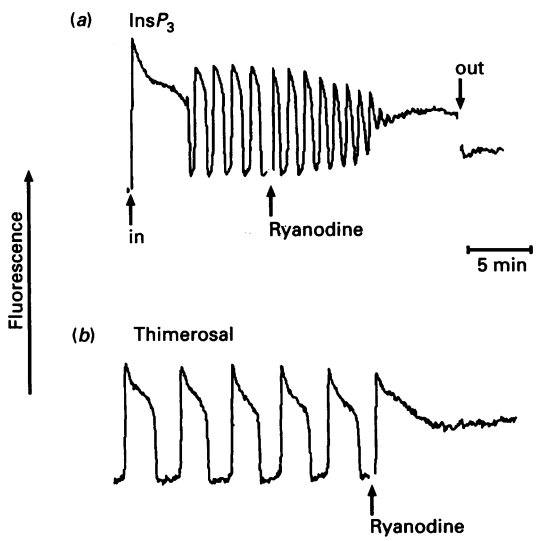

Fig. 6. Ryanodine inhibition of $[Ca^{2+}]_i$ oscillations triggered by different stimuli

In (*a*) an egg was stimulated to undergo $[Ca^{2+}]_i$ oscillations by inserting a pipette (at the arrow) containing 1 mM-InsP_3 and then 1 mM-ryanodine was added to the medium later. In (*b*) 100 μM-thimerosal was added to the egg about 30 min before the start of the trace, and then 1 mM-ryanodine was added extracellularly (at the arrow). Each record is representative of three experiments.

been treated with 1 mM-ryanodine, and any oscillations ceased, insertion of the Ca^{2+} pipette again triggered a brief $[Ca^{2+}]_i$ transient, but within 1–5 min this was followed in 4/4 eggs by a series of high-frequency $[Ca^{2+}]_i$ oscillations, and eventually a sustained increase in $[Ca^{2+}]_i$ that even persisted after the Ca^{2+} pipette was removed (Figs. 4*b* and 4*c*). These data show that ryanodine sensitizes the egg to Ca^{2+}-injection-induced Ca^{2+} release.

Thimerosal has been found to potentiate CICR in hamster eggs, where it increases the sensitivity of CICR (Swann, 1990). Thimerosal also potentiated the effect of Ca^{2+} injection in mouse eggs. When eggs were incubated in 10–20 μM-thimerosal no Ca^{2+} oscillations were seen, but after inserting a Ca^{2+}-containing pipette a series of $[Ca^{2+}]_i$ oscillations occurred that led to prolonged increases in $[Ca^{2+}]_i$ in 4/4 eggs (Fig. 4*d*). In contrast, caffeine, which enhances CICR in muscle and nerve cells, failed to enhance the effects of Ca^{2+} injection in mouse eggs and did not trigger $[Ca^{2+}]_i$ oscillations on its own; only a slow increase in fluo3 fluorescence was observed (results not shown). These data show that Ca^{2+}-injection-induced $[Ca^{2+}]_i$ oscillations are enhanced by ryanodine and by the thiol reagent thimerosal.

Ryanodine inhibits $[Ca^{2+}]_i$ oscillations triggered by other stimuli

The potential role of a ryanodine-sensitive release mechanism in generating $[Ca^{2+}]_i$ oscillations was investigated by adding ryanodine to eggs in which $[Ca^{2+}]_i$ was oscillating in response to the three stimuli that reliably triggered responses; the sperm factor, thimerosal and InsP_3 injection. Fig. 5(*a*) shows the effect of adding 1 mM-ryanodine to eggs undergoing $[Ca^{2+}]_i$ oscillations after sperm-extract injection. In all such cases ryanodine caused a progressive increase in frequency, but a decrease in amplitude, of oscillations until eventually oscillations stopped and a $[Ca^{2+}]_i$ level was maintained between the maxima and minima of oscillations. Lower concentrations of ryanodine (100 μM) that did not cause any change in $[Ca^{2+}]_i$ in uninjected eggs also caused a change in oscillation pattern by decreasing oscillation amplitude and increasing baseline $[Ca^{2+}]_i$ (Fig. 5*b*). A similar effect of ryanodine was seen after it was applied to eggs oscillating in response to InsP_3, where an alteration in the pattern led to a progressive decrease in amplitude and finally a cessation of oscillatory Ca^{2+} release (Fig. 6*a*). Ryanodine also inhibited thimerosal-induced $[Ca^{2+}]_i$ oscillations in a similar manner, causing a very rapid shift to intermediate $[Ca^{2+}]_i$ levels (Fig. 6*b*). Whatever the stimulus, the rate at which ryanodine caused the decline in amplitude of oscillations varied between different eggs, with the more rapid running-down occurring after ryanodine was applied to higher-frequency oscillators. These data show that ryanodine modifies and inhibits $[Ca^{2+}]_i$ oscillations in mouse eggs regardless of whether responses were triggered by the sperm factor, by thimerosal or by InsP_3.

DISCUSSION

Different triggers for $[Ca^{2+}]_i$ oscillations

The present data show that $[Ca^{2+}]_i$ oscillations in mouse eggs can be triggered by a number of stimuli, such as a cytosolic sperm factor, InsP_3, GTP[S] plus an external agonist, and thimerosal. These types of stimuli have previously been shown to trigger repetitive Ca^{2+}-dependent membrane responses in unfertilized hamster eggs (Swann *et al.*, 1989; Swann, 1990, 1991). In hamster eggs, thimerosal-, GTP-analogue- and InsP_3-induced $[Ca^{2+}]_i$ oscillations are less persistent than those seen after fertilization or sperm-factor injection (Swann *et al.*, 1989; Swann, 1990, 1991). In contrast, in mouse eggs most stimuli can cause longer-lasting $[Ca^{2+}]_i$ oscillations. Although there are variations in frequency, and occasionally in the shape of the initial $[Ca^{2+}]_i$ increase, each of these stimuli causes $[Ca^{2+}]_i$ oscillations in mouse eggs that are broadly similar to each other and to those occurring during fertilization *in vitro* (Cuthbertson & Cobbold, 1985; Kline & Kline, 1992). In this sense mouse eggs may be less useful than hamster eggs for discriminating which stimuli best mimic fertilization, but they do offer advantages for studies on the mechanisms of oscillations.

The finding that prolonged injection of InsP_3 causes $[Ca^{2+}]_i$ oscillations in mouse eggs is similar to the results seen in other types of oocyte and somatic cells (Berridge & Galione, 1988; Berridge, 1991; Wakui *et al.*, 1990). The synergistic effect of GTP[S] and carbachol in triggering oscillations further suggests that mouse eggs appear to have muscarinic receptors linked to

G-proteins that control phosphoinositide turnover. This idea is consistent with reports that small membrane responses are elicited by acetylcholine in mouse eggs (Eusebi *et al.*, 1979), and with the findings that carbachol-stimulated receptors are linked to G-proteins and InsP_3 production in several somatic cell types (Cockcroft & Stutchfield, 1988). The potentiating effect of GTP[S] on carbachol-induced Ca^{2+} release is similar to that described for 5-hydroxytryptamine-induced $[Ca^{2+}]_i$ transients in hamster eggs (Miyazaki *et al.*, 1990). Although these data suggest that the receptor/G-protein model of fertilization has potential validity in mammalian eggs, there are still no reports of any sperm-bound ligands for external receptors in mammalian eggs that might interact with G-proteins.

The alternative hypothesis for fertilization is that the sperm causes Ca^{2+} release by introducing a cytosolic sperm factor into the egg. This idea is fully supported by the present results. Once inside the egg, some cytosolic factor isolated from mammalian sperm can clearly trigger sustained $[Ca^{2+}]_i$ oscillations in mouse eggs as well as hamster eggs (Swann, 1990). The mechanism of the sperm factor may not involve the same components of the receptor–G-protein/InsP_3 pathway. For example, thimerosal was highly effective in triggering $[Ca^{2+}]_i$ oscillations in mouse eggs, and yet it does not cause any increase in phosphoinositide turnover in other cell types where it triggers intracellular Ca^{2+} release (Hecker *et al.*, 1989). Thimerosal's actions appeared to involve CICR, since it increased the sensitivity of mouse and hamster eggs to Ca^{2+} injection (Swann, 1991). The order-of-magnitude enhancement of CICR sensitivity appears to be a common property of the actions of thimerosal, the sperm factor and fertilization (Swann, 1990, 1991). It is important to establish the nature of the CICR system.

A ryanodine-sensitive Ca^{2+}-release mechanism

In mouse eggs the alkaloid ryanodine, as well as InsP_3, triggered oscillatory $[Ca^{2+}]_i$ release. Ryanodine has been used as a specific activator, or inhibitor, of CICR and binds with high affinity to identified CICR channels in sarcoplasmic reticulum of muscle, or endoplasmic reticulum of neurons (Lai *et al.*, 1989; McPherson *et al.*, 1991). Ryanodine does not appear to open or bind to the InsP_3-sensitive Ca^{2+}-release channel, or to affect Ca^{2+} pumps (Palade *et al.*, 1989; Bezprozvanny *et al.*, 1991; Bazotte *et al.*, 1991). Consequently the present data suggest the presence of distinct InsP_3 and ryanodine-sensitive release channels in mouse eggs. The finding that ryanodine triggers $[Ca^{2+}]_i$ oscillations may be explained by increased opening of Ca^{2+}-release channels (Lai *et al.*, 1989). The demonstration of a potentiation of oscillations by injection of ryanodine plus Ca^{2+} is consistent with ryanodine enhancing CICR in sarcoplasmic-reticulum vesicles (Meissner, 1986).

The concentrations of ryanodine that are required to cause clear effects in mouse eggs are higher than those described in muscle and nerve cells (Fabiato, 1985; Palade *et al.*, 1989). One reason for this appeared to be the relatively poor uptake of the drug by mouse eggs, since intracellular concentrations of 200–400 μM were effective, compared with millimolar concentrations when the drug was added outside the eggs. There are other likely reasons for the requirement of relatively high concentrations of ryanodine to cause effects in mouse eggs. Ryanodine binding to muscle Ca^{2+} channels is dependent on the free Ca^{2+} concentration, and even at high Ca^{2+} levels the binding is very slow (Lai *et al.*, 1989). One might expect high concentrations of ryanodine to be necessary to see rapid effects in mouse eggs, which have a rather low resting $[Ca^{2+}]_i$ of around 50 nM (Cuthbertson *et al.*, 1981). Alternatively, other factors that may be different in eggs, such as the cell redox state, may alter ryanodine binding to the Ca^{2+} channel (Zaida *et al.*, 1989; Pessah *et al.*, 1987). Also, it was found that intracellular perfusion of 100 μM-ryanodine is required to cause Ca^{2+} release in CHO cells expressing the skeletal isoform of the ryanodine-sensitive channel (Penner *et al.*, 1989), and that 100–200 μM-ryanodine is required to trigger Ca^{2+} release in homogenates of sea-urchin eggs (Galione *et al.*, 1991). Such intracellular concentrations of ryanodine are similar to those used in the present study.

In contrast with muscle cells, caffeine does not appear to have a marked affect on CICR or trigger $[Ca^{2+}]_i$ oscillations in mouse or hamster eggs (Swann, 1990; Miyazaki, 1991). This may be because eggs have a distinct subtype of ryanodine-sensitive Ca^{2+} channel. For example, caffeine is very effective at activating a ryanodine-sensitive channel and affecting Ca^{2+} release in neurons and cardiac cells (Palade *et al.*, 1989; Sutko *et al.*, 1985). In contrast, the liver cell ryanodine-sensitive Ca^{2+} channel appears to be a different protein, is not activated by caffeine, and shows a distinct pharmacology (Shoshan-Barmatz *et al.*, 1991). As an alternative drug to caffeine, thimerosal does enhance CICR and cause $[Ca^{2+}]_i$ oscillations in mammalian eggs. Thiol reagents have been suggested to act on either the InsP_3- or the ryanodine-sensitive release channels (Rooney *et al.*, 1991; Missiaen *et al.*, 1991; Swann, 1991). An effect of thimerosal on a ryanodine-sensitive channel seems more likely, for several reasons. In mouse oocytes microinjection of the InsP_3-receptor antagonist heparin fails to block thimerosal-induced Ca^{2+} oscillations (Carroll & Swann, 1992), and in insulin-secreting cells thimerosal has been shown to release Ca^{2+} from an InsP_3-insensitive store (Islam *et al.*, 1992). In addition, thiol reagents similar to thimerosal trigger Ca^{2+} release via a ryanodine-sensitive channel in sarcoplasmic reticulum (Nagura *et al.*, 1988; Zaida *et al.*, 1989).

Mechanisms of $[Ca^{2+}]_i$ oscillations in mouse eggs

Several types of models to describe cellular Ca^{2+} oscillations have been proposed, based on InsP_3- and Ca^{2+}-induced Ca^{2+} release (Berridge & Galione, 1988; Tsien & Tsien, 1990). Both InsP_3 and CICR mechanisms exist in mammalian eggs (Miyazaki, 1991). The existence of a CICR mechanism could be explained either by the InsP_3 receptor being sensitized to Ca^{2+} or by the presence of a distinct ryanodine-sensitive CICR channel (Finch *et al.*, 1991; Bezprozvanny *et al.*, 1991). The present data support the latter idea of a distinct CICR channel, since ryanodine releases Ca^{2+} and sensitizes the overt signs of CICR in mouse eggs. Ryanodine also inhibits oscillations, apparently by potentiation of Ca^{2+} release to the point where the internal store became depleted and a constant elevation of $[Ca^{2+}]_i$ occurred. This affect is consistent with the ability of ryanodine to open CICR release channels (Lai *et al.*, 1989). Ryanodine appears to act on CICR channels involved in the oscillatory release, because ryanodine binding is enhanced by channel opening (Lai *et al.*, 1989), and concentrations of ryanodine that cause no increase in resting $[Ca^{2+}]_i$ in uninjected eggs nevertheless affected $[Ca^{2+}]_i$ in oscillating eggs. These effects of ryanodine in mouse eggs on $[Ca^{2+}]_i$ oscillations are similar to those in cardiac muscle, adrenal chromaffin cells and parotid acinar cells (Fabiato, 1985; Malgaroli *et al.*, 1990; Foskett & Wong, 1991). The data suggest that any type of oscillatory Ca^{2+} release in mouse eggs is from a ryanodine-sensitive store.

The InsP_3-induced $[Ca^{2+}]_i$ oscillations in mouse eggs may be explained by the two-store model, in which InsP_3 causes Ca^{2+} release from an InsP_3-sensitive store that leads to cycles of CICR from an InsP_3-insensitive store (Berridge & Galione, 1988; Dupont *et al.*, 1991). This two-store model has already received experimental support in pancreatic and parotid acinar cells, and in *Xenopus* oocytes (Wakui *et al.*, 1990; Foskett & Wong, 1991; Berridge, 1991). The CICR store could be the ryanodine-sensitive store in eggs which periodically releases Ca^{2+} as a result of

overloading with Ca^{2+}. Such 'Ca^{2+}-overload'-induced Ca^{2+} release from a ryanodine-sensitive store has been described in muscle sarcoplasmic reticulum and in endoplasmic reticulum of sea-urchin eggs and adrenal chromaffin cells (Fabiato, 1985; Nelson & Nelson, 1990; Galione *et al.*, 1991; Cheek *et al.*, 1991).

One of the implications of the two-store model of $[Ca^{2+}]_i$ oscillations is that oscillations can occur via CICR without the involvement of $InsP_3$. This may be how thimerosal works, and may be important at fertilization. Recent experiments in sea-urchin eggs argue against the direct involvement of an $InsP_3$-sensitive channel in the initial Ca^{2+} release in eggs at fertilization (Rakow & Shen, 1990; Swann & Whitaker, 1990; Crossley *et al.*, 1991). The sperm factor is one of several alternative Ca^{2+}-releasing agents (Swann & Whitaker, 1990). In mammals this protein factor may be analogous to a peptide oscillogen that causes cycles of Ca^{2+} release in skinned skeletal muscle by activating a ryanodine-sensitive Ca^{2+}-release channel (Herrmann-Frank & Meissner, 1989).

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