The effects of a Ca²⁺ chelator and heavy-metal-ion chelators upon Ca²⁺ oscillations and activation at fertilization in mouse eggs suggest a role for repetitive Ca²⁺ increases

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During fertilization in mouse eggs, the sperm triggers a series of intracellular Ca²⁺ oscillations that lead to egg activation, as indicated by pronuclear formation. We show that Ca²⁺ oscillations in fertilized mouse eggs can be inhibited by addition of either the Ca²⁺ chelator 1,2-bis-(o-aminophenoxy)ethane-N,N,N',N'-tetra-acetic acid acetoxymethyl ester (BAPTA-AM) or the heavy-metal-ion chelator N,N,N',N'-tetrakis-(2-pyridylmethyl)ethylenediamine (TPEN) plus dithiothreitol (DTT). Both treatments inhibited Ca²⁺ oscillations, but they had different effects upon egg activation. Blocking Ca²⁺ oscillations with BAPTA-AM after the occurrence of just two Ca²⁺ spikes resulted in most eggs forming pronuclei. However, we found that BAPTA-AM-treated fertilizing eggs showed a decreased rate of protein synthesis, which by itself can promote egg activation. In contrast, blocking Ca²⁺ oscillations with TPEN plus DTT was accompanied by the inhibition of egg activation with no significant effect on protein synthesis. In eggs that were fertilized and then treated with TPEN plus DTT, there was a correlation between the number of Ca²⁺ spikes and the proportion of eggs that formed pronuclei, as well as between the number of Ca²⁺ spikes and the time taken for pronuclear formation and the first mitosis to occur. The addition of TPEN plus DTT did not block the generation of Ca²⁺ spikes or pronuclear formation when eggs were artificially stimulated by electroporation pulses. These data suggest that TPEN plus DTT inhibits pronuclear formation in fertilizing eggs via the inhibition of Ca²⁺ oscillations and that the number of Ca²⁺ spikes may regulate egg activation.

INTRODUCTION

Repetitive spikes, or oscillations, in the intracellular free Ca²⁺ concentration are known to occur in a variety of different cell types [1,2]. Such cytosolic Ca²⁺ oscillations can occur in response to hormones or to many other extracellular stimuli that lead to the generation of inositol 1,4,5-trisphosphate (InsP₃). The precise function of oscillations in Ca²⁺, as opposed to a monotonic rise in Ca²⁺, is not entirely clear [1,2]. However, it has been suggested that cellular responses may be modulated by the frequency, or the number, of Ca²⁺ oscillations [2,3]. One of the first examples of cytosolic Ca²⁺ oscillations to be described was that associated with fertilization in mouse eggs [4,5]. These Ca²⁺ oscillations last for several hours after sperm–egg interaction [5–7]. They appear to stop around the time of the formation of pronuclei, which occurs 4–6 h after sperm penetration and is one of the first visible indicators of egg activation in mammals [6,8]. Ca²⁺ oscillations similar to those in mouse eggs have now been described at fertilization in a number of different mammalian, and some non-mammalian, species [7,9,10].

The mechanisms that lead to the generation of changes in Ca²⁺ concentration at fertilization are not fully understood [7,11]. Nevertheless it is clear that some form of increase in Ca²⁺ at fertilization is essential for egg activation in the mouse. Pre-loading eggs before fertilization with the Ca²⁺ chelator 1,2-bis-(o-aminophenoxy)ethane-N,N,N',N'-tetra-acetic acid (BAPTA) to block all Ca²⁺ oscillations prevents meiotic resumption and

pronuclear formation after sperm entry [12]. In addition, it is known that increasing the Ca²⁺ concentration within mammalian eggs can lead to the activation of development [7,13,14]. Such parthenogenetic egg activation can be achieved by agents that cause either a single prolonged [13–15], or an oscillatory [12–17], Ca²⁺ increase. Previous work has shown that parthenogenetically stimulated mouse eggs can respond differently to different patterns of Ca²⁺ spikes that are imposed artificially by a series of electroporation pulses [16,17]. However, it is not known if the fertilized mouse egg also responds differentially to the different patterns of Ca²⁺ oscillations generated by the sperm. It is also uncertain whether the initial Ca²⁺ increase at fertilization is sufficient to lead to pronuclear formation and cell-cycle progression [7,11,15]. These question are difficult to answer, because one needs to inhibit Ca²⁺ oscillations at fertilization in a way that selectively allows different numbers of Ca²⁺ spikes to occur.

In the present study we have inhibited Ca²⁺ oscillations at fertilization by applying the Ca²⁺ chelator BAPTA, which was introduced into eggs in the acetoxymethyl ester (AM) form. In contrast with previous studies, where BAPTA was introduced before fertilization, we added BAPTA-AM after Ca²⁺ oscillations had been initiated by the sperm. This was done in order to investigate the effects of different numbers of Ca²⁺ spikes on egg activation. In the course of studying the effects of different ion chelators upon Ca²⁺ spikes in eggs, we discovered that some heavy-metal-ion chelators also inhibited other Ca²⁺ changes at fertilization. In particular, the membrane-permeable heavy-

metal-ion chelator N,N,N',N'-tetrakis-(2-pyridylmethyl)ethyl-enediamine (TPEN) [18] inhibited Ca^{2+} oscillations at fertilization in mouse eggs. Such an inhibitory effect in mouse eggs is consistent with the finding that TPEN can inhibit hormone-induced Ca^{2+} increases in pancreatic acinar cells [19]. In the present study we have compared the inhibitory effects of TPEN, combined with a reducing agent, with those of the membrane-permeable Ca^{2+} chelator BAPTA-AM. Despite the fact that the two different treatments both inhibited Ca^{2+} oscillations, our data show that there are different consequences for mouse egg activation. These differences may be explained by an effect of BAPTA loading on protein synthesis [20]. We discuss the differential effects of TPEN and reducing agents upon egg activation in the context of the role of repetitive Ca^{2+} spikes at fertilization in mammals.

MATERIALS AND METHODS

Media and reagents

Experiments on mouse eggs were carried out in M2 medium (Hepes-buffered) supplemented with 4 mg/ml BSA (crystalline; ICN) [14]. To capacitate sperm, a T6 medium supplemented with 15 mg/ml BSA (fraction V; Sigma) was used [21]. The chemicals TPEN, dithiothreitol (DTT), 1,10-phenanthroline and desferrox-amine were purchased from Sigma. BAPTA-AM was from Novabiochem. TPEN, BAPTA-AM, phenanthroline and des-ferroxamine were made up as stock solutions in DMSO and diluted into M2 medium for experiments. The final concentration of DMSO was less than 0.5%. DTT was dissolved in M2 medium on the day of use.

Preparation and handling of gametes

Female MF1 mice were superovulated and eggs were collected 15–17 h after injection of human chorionic gonadotropin, as described previously [14,22]. The eggs were maintained in 200 μl drops of M2 medium under light liquid paraffin at 37 °C. Just before each experiment, the zonae pellucidae were removed by brief treatment with acid Tyrode solution (pH 2.5) and the eggs were then washed several times in M2 medium. Sperm from CBA male mice were capacitated in T6 medium in a humidified 5% CO_2 incubator at 37 °C [21]. Sperm were added to eggs after at least 2 h of capacitation. The activation of eggs was assessed by observing gametes to check for either second polar body or pronuclear formation. The female pronucleus was discriminated from the male pronucleus because it is smaller in size and usually forms in close proximity to the second polar body. The activation stages of eggs were scored towards the end of experiments (> 6 h), and eggs were regarded as fully activated when the normal complement of two pronuclei was observed.

Measurement of Ca^{2+} changes in eggs

Intracellular Ca^{2+} changes were measured with the AM derivatives of fura red (Molecular Probes) or indo-1 (Molecular Probes). Fura red-AM and indo-1-AM were made up as 2 mM stocks in DMSO plus 5% Pluronic F127 (Molecular Probes), which were diluted 1000-fold into M2 medium to form the loading buffer. The loading medium was also supplemented with 250 μM of the anion-exchange inhibitor sulfinpyrazone (ICN), which helps to prevent compartmentalization and extrusion of the dye [23]. Sulfinpyrazone did not affect the frequency of Ca^{2+} spikes or the timing of pronuclear formation in fertilized eggs (Y. Lawrence and K. Swann, unpublished work). Eggs were incubated for 10–12 min in 2 μM fura red-AM or indo-1-AM before being placed in the chamber on the microscope stage. For Ca^{2+}

measurements in fertilizing eggs, these were placed in a 1 ml bath of medium that was heated to 37 °C and covered with mineral oil to prevent evaporation. Eggs adhered to a polylysine-coated coverslip that formed the base of the bath. Fertilization was initiated by adding 50 μl drops of the capacitated sperm preparation into the bath containing the eggs.

For fertilization experiments, the Ca^{2+} concentration was monitored by measuring the fluorescence from individual eggs loaded with fura red-AM. The microscope used was a Nikon Diaphot 200 equipped with epifluorescence optics and either a × 20, 0.75 numerical aperture or a × 40, 0.8 numerical aperture objective lens to view and excite the oocytes. A 100 W Xenon lamp was the source of excitation light that was filtered by neutral-density filters and the 10 nm bandpass filters. A cooled CCD camera was used to measure emitted light. The excitation filters, camera shutter and data collection were controlled by a computer using the Newcastle Photometrics Multipoint System (Newcastle upon Tyne, U.K.). This system allowed for simultaneous recording from up to 16 eggs. For fura red-AM-loaded egg, the excitation wavelength was altered from 440 nm to 490 nm and the emitted fluorescent light was measured through a 590 nm longpass filter [24]. Recordings are presented as fluorescence ratios against time because of uncertainties about the calibration of dye in the cytoplasm [24].

Assay of protein synthesis

Protein synthetic activity was assayed as described previously [25]. Briefly, zona-intact mouse eggs were fertilized and then, after 1 h, groups of 20 eggs were exposed to TPEN plus DTT or to BAPTA-AM for 1 h while being incubated in M2 medium containing L-[4,5-³H]leucine (50 μCi/ml; Amersham). After incubation the eggs were washed three times in M2 containing unlabelled leucine before lysis by freeze/thaw cycles. BSA was added to the samples as carrier, and proteins were precipitated and washed with trichloroacetic acid and then dissolved overnight in 1 M NaOH before transfer into Optiphase scintillation fluid (Wallac) to count radioactivity. Background radioactivity was obtained from the final trichloroacetic acid wash. We verified that incubation of eggs under these conditions with 10 μM of the protein synthesis inhibitor cycloheximide led to > 95% inhibition of incorporation of [4,5-³H]leucine [25,26].

Electrical field (EF) pulse-induced egg activation protocols

Mouse eggs previously loaded with indo-1-AM were set up for Ca^{2+} measurements during EF pulse stimulation. Individual eggs were held by a suction pipette between two platinum electrodes in a perfusion chamber containing M2 medium maintained at 37 °C and placed on the stage of an inverted microscope [17,27]. An EF radio-frequency pulse of 1.5 kV/10 kHz lasting for 300 μs was applied at 10 min intervals, and the intracellular Ca^{2+} changes were measured and recorded using a Newcastle Photometrics System. At 10 s before each EF stimulation, the medium was rapidly exchanged with a glucose solution (0.3 M) containing 100 μM Ca^{2+}. At the end of each EF pulse the glucose solution was rapidly exchanged for M2 medium. The use of a rapid perfusion system facilitates the decrease in Ca^{2+} concentration after an EF pulse, and is particularly necessary during repetitive pulse stimulation. Five recordings were performed for each treatment, and the average Ca^{2+} changes were computed and charted using SIGMA plot software 3.2.

A large chamber was used to study the time course of pronuclear formation in groups of eggs following treatment with TPEN and DTT. The protocols for perfusion and EF stimulation in these activation experiments were identical to those used in

corresponding Ca²⁺ measurements, i.e. one EF pulse every 10 min for 4 h. The time course of pronuclear formation after treatment was monitored visually using an inverted microscope equipped with Nomarski optics as described elsewhere [16,17].

RESULTS

Inhibition of Ca²⁺ oscillations in fertilizing eggs

Figure 1(A) shows a typical series of Ca²⁺ oscillations, as monitored by fura red fluorescence, after *in vitro* fertilization in a mouse egg. In 47 fertilized eggs, a mean ($\pm$S.D.) of 15.8 ± 8.6 Ca²⁺ spikes occurred at an interval of 15.1 ± 8.6 min. This pattern of Ca²⁺ oscillation is similar to that reported with other fluorescent dye indicators in fertilizing mouse eggs [7,12,28]. In order to examine the effects of different numbers of Ca²⁺ spikes upon egg activation, we sought means of rapidly inhibiting Ca²⁺ oscillations after they had been initiated by sperm. One method for inhibiting Ca²⁺ oscillations in mature eggs and immature oocytes is to add BAPTA-AM. Figure 1(B) shows that addition of the membrane-permeable Ca²⁺ chelator BAPTA-AM after the start of Ca²⁺ oscillations effectively inhibited Ca²⁺ spikes. Such inhibition was seen in most eggs at 5 μM BAPTA-AM and in all eggs when the chelator was used at concentrations of 10–20 μM (Table 1).

The use of BAPTA-AM provided one means of rapidly inhibiting Ca²⁺ oscillations. Another method of inhibiting Ca²⁺ oscillations in mouse eggs made use of a fortuitous novel finding. Some of the dyes used to monitor Ca²⁺ can be affected by heavy-metal ions [18]. In the course of testing for a contribution of heavy-metal ions to fluorescence signals in mouse eggs, we found that the membrane-permeable chelator TPEN inhibited Ca²⁺ spikes. Figure 1(C) shows that the addition of 40 μM TPEN to

Table 1   Effects of different metal-ion chelators on Ca²⁺ oscillations and activation of oocytes

[Chelator] (μM)	No. of eggs	Eggs in which Ca²⁺ spikes stopped (%)	Activation (pronuclear formation) (%)
BAPTA-AM			
5	96	97	94
10	32	100	100
20	30	100	100
TPEN			
10	14	29	80
20	16	40	72
40	44	70	66
40 (+1 mM DTT)	242	92	71
Phenanthroline			
10	31	81	46
20	16	100	51*
40	15	100	0*

* Egg lysis occurred in non-activated eggs.

mouse eggs was effective in inhibiting Ca²⁺ oscillations after they had been initiated by sperm–egg interaction. We also found that phenanthroline was effective at inhibiting Ca²⁺ oscillations in eggs. However, unlike TPEN, phenanthroline caused a slow elevation of Ca²⁺ levels, and lysis occurred within a few hours of application (Table 1). Since TPEN did not cause any progressive elevation of Ca²⁺ levels, nor any signs of cell lysis, further experiments were restricted to investigations of TPEN.

The addition of TPEN to fertilizing eggs was not as effective at inhibiting Ca²⁺ oscillations as was addition of 5–20 μM
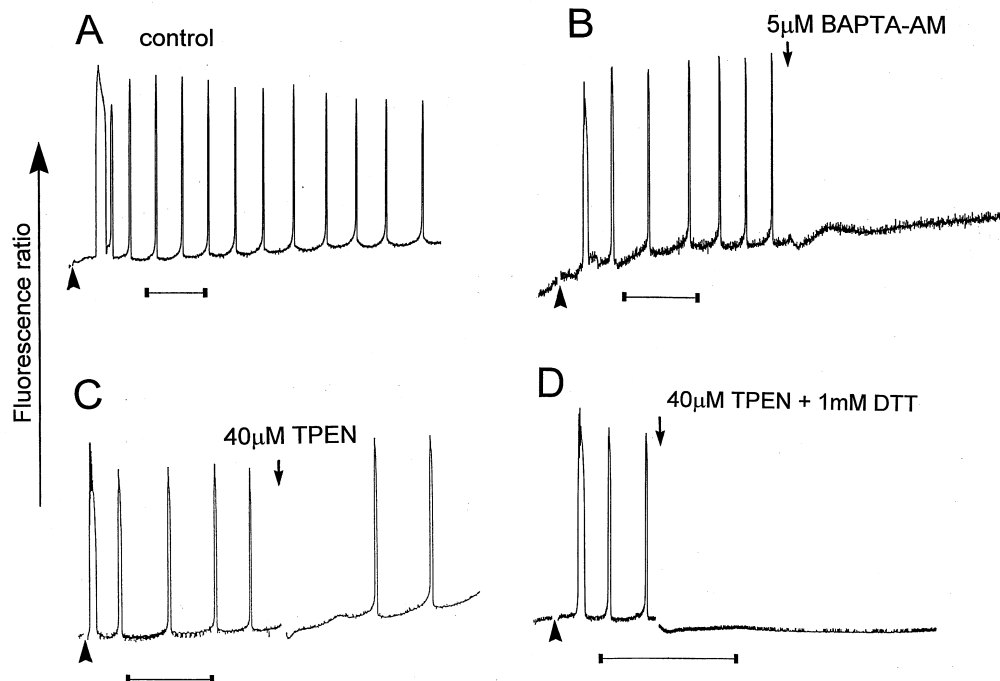

Figure 1   Addition of membrane-permeable metal-ion chelators after the start of Ca²⁺ oscillations in fertilizing mouse eggs

Ca²⁺ was monitored with fura red-AM. (**A**) Typical response in a control fertilized egg; (**B**) addition of 5 μM BAPTA-AM inhibited Ca²⁺ oscillations; (**C**) 40 μM TPEN reduced the frequency of Ca²⁺ oscillations; (**D**) Ca²⁺ oscillations were blocked by the simultaneous addition of TPEN (40 μM) and DTT (1 mM). The time bars indicate 30 min.

A
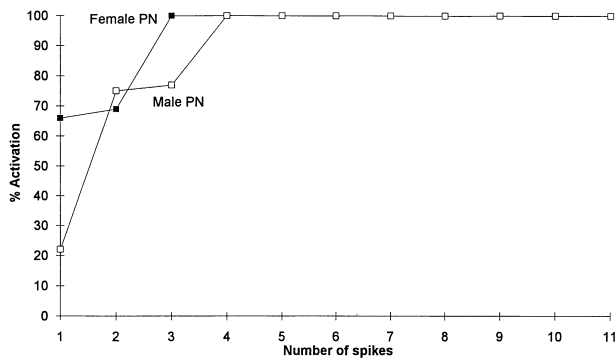
B

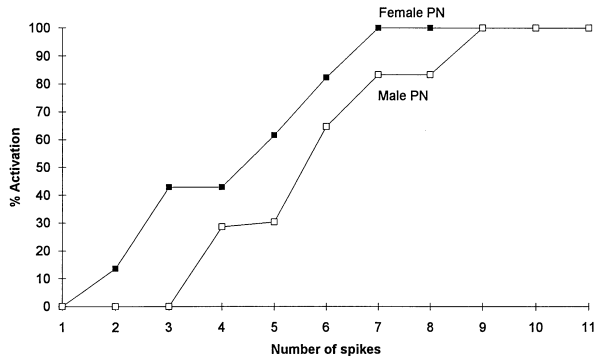**Figure 2   Relationship between the percentage of mouse eggs forming male and female pronuclei and the number of Ca²⁺ spikes that they had undergone before addition of (A) BAPTA-AM or (B) TPEN plus DTT**

Mouse eggs in which the number of Ca^{2+} oscillations had been measured were treated with BAPTA-AM or TPEN plus DTT for 1 h as in Figure 1, and were scored for pronuclei (PN). Data are plotted for eggs in which oscillations were completely blocked by the addition of drugs. The percentage activation is calculated from the number of eggs that formed either a female (■) or a male (□) pronucleus. These curves were compiled using data from 85 eggs for (**A**) and 242 eggs for (**B**).

BAPTA-AM. However, it proved possible to increase the efficiency of inhibition by TPEN by adding a thiol reducing agent. There were two reasons for initially examining the effects of thiol reagents upon Ca^{2+} oscillations in mouse eggs. Firstly, it has been shown that the thiol reducing agent DTT can enhance the frequency of Ca^{2+} oscillations in fertilizing eggs [28]. Thiol reagents can also either enhance or inhibit the ability of heavy-metal ions to trigger Ca^{2+} release from intracellular stores in muscle [29]. We confirmed that DTT increased the frequency of oscillations at fertilization (results not shown). However, rather than stimulating oscillations, Figure 1(D) and Table 1 indicate that the addition of DTT along with TPEN to fertilized eggs enhanced the ability of TPEN to inhibit Ca^{2+} oscillations. The combination of TPEN and DTT proved almost as effective as BAPTA-AM in blocking the sperm-induced Ca^{2+} oscillations (Table 1). Since both treatments rapidly inhibited Ca^{2+} oscillations, further studies on fertilizing mouse eggs were restricted to comparing the effects of TPEN plus DTT with those of BAPTA-AM.

Egg activation after inhibition of Ca²⁺ oscillations

The Ca^{2+} oscillations seen at fertilization are responsible for activating the mammalian egg [7,12]. The initial signs of egg activation consist of the extrusion of the second polar body, with full activation being indicated by the formation of both female and male pronuclei [8]. The activation of eggs was assessed 4–6 h after fertilized eggs had been treated with either BAPTA-AM or TPEN plus DTT. Eggs were exposed to either of these inhibitory treatments for 1 h before they were washed out; the activation data in Table 1 were compiled from eggs in which Ca^{2+} was monitored. There was a clear difference in the activation rates obtained with BAPTA-AM and TPEN plus DTT. We found that nearly all the eggs that had been treated with BAPTA-AM after Ca^{2+} transients had been initiated formed both female and male pronuclei. In contrast, a proportion of the eggs that were exposed in a similar way to TPEN plus DTT did not form pronuclei.

The different effects of BAPTA-AM and TPEN plus DTT upon egg activation are illustrated in Figure 2, which again shows data from eggs that were treated with these agents after Ca^{2+} spikes had been initiated by sperm. Figure 2 shows the percentage of eggs that were activated, as judged by the formation of male and female pronuclei, against the number of Ca^{2+} spikes that an egg had undergone before addition of agents that blocked Ca^{2+} oscillations. All eggs that showed at least one Ca^{2+} transient, before application of either inhibitor, underwent second polar body extrusion. For both treatments there was also a correlation between the number of Ca^{2+} spikes that occurred in eggs and the activation rate, as judged by pronuclear formation. In particular, more Ca^{2+} spikes were associated with a greater pronuclear formation rate. However, there were differences in the number of Ca^{2+} spikes associated with pronuclear formation that depended upon the inhibitor used. Figure 2(A) shows that most eggs treated with BAPTA-AM formed both pronuclei after the occurrence of two Ca^{2+} spikes, and the occurrence of four Ca^{2+} spikes was always accompanied by complete egg activation (the formation of both pronuclei). In contrast, when eggs had undergone between one and four Ca^{2+} spikes before treatment with TPEN plus DTT, the majority did not form either pronucleus. When more Ca^{2+} spikes had occurred in the TPEN + DTT-treated eggs, there was an increase in the percentage of eggs forming pronuclei. Eggs that had undergone nine or more Ca^{2+} spikes before addition of TPEN + DTT all formed two pronuclei (Figure 2B). Both of these sets of data suggest that egg activation at fertilization is correlated with the number of Ca^{2+} spikes that have occurred in eggs. However, there are differences in the number of Ca^{2+} spikes that is correlated with formation of pronuclei, depending upon the inhibitor used.

In addition to the correlation between the number of Ca^{2+} spikes and the percentage pronuclear formation, we also found correlations with the timing of events in the first cell cycle. Figure 3(A) shows the time taken for the formation of pronuclei in different groups of eggs that displayed two particularly common values (10 and 21) for the number of Ca^{2+} spikes before the addition of TPEN plus DTT. We also looked at the formation of pronuclei in control (not treated with TPEN, DTT or BAPTA-AM) eggs that underwent either 10 or 21 Ca^{2+} spikes before spontaneously stopping oscillations. Figure 3(A) shows that there was a significant difference in the time taken to form pronuclei in these two groups of eggs, such that the greater number of Ca^{2+} spikes was associated with a more rapid rate of pronuclear formation. These differences in timing of events also extended into the first mitosis. Figure 3(B) shows that the time taken to undergo nuclear envelope breakdown during entry into mitosis was significantly shorter for the eggs that displayed 21
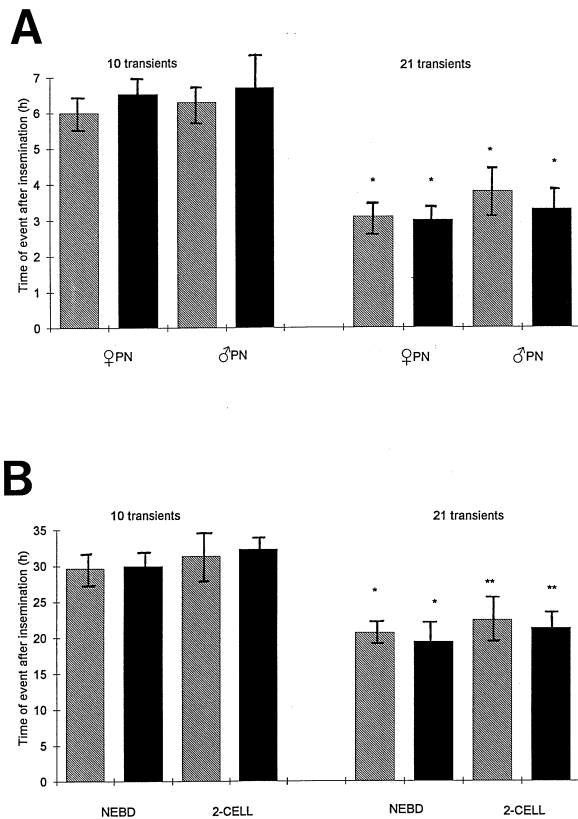
A

B

Figure 3 **Rates of activation in mouse eggs that generated 10 transients and those that generated 21 transients**

The eggs that were treated with TPEN plus DTT and generated either 10 or 21 transients are represented by the solid bars. Control eggs that generated either 10 or 21 transients are represented by hatched bars. (**A**) The time taken for male and female pronucleus (PN) formation is indicated for the four different groups of eggs. (**B**) The time taken for nuclear envelope breakdown (NEBD) and cleavage to the two-cell stage is shown for each of the four groups of eggs. Data are from 14 control eggs that generated 10 transients, 14 control eggs that generated 21 transients, 15 TPEN + DTT-treated eggs that generated 10 transients, and 12 TPEN + DTT-treated eggs that generated 21 transients. Significance of differences compared with eggs generating 10 transients: *$P < 0.05$; **$P < 0.001$ (Student's t-test).

Ca^{2+} spikes than for those that displayed 10 spikes. The time to cleavage to the two-cell stage was also shorter for eggs with the greater number of Ca^{2+} spikes. These differences in timing of the events of pronuclear formation and the first mitosis existed in control eggs as well as in eggs that were exposed to TPEN plus DTT. These data suggest that there is a correlation between the number of Ca^{2+} spikes and the timing of activation and the first cell cycle after fertilization in the mouse.

Effects of ion chelators on protein synthesis

Our data suggest there are correlations between Ca^{2+} spikes and activation events. However, we saw differences in the number of Ca^{2+} spikes correlated with pronuclear formation, depending upon the agent used to inhibit Ca^{2+} oscillations. One possible reason for the different effects on egg activation of TPEN and BAPTA is the level of protein synthesis. It has been shown that BAPTA-AM loading can inhibit protein synthesis in somatic cells [20], and the inhibition of protein synthesis is known to enhance Ca^{2+}-induced egg activation in mammals [25,26]. We investigated the effects of BAPTA-AM and TPEN plus DTT

Table 2 **Comparison of the effects of BAPTA-AM and TPEN plus DTT on the incorporation of L-[³H]leucine into proteins in fertilized mouse eggs**

Zona-intact mouse eggs were fertilized, and after 1 h the agents shown were added for a further 1 h period. Results are expressed as a percentage of the values obtained with control eggs that were fertilized on the same day, and are means ± S.D. of six separate experiments.

Agents	L-[³H]Leucine incorporated (%)
40 μM TPEN	85.4 ± 8.6
40 μM TPEN + 1 mM DTT	87.2 ± 5.4
10 μM BAPTA-AM	34.8 ± 11.2

upon protein synthesis in fertilized mouse eggs under the same experimental conditions as were used to inhibit Ca^{2+} oscillations. Table 2 shows the rate of leucine incorporation into mouse eggs after fertilization. Compared with control eggs (fertilized but not exposed to any drugs), the eggs treated with BAPTA-AM showed a decrease in leucine incorporation. In contrast, the addition of TPEN alone, or TPEN in combination with DTT, did not have a great effect upon leucine incorporation. These data suggest that BAPTA-AM treatment inhibits protein synthesis in mouse eggs after fertilization. This dual action on Ca^{2+} oscillations and on protein synthesis suggests that the results of experiments using BAPTA to block Ca^{2+} oscillations may be more difficult to interpret than those obtained using TPEN and DTT.

Effects of TPEN plus DTT on artificial activation

Since addition of TPEN plus DTT to fertilizing eggs did not appear to affect protein synthesis, it is possible that the correlation between the number of Ca^{2+} spikes and the activation rate in Figure 2(B) represents a relationship between the pattern of Ca^{2+} oscillations and the degree of egg activation. However, before this conclusion can be made, it was important to establish that TPEN plus DTT did not affect any events downstream of Ca^{2+} spikes. This possibility was tested by examining the rate of pronuclear formation in eggs that were induced to undergo Ca^{2+} spikes despite the presence of TPEN and DTT. Previously we have shown that the pattern of Ca^{2+} spikes at fertilization can be mimicked by repeated application of electroporation pulses in the presence of Ca^{2+} [17]. Each electroporation pulse leads to an influx of Ca^{2+}, and the series of repeated pulses mimics the way in which the sperm activates the egg, resulting in high rates of pronuclear formation [16,17]. Figure 4 shows the Ca^{2+} spikes initiated in unfertilized mouse eggs that were exposed to electroporation pulses. The pattern of Ca^{2+} spikes initiated by this artificial method was not altered when eggs were stimulated in the presence of TPEN and DTT (Figure 4). This indicates that TPEN plus DTT was not buffering Ca^{2+} in the egg, or affecting the recovery from Ca^{2+} increases.

We incubated eggs in TPEN plus DTT for 1 h, exposed them to the pattern of pulses as in Figure 4, and then examined the formation of pronuclei. Table 3 shows the percentage pronuclear formation in electroporation-treated eggs in the presence and absence of TPEN plus DTT. Since these eggs are activated in the absence of sperm, the formation of a female pronucleus is the assay for egg activation. Table 3 indicates that the control and treated groups of eggs showed similar rates of egg activation. The time taken to form pronuclei was also similar. The period of exposure of eggs to TPEN plus DTT in these experiments was as long as in the fertilization experiments, and began at the very beginning of the activation protocol. These data
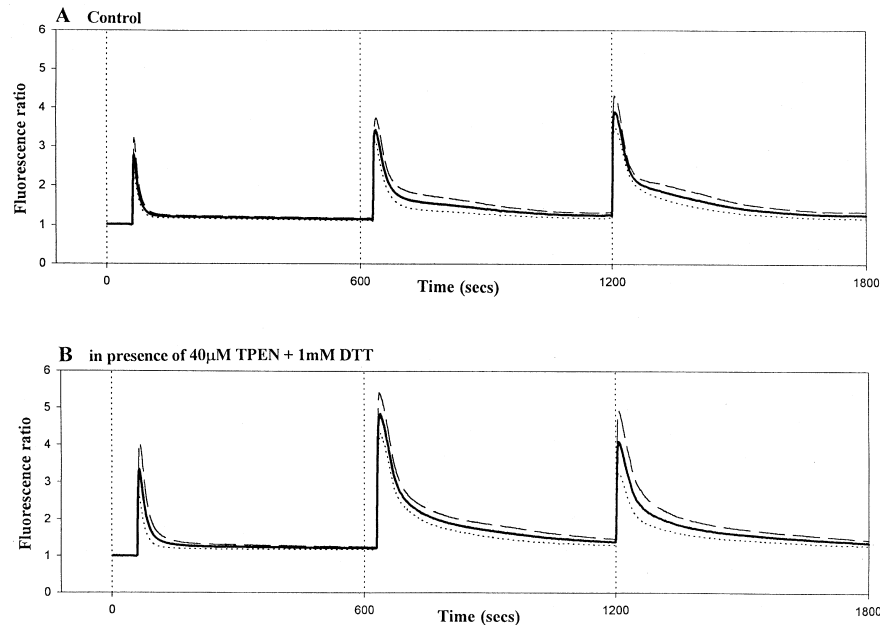
Figure 4 EF pulse-induced increases in intracellular Ca²⁺ concentration ([Ca²⁺]ᵢ) recorded with indo-1-AM

(**A**) Typical control responses; (**B**) Ca²⁺ response after addition of 40 μM TPEN plus 1 mM DTT. The dotted and dashed lines indicate the full range of variability, by showing eggs that had the most different responses from a typical one (solid line).

Table 3 Rates of pronuclear formation in mouse eggs triggered by stimulation of EF pulse-induced Ca²⁺ oscillations

TPEN and DTT were used at concentrations of 40 μM and 1 mM respectively.

| Time after start of EF stimulation (h) | Pronuclear formation (%) | |
	Control eggs ($n = 53$)	TPEN + DTT-treated eggs ($n = 65$)
4	19	22
5	72	52
6	88	78
7	91	92
8	91	94

suggest that TPEN plus DTT does not have a significant effect upon events downstream of Ca²⁺ signals that lead to pronuclear formation, even when the agents are present from the very start of the activation process.

DISCUSSION

We show here that chelators of heavy-metal ions and a chelator of Ca²⁺ can inhibit Ca²⁺ oscillations after they have been initiated in mammalian eggs. However, the consequences of the inhibition of Ca²⁺ oscillations for egg activation, as defined by pronuclear formation, depend on the nature of the agent used to inhibit Ca²⁺ responses.

While it is expected that BAPTA will block Ca²⁺ oscillations due to its ability to chelate Ca²⁺, it is not clear why TPEN was able to inhibit Ca²⁺ oscillations in mouse eggs. At the concentrations that we used, TPEN would not be expected to chelate significant amounts of cytosolic Ca²⁺ [18,30]. A lack of effect of

TPEN plus DTT on general Ca²⁺ buffering is also supported by the finding that the time course of electroporation-induced Ca²⁺ increases was unaltered by these agents. TPEN can also chelate Ca²⁺ inside the endoplasmic reticulum of cells, and this might offer one explanation for its actions in eggs [30], although higher concentrations (> 100 μM) chelated Ca²⁺ in the endoplasmic reticulum [30] than were used in the present studies. TPEN chelates a number of heavy-metal ions, including Zn, Fe, Mn and Cu, more effectively than it chelates Ca²⁺ [18]. Chelation of Fe ions does not seem to be relevant, since the selective Fe chelator desferroxime was not effective at inhibiting Ca²⁺ oscillations (results not shown). An effect of TPEN on other heavy-metal ions, such as Zn, Fe or Mn, is a possible explanation, because we found that Ca²⁺ oscillations could also be inhibited by another membrane-permeable heavy-metal-ion chelator, phenanthroline [31]. Phenanthroline was clearly toxic to eggs and was not used in our studies. However, TPEN caused no visible damage to eggs, did not affect protein synthesis, and did not inhibit events leading to pronuclear formation in parthenogenetically stimulated eggs. If the inhibitory effect of TPEN is explained by chelation of another heavy-metal ion, then the effects may not be specific to fertilization, since it has been shown that TPEN inhibits Ca²⁺ increases generated by low doses of cholecystokinin in pancreatic acinar cells [19]. Such low concentrations of cholecystokinin can trigger large-amplitude Ca²⁺ oscillations that are similar in form to those seen in fertilizing mammalian eggs [5,7,12,28,32].

It is also possible that the effects of TPEN are related to the findings that heavy-metal ions can trigger Ca²⁺ release in muscle and other cell types [29]. We have also found that extracellular addition of some heavy metals such as Cd triggers large Ca²⁺ increases in mouse eggs (Y. Lawrence and K. Swann, unpublished work). These effects of heavy-metal ions are proposed to be on protein thiol groups [29]. Whether this has direct relevance to our finding that DTT enhanced the inhibitory effects of TPEN is

unclear. Nevertheless, both $InsP_3$ and ryanodine receptor Ca^{2+} channels are present in mouse eggs, and they are known to possess critical thiol groups that are involved in effecting Ca^{2+} release [1,29,33]. It is possible that endogenous heavy-metal ions associated with these thiol groups can modulate Ca^{2+} release in mouse eggs.

Regardless of the precise mechanism, our data do show that TPEN plus DTT can effectively inhibit Ca^{2+} oscillations at fertilization. Previous studies have shown that loading mouse eggs with BAPTA using BAPTA-AM prevents all Ca^{2+} oscillations and signs of egg activation, including pronuclear formation [12]. We have used a different protocol to inhibit Ca^{2+} oscillations, adding BAPTA-AM after oscillations had been initiated by the sperm. Our data show that BAPTA loading of eggs is not very effective at inhibiting egg activation to the pronuclear stage if it is applied after between one and four Ca^{2+} spikes have occurred. This may mean that the first few Ca^{2+} oscillations at fertilization are able to fully activate the mouse egg. This would be consistent with the finding that application of the Ca^{2+} ionophore A23187, which causes a single Ca^{2+} increase, is able to activate mouse eggs [7,11,15]. However, when only one to three Ca^{2+} spikes have occurred in BAPTA-AM-treated eggs, or when a single large rise is induced by ionophore treatment, the extent of egg activation is often approx. 50 % [15]. In addition, the effects of both A23187 and BAPTA-AM are complicated by their inhibition of protein synthesis.

It has been shown that the combination of Ca^{2+}-free media and A23187 is the most efficient way to activate mammalian eggs [13,15]. However, under these conditions A23187 is nearly as effective at inhibiting protein synthesis as is cycloheximide [25]. Protein synthesis inhibitors such as cycloheximide can activate mammalian eggs [26]. It has also been shown that protein synthesis inhibitors plus a Ca^{2+} stimulus can act synergistically to cause high rates of pronuclear formation in mammalian eggs [25]. It is possible that agents such as A23187 have a dual action in activating mammalian eggs. The activation of some fertilizing eggs after BAPTA-AM treatment may be due in part to an effect on protein synthesis, since we found that BAPTA-AM inhibits protein synthesis. This is consistent with finding that BAPTA loading of HeLa cells depletes Ca^{2+} stores and leads to an inhibition of protein synthesis [20]. Even though BAPTA-AM does not appear to lower protein synthesis to the levels seen with $10 \mu M$ cycloheximide, it may still be difficult to draw a clear conclusion about the role of different numbers of Ca^{2+} spikes in mouse fertilization using Ca^{2+} chelators, such as BAPTA, if a single Ca^{2+} spike is accompanied by some inhibition of protein synthesis.

The effects of TPEN plus DTT on mouse egg activation contrasted with those of BAPTA, in that inhibition of Ca^{2+} oscillations was associated with an inhibition of pronuclear formation. We cannot rule out a possible effect of TPEN upon pronuclear formation itself, since high (1 mM) concentrations of TPEN have been reported to inhibit the assembly of the nuclear lamina in *Xenopus* eggs [34]. However, our control experiments indicated that TPEN plus DTT was unable to prevent Ca^{2+} spikes or egg activation stimulated by electroporation pulses. These pulses are a close mimic of the pattern of Ca^{2+} oscillations seen at fertilization in mouse eggs [7,17]. The lack of inhibition by TPEN plus DTT on the rate or timing of the formation of pronuclei in response to such parthenogenetic stimuli suggests that events downstream of Ca^{2+} signalling that lead to pronuclear formation are not critical to the inhibitory effect of these agents upon fertilization. Furthermore, since TPEN plus DTT did not significantly affect protein synthesis in fertilizing mouse eggs, the simplest explanation of why TPEN plus DTT inhibits pronuclear formation is because this combination of agents effectively inhibits Ca^{2+} oscillations.

Given that TPEN plus DTT inhibits pronuclear formation via an effect on Ca^{2+} oscillations, our data suggest that there is a relationship between the number of Ca^{2+} spikes that have occurred and the chances of the mouse egg undergoing activation. A greater number of Ca^{2+} spikes was associated with a larger proportion of eggs forming both male and female pronuclei and with a more rapid entry into the first cell cycle during development. The idea that the number of Ca^{2+} spikes is important for activation is evident from previous experiments in mammalian eggs. For example, with TPEN plus DTT we found that eggs undergoing only the initial Ca^{2+} spike at fertilization did not go on to form pronuclei. This result is similar to the finding that injecting $InsP_3$ or Ca^{2+} solutions into mouse eggs, in order to generate a single physiological Ca^{2+} spike, can lead to exocytosis, but is unable to induce (or is inefficient at inducing) pronuclear formation in recently ovulated eggs [35,36]. Brief periods of ionophoretic injection of Ca^{2+}, or a single electroporation pulse in the presence of Ca^{2+}, are also inefficient in triggering pronuclear formation in recently ovulated mouse eggs [14,17]. In contrast, multiple electroporation pulses causing multiple Ca^{2+} spikes can readily activate eggs of any post-ovulatory age [16,17]. After the initial Ca^{2+} spike at fertilization, there appears to be a relationship between the number of spikes and the chances of the egg forming pronuclei. The occurrence of nine or more Ca^{2+} spikes at fertilization seems to be the minimum number associated with formation of two pronuclei, which is indicative of full egg activation. In addition, we also found that the number or frequency of Ca^{2+} spikes was correlated with the timing of pronuclear formation and mitosis in mouse eggs. This difference in timing did not appear to be due to a spurious effect of TPEN or DTT, since it was also seen in control, untreated eggs. These data raise the possibility that some of the variation normally seen in the timing of pronuclear formation and cell division in eggs is due to variable patterns of Ca^{2+} signals.

The current experiments were carried out on fertilizing mammalian eggs. The suggestion that the number of Ca^{2+} spikes plays some causal role in determining the rate of egg activation was made in previous studies using parthenogenetically stimulated eggs [16,17,27]. By using electroporation, it has been shown that the number or frequency of Ca^{2+} spikes can affect the proportion of eggs that form pronuclei, as well the timing of pronuclear formation [17]. The amplitude of a fixed frequency of artificially generated Ca^{2+} spikes can also exert a significant effect upon the timing of egg activation and of the first cell division in mouse embryos [16]. Our data suggest that some of these effects may apply to fertilization. The mouse egg may be able to respond to either the number, or the amplitude, of Ca^{2+} oscillations in a manner that has been suggested for many somatic cells, and recently demonstrated for the actions of isolated enzymes, such as Ca^{2+}/calmodulin-dependent protein phosphorylation [2,3,37].

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