Stimulation of repetitive calcium transients in mouse eggs

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1. We have combined cell membrane electroporation by electrical field (EF) stimulation with a rapid perfusion system in order to stimulate repetitive increases in cytoplasmic free [Ca^{2+}] ([Ca^{2+}]$_i$) in mouse eggs. [Ca^{2+}]$_i$ was monitored by ratio fluorescent measurements of intracellular indo-1 on individual eggs. The conditions required to cause different types of [Ca^{2+}]$_i$ increases were established and the effects of these [Ca^{2+}]$_i$ changes upon egg activation examined.
2. The rapid perfusion of non-ionic medium caused a single [Ca^{2+}]$_i$ increase. However, to generate repetitive [Ca^{2+}]$_i$ increases, eggs were exposed to EF pulses in the presence of Ca^{2+} and then washed rapidly with culture medium. Sequential EF pulse application led to prolonged elevation of [Ca^{2+}]$_i$ levels and eventual cell lysis unless rapid reperfusion with culture medium was achieved. Transient increases in [Ca^{2+}]$_i$ in eggs could also be generated by EF pulses in the presence of inositol 1,4,5-trisphosphate (InsP_3).
3. In response to EF stimulation fertilized eggs showed [Ca^{2+}]$_i$ increases that were enhanced relative to unfertilized eggs. The responses in these fertilized eggs were often followed by repetitive [Ca^{2+}]$_i$ oscillations, despite the fact that the [Ca^{2+}]$_i$ oscillations associated with sperm penetration had ceased by this stage.
4. In unfertilized mouse eggs the [Ca^{2+}]$_i$ increases appeared to be due to direct cation influx since repeated EF pulses caused repeated influx of Mn^{2+} as monitored by quenching of fluorescence of fura-2 loaded eggs.
5. Under conditions that stimulated reproducible patterns of [Ca^{2+}]$_i$ transients we found that a single large [Ca^{2+}]$_i$ transient did not cause significant egg activation, but that inducing repetitive [Ca^{2+}]$_i$ transients was effective in activating eggs. The speed of activation as judged by the rate of pronuclear formation was also dependent upon the frequency of pulse application.
6. These data show that combining EF pulses with a rapid and precise sequential perfusion system can be used to manipulate [Ca^{2+}]$_i$ levels in mammalian eggs. This provides a means of artificial mimicry of the [Ca^{2+}]$_i$ transients seen after fertilization. It appears that Ca^{2+} influx during EF pulses does not cause significant Ca^{2+} release from internal stores in unfertilized eggs, but after fertilization Ca^{2+} influx does induce Ca^{2+} release. It is also apparent that mouse eggs are more successfully activated by repetitive [Ca^{2+}]$_i$ increases than by single large [Ca^{2+}]$_i$ rises. We suggest that our data provide direct evidence for the hypothesis that a cellular response to oscillations of intracellular [Ca^{2+}]$_i$ can be distinct from that to monotonic rises in [Ca^{2+}]$_i$.

Repetitive rises in the cytoplasmic free Ca^{2+} concentration, so-called [Ca^{2+}]$_i$ oscillations, are observed in many different cell types when stimulated by hormones, neurotransmitters or growth factors (Berridge & Galione, 1988; Tsien & Tsien, 1990; Meyer & Stryer, 1991). It is thought that these [Ca^{2+}]$_i$ oscillations are a means by which extracellular signals are converted into intracellular information in a way that may determine the degree and nature of a cellular response. The fact that the number of [Ca^{2+}]$_i$ transients per unit time (frequency) has been found to vary with hormone concentration has led to the proposal that [Ca^{2+}]$_i$ oscillations might form part of a digitally based cell signalling system (Berridge & Rapp, 1979; Goldbeter, Dupont & Berridge, 1990; Meyer & Stryer, 1991). Cells may be able to respond differently to repetitive than to monotonic [Ca^{2+}]$_i$ increases (Tsien & Tsien, 1990; Meyer & Stryer, 1991). Such digitally encoded information may play an important role in many forms of cellular signalling

(Berridge & Rapp, 1979). However, there are very few direct demonstrations of cellular reactions that are specifically elicited in response to pulsed $[Ca^{2+}]_i$ signals applied independently of the physiological or hormonal stimulus (Tsien & Tsien, 1990). The difficulty entailed in directly manipulating cell $[Ca^{2+}]_i$ has meant that the analysis of how cells would respond specifically to oscillations has remained mainly theoretical (Goldbeter *et al.* 1990; Meyer & Stryer, 1991).

Mammalian eggs have proved to be a useful cell type for studying the mechanisms and functions of repetitive $[Ca^{2+}]_i$ oscillations. At fertilization, in all mammalian species studied, the fusion of the sperm triggers a series of prolonged $[Ca^{2+}]_i$ oscillations (Miyazaki, 1991; Swann & Ozil, 1994). In mouse eggs, at least five independent studies have confirmed the presence of such $[Ca^{2+}]_i$ oscillations during fertilization (Cuthbertson & Cobbold, 1985; Kline & Kline, 1992; Shiina, Koneda, Matuyama, Tanaka, Hirio & Doi, 1993; Cheek, McGuinness, Vincent, Moreton & Berridge, 1993; Swann & Ozil, 1994). As with non-mammalian eggs, the increases in $[Ca^{2+}]_i$ in mammalian eggs are responsible for the activation of development (Fulton & Whittingham, 1978; Kline & Kline, 1992; Swann & Ozil, 1994). Normal activation in mammalian eggs involves exit from metaphase II arrest and entry into interphase as marked by the formation of pronuclei (Whittingham, 1980). Whilst a critical role for $[Ca^{2+}]_i$ in mammalian egg activation is established, the meaning and precise function of different pattern of oscillations has been less clear.

A specific function for repetitive $[Ca^{2+}]_i$ increases in egg activation has been suggested from experiments that generated parthenogenetic embryos in mice and rabbits. It was found that repetitive electrical field (EF) stimulation in the presence of Ca^{2+} was a more effective trigger for pre- and post-implantation development than other parthenogenetic stimuli that are reported to cause a monotonic increase in $[Ca^{2+}]_i$ (Ozil, 1990; Vitullo & Ozil, 1992). Such EF pulses have been shown to cause pore formation and Ca^{2+} influx in eggs (Baker, Knight & Whitaker, 1980; Rossignol, Decker, Lennarz, Tsong & Teissie, 1981; Knight, 1981). Hence it appears that repeated Ca^{2+} transients are a more effective trigger for parthenogenetic mammalian embryo development. However, these initial studies only examined the effects of fixed frequency of stimulation on activation; the amplitude of stimulation was varied. In addition the actual form of $[Ca^{2+}]_i$ increase induced by EF pulses in these parthenogenetic studies was not examined. In particular it was unclear how effectively the stimulation pattern used mimicked the $[Ca^{2+}]_i$ oscillations seen at fertilization. This is of some concern since recent studies have confirmed that EF pulses can generate $[Ca^{2+}]_i$ increases in mammalian eggs, but found that the $[Ca^{2+}]_i$ responses after EF stimulation were generally of longer duration and showed a slower recovery to baseline than those seen after fertilization (Rickords & White, 1992; Sun, Hoyland, Huang, Mason & Moor, 1992; Collas, Sullivan & Barnes, 1993*a*; Collas, Fissore, Robl, Sullivan & Barnes, 1993*b*). It was also noted in those studies that repeated EF stimulation did not improve activation rates by as much as previously reported (Ozil, 1990). This may have been because the studies that measured $[Ca^{2+}]_i$ in eggs did not explore the effect on activation of more than six repeated EF pulses. This is somewhat less than the range 10–100 $[Ca^{2+}]_i$ transients seen after fertilization in rodent eggs (Swann & Ozil, 1994).

In the present study we have combined EF pulse stimulation with a fast perfusion system to explore the conditions for inducing a prolonged series of repetitive $[Ca^{2+}]_i$ transients that closely mimic the pattern of Ca^{2+} oscillations seen after sperm penetration during *in vitro* fertilization of mouse eggs. We then examine the activation response of the egg when exposed to Ca^{2+} stimulations of varying frequencies. Since a future strategy to study the effect of $[Ca^{2+}]_i$ changes on development may involve fertilized eggs we have also examined the response of fertilized eggs to EF stimulation. These and other experiments suggest that fertilized eggs respond fundamentally differently from unfertilized eggs to EF-generated Ca^{2+} influx. The technology that we describe here in detail allows us direct access to the cytoplasm during earlier stages of development and suggests a new approach for studying how epigenetic signals, such as repetitive $[Ca^{2+}]_i$ oscillations, may affect later developmental processes. Some the findings in this study were presented as an abstract to the Physiological Society (Ozil & Swann, 1993).

METHODS

Recovery of oocytes

F1 (C57BL/6XCBA) hybrid female immature mice were superovulated at ages of 3–4 weeks by an I.P. injection of 5 i.u. pregnant mares serum gonadotrophin (PMSG) followed 48 h later by 7 i.u. of human chorionic gonadotrophin (hCG). Mice were killed by cervical dislocation, metaphase II stage oocytes (referred to henceforth as eggs) were released from the oviducts 12–18 h after hCG injection and the cumulus cells were removed with hyaluronidase (300 U ml^{-1}). Egg were washed, maintained and cultured at 37 °C in M16 medium (Fulton & Whittingham, 1978) supplemented with 4 mg ml^{-1} of BSA (fraction V; Sigma). All electrical permeabilizations were carried out on eggs perfused with a low ionic strength solution containing additives as described in the Results.

Media used

The eggs were maintained in an embryo culture medium that is referred to as M16 and is described in Fulton & Whittingham (1978) and Igusa & Miyazaki (1983). M16 contains 1·7 mM Ca^{2+} and for many for the experiments we describe, the M16 medium was modified by omitting the Ca^{2+} and replacing the missing ions of $CaCl_2$ with NaCl (see Igusa & Miyazaki, 1983). In some experiments the M16 medium was supplemented with an ATP regeneration system. This consisted of 4 mm ATP,

5 mM phosphocreatine (Sigma) and 3 U ml^{-1} creatine phosphokinase (Sigma). The non-ionic medium used for the majority of experiments consisted of 0·3 M glucose (Aristar grade, BDH) in reverse osmosis water (Elgarstat, 18 MΩ cm). InsP_3 (Sigma) or cations (Ca^{2+}, Mn^{2+} and K^+) were added directly to this medium at the stated concentrations. Unless indicated otherwise all chemicals were Analar grade and obtained from BDH.

Measurements of intracellular free calcium concentrations

Intracellular Ca^{2+} was measured using the Ca^{2+}-sensitive dye indo-1 (Grykiewicz, Poenie & Tsien, 1985; from Molecular Probes, Eugene, OR, USA). Mn^{2+} influx was monitored with fura-2 (Molecular Probes). To load the dyes eggs were incubated for 30 min at 37 °C with 50 μM of the acetoxymethyl (AM) form of the dyes made up in M16 with 0·02% pluronic F-127 (Molecular Probes). After loading, eggs were placed in a special chamber on the stage of an inverted epifluorescence microscope (Nikon Diaphot) fitted with a ×100 oil immersion fluorescence objective of numerical aperture 1·3 (see Fig. 1). For excitation of the indo-1, a collimated light beam from a mercury arc lamp (100 W Hg) was passed through neutral filters to reduce the light intensity by 1024 times and reflected off a dichroic mirror (380 nm). Emitted fluorescence signal was directed to another dichroic mirror (455 nm), and transmitted light was filtered at 485 nm and reflected light filtered at 405 nm. The intensity of light was recorded by two separated photometers (type 9924B) connected to current-to-voltage amplifier–converters (Thorn EMI, Ruislip, UK). Analog voltage signals were collected, stored and analysed on a Compaq SLT20s computer using the UMANS system (C. Regen, PO Box 361, Urbana, IL 61801, USA). The mean background fluorescence recorded with separate unloaded eggs was first subtracted from each recording. Under these experimental conditions the ratio $R = F_{405}/F_{485}$ was recorded on-line as a voltage signal and expressed as intracellular Ca^{2+} using the formula derived by Grynkiewicz *et al.* (1985):

$$[Ca^{2+}]_i = K_d F_{485,min}/F_{485,max}(R - R_{min})/(R_{max} - R),$$

where R_{max} is the value of R at saturating Ca^{2+}, R_{min} the value of R at limiting low Ca^{2+}, and $F_{485,min}/F_{485,max}$ the ratio of F_{485} at minimal to that at maximal Ca^{2+}. The value of asymptotic R values was estimated from *in vitro* calibration using a 120 mM KCl–20 mM Hepes solution with 1 mM $CaCl_2$ for estimation of R_{max} and the same solution supplemented with 5 mM EGTA for estimation of R_{min}. The values of the ratios R_{min}, R_{max} and the ratio of $F_{485,min}/F_{485,max}$ averaged 6·5, 0·2 and 7·241, respectively. These values and the relationship between Ca^{2+} and ratio R allowed us to estimate that the calcium change occurs within physiological limits of Ca^{2+}, 300 nM to 2·8 μM. Since we are ultimately concerned with comparing a signal with that at fertilization and since there are difficulties in extrapolating absolute estimates of $[Ca^{2+}]$ using indo-1 to measurements in intact cells (Owen & Shuler, 1989), the data in this paper are presented as fluorescence ratios. All $[Ca^{2+}]_i$ measurement experiments are typical of at least three separate experiments.

Electrical field stimulation procedures

For all fluorescence measurement experiments single eggs were held by a suction pipette between two platinum electrodes 0·3 mm apart (0·2 mm high and 1 mm long) in a chamber that was placed on the stage of the inverted microscope (see Fig. 1). The design of the stimulation chamber is similar to that previously described (Ozil, 1990). On each side of the chamber two small tubes allowed injection of either culture or glucose medium. In most experiments, shortly before the EF pulse the culture medium was removed in two steps. The glucose medium was perfused at 70 ml h^{-1} for 5 s, and then at 200 ml h^{-1} for 5 s. The conductance of the medium before and during the pulse was monitored by measuring the amplitude of current generated by defined voltage pulse with a Tektronix 7704 oscilloscope mounted with a 7D20 programmable digitizer and a 7A22 differential amplifier (Tektronix, 91 941 Les Ulis, France). This measurement enabled us to confirm that the culture medium had been efficiently removed before EF pulse application since the conductance reached an asymptotic low level after perfusion in non-ionic medium. After each EF pulse the culture medium was immediately reperfused into the chamber. At the maximum perfusion flux used, the conductance increase implied that pulsating medium was replaced by culture medium in the chamber within 100–300 ms. The rapid washing procedure for replacement of the culture medium and glucose solution was by remote infusion and a withdrawal syringe pump (Type 22, Harvard Apparatus, Ealing France, 91 941 Les Ulis, France).

The main improvement on the previous technique (Ozil, 1990) was that four perfusion channels were used simultaneously. Excess medium in the chamber was pumped out with a peristaltic pump whose speed of rotation was coupled with the injection flux. All equipment was digitized and the speeds of the pump, electronic valves and stimulator were controlled by a Compaq Deskpro 25E via a customized BIONIC interface (M. Rocheman, Montlhery, France) with an Asyst-written software program (McMillan Software Co., New York).

Studies of egg activation

The chamber used for egg activation was slightly different from the one used for $[Ca^{2+}]_i$ measurements because large batches of eggs had to be stimulated at a time. Eggs were therefore held in the chamber by suction through a small longitudinal slit (20 μm) in the bottom of a long glass chamber, equidistant from two platinum electrodes (15 mm long instead of 1 mm for the measured chamber) (see Ozil, 1990). In order to retain eggs in the slit during rapid perfusion the suction was made proportional to the rate of perfusion of the medium into the chamber. Consequently more than 100 eggs could be simultaneously exposed to a series of EF pulses with various sequences of perfusion (INRA–SNEA patented, 'Process for artificial stimulation of cells and oocytes', Patent no. 9003108, Paris). The total volume of the chamber was 7 μl. The protocols for perfusion and EF stimulation in these activation experiments were identical to those used in corresponding $[Ca^{2+}]_i$ measurement experiments. We assume that the slight difference in the structure of the chamber did not alter the pattern of $[Ca^{2+}]_i$ responses seen in the measuring chamber. The time course of pronuclear formation after egg activation was monitored visually using an inverted Nikon Diaphot microscope equipped with Nomarski optics as described elsewhere (Vitullo & Ozil, 1992).

Fertilization of mouse oocytes *in vitro*

Sperm were obtained from the cauda epididymis of 16-week-old male mice. Sperm, at 2–5 × 10^6 sperm ml^{-1}, were incubated for 1–2 h in T-6 medium (Quinn, Barros & Whittingham, 1982) supplemented with 15 mg BSA (fraction V) at 37 °C in 5% CO_2. Egg were loaded with indo-1 and freed from zona pellucida by brief treatment with acid Tyrode solution. Zona-

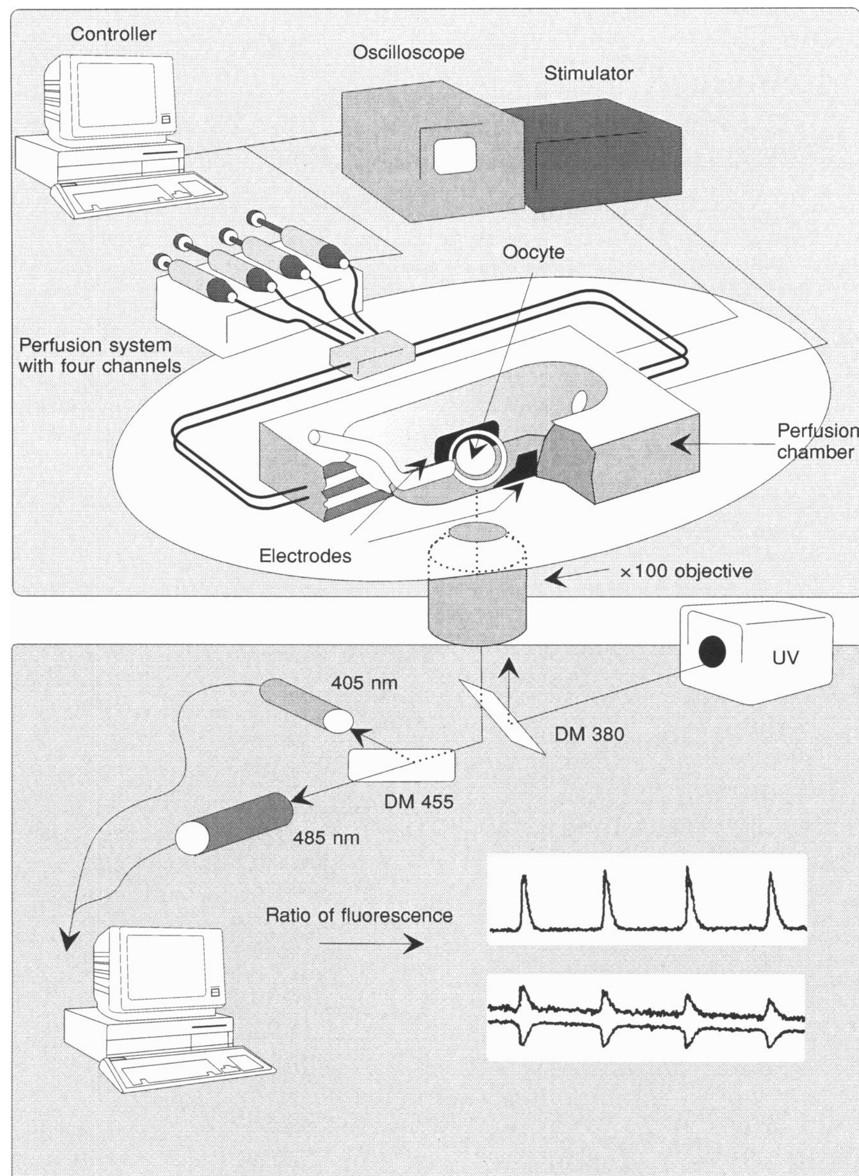

Figure 1. A schematic diagram of the apparatus used for all experiments involving measurements of $[Ca^{2+}]_i$

free eggs were put on the bottom of the perfusion chamber, previously coated with polylysine (100 μg ml^{-1}), in order to attach the eggs to the bottom of the drop where eggs were inseminated. Fluorescence was measured as described above. Figure 2*A* shows the start of $[Ca^{2+}]_i$ changes after fertilization in a single mouse egg as measured by the indo-1 fluorescence ratio.

RESULTS

The effects of rapid perfusion and EF stimulation on calcium in unfertilized eggs

In previous studies of egg activation the standard culture medium was replaced by a non-conductive medium before exposing cells to EF stimulation (Onodera & Tsunoda, 1989; Sun *et al.* 1992; Collas *et al.* 1993*b*). To completely remove the culture medium, according to the criterion of minimum conductivity, rapid perfusion of eggs was necessary (see Methods). However, we found that under certain conditions the rapid perfusion of non-ionic medium itself affected the $[Ca^{2+}]_i$ levels in eggs. When eggs were maintained in the chamber under perfusion with culture medium at 37 °C, and then washed for 3 min in a non-ionic solution containing 0·3 M glucose, mannitol, 2-deoxy-D-glucose or saccharose, a single $[Ca^{2+}]_i$ increase occurred in almost all eggs (Fig. 2*A*–*D*). Any one egg only ever showed this type of response once. Often, on top of the main response curve, a series of small oscillations occurred as shown on Fig. 2*C* and *E*. This pattern of response is similar to the first $[Ca^{2+}]_i$ increase following fertilization (Fig. 2*A*). These $[Ca^{2+}]_i$ increases occurred in medium in which there

was no added calcium. The mechanism responsible is unclear. Perfusing with different sugars, such as mannitol, or even non-metabolizable analogues such as 2-deoxy-D-glucose all gave the same response. Nevertheless, it was notable that we could mimic the initial $[Ca^{2+}]_i$ increase at fertilization merely by rapid perfusion in appropriate medium. However, to avoid generating such responses before applying EF pulses, in the next series of experiments we set up a perfusion protocol that allowed for removal of culture medium and replacement with 0·3 M glucose medium in 10 s. Under such conditions repetitive washes in non-ionic medium did not by itself cause any intracellular $[Ca^{2+}]_i$ changes.

When eggs were submitted every 4 min to a bipolar EF pulse of 1·25 kV cm^{-1} and 1 ms duration in glucose solution without Ca^{2+}, no $[Ca^{2+}]_i$ increase occurred (Fig. 3*A*). Even the immediate post-pulse perfusion with culture medium (which contains 1·7 mM Ca^{2+}) did not cause any $[Ca^{2+}]_i$ increase. The 10 s wash with glucose before the pulse was strong enough (perfusion at 200 ml h^{-1}) to remove all ions from the culture medium because the electrical current generated by the pulse reached its minimal asymptote.

However, if the culture medium was not properly removed before the pulse, either due to slow perfusion or to turbulence of the medium in the chamber, the current intensity during the pulse was not minimized and $[Ca^{2+}]_i$ increases occurred after each EF pulse and failed to recover. This eventually led to egg lysis (Fig. 3*A*). This result demonstrated that a rapid and complete perfusion protocol is critical for repetitive EF stimulation to be successful. In the following experiments a 10 s rapid wash protocol was used and the EF pulse parameters were set at 1·25 kV cm^{-1} and 1 ms duration.

The effects of EF stimulation on unfertilized eggs in the presence of non-ionic medium containing calcium

When 50 μM $CaCl_2$ was added in the pulsating medium a sharp rise of the $[Ca^{2+}]_i$ was observed after each EF pulse. Repeated stimuli at 4 min intervals caused similar patterns of $[Ca^{2+}]_i$ increase suggesting that there was no substantial change of the membrane permeabilization and resealing processes after multiple stimuli (Fig. 3*B*). The changes in $[Ca^{2+}]_i$ typically showed a three-phase response pattern. In the first phase the $[Ca^{2+}]_i$ increased rapidly in response to the EF pulse, reaching a peak amplitude in 2–3 s. This was followed by a second phase with a rapid decay to one-third of the amplitude, and then a third phase consisting of a slow decay to the resting level which lasted more than 2 min (Fig. 3*B*). This pattern of response in $[Ca^{2+}]_i$ does not match that seen after fertilization in mouse eggs (see Fig. 2*A* and Kline & Kline, 1992; Shiina *et al.* 1993; Cheek *et al.* 1993; Swann & Ozil, 1994). The Ca^{2+} increases at fertilization stay elevated for more than 1 min and decrease very rapidly in one phase. In order to increase the match between the EF pulse-stimulated $[Ca^{2+}]_i$ increases and those seen at fertilization we have concentrated on methods to increase the rate of recovery from EF pulse-stimulated $[Ca^{2+}]_i$ increases.

One simple method we found of increasing the rate of recovery from $[Ca^{2+}]_i$ increases was to reperfuse the eggs with a Ca^{2+}-free M16 medium. Figure 3*B* illustrates the difference in recovery of $[Ca^{2+}]_i$ increases with eggs in normal M16 medium and Ca^{2+}-free M16 medium. Another method that improved the rate of recovery involved adding ATP. Studies on hamster eggs have suggested that recovery from $[Ca^{2+}]_i$ increases in mammalian eggs relies on a plasma membrane Ca^{2+}-ATPase. It has also been shown that EF stimulation in sea urchin eggs promotes leakage of ATP (Swezey & Epel, 1989). It is therefore possible that a transient decline in ATP levels confounds the recovery of EF-stimulated $[Ca^{2+}]_i$ increases. We investigated this by adding ATP, in an ATP regenerating system, to the culture medium that was perfusing the eggs during the recovery phase. Figure 3*C* shows that when eggs were stimulated with an EF pulse in glucose medium containing 50 μM Ca^{2+} and then immediately washed with culture medium enriched with 4 mM ATP for 1 min, the $[Ca^{2+}]_i$ recovered more rapidly to the baseline levels.

Studies in somatic cells have suggested that electrical permeabilization using an oscillating (or radio frequency) field pulse causes less long-term damage to cells than application of a simple bipolar pulse (Chang, 1989). We have tested this hypothesis in mouse eggs by submitting eggs to an oscillating field superimposed on the bipolar pulse of 1·25 kV cm^{-1} and 1 ms duration. The waveform of the oscillating electric field pulse is shown next to Fig. 3*D* and *E*. For 10 kHz the pulse is composed of ten bipolar pulses of 45 μs at 5 μs intervals. The records of Fig. 3*D* show clearly that applying an RF pulse (10 kHz) in Ca^{2+}-containing glucose medium caused $[Ca^{2+}]_i$ changes of similar amplitude to those seen with a bipolar pulse. However, with radio frequency pulses the $[Ca^{2+}]_i$ transient recovery to baseline was more rapid.

The source of the calcium increase for EF-induced responses

Previous studies have used large batch numbers of sea urchin eggs to demonstrate that the EF pulse can cause Ca^{2+} influx. In order to investigate Ca^{2+} fluxes in single mouse eggs we have adopted the Mn^{2+} quench technique that uses Mn^{2+} as a surrogate tracer for Ca^{2+} (Jacob, 1990). Mn^{2+} is able to quench the fluorescence of the fura-2 and this process can be monitored independently of $[Ca^{2+}]_i$ changes by measuring fura-2 fluorescence at 360 nm wavelength excitation. We loaded fertilized and unfertilized eggs with fura-2 and used 40 μM Mn^{2+} instead of Ca^{2+} in the glucose solution; intensity of fluorescence was observed with excitation light at 360 nm. The records of Fig. 4*A* show that when an egg was washed in glucose solution alone, no decrease in fluorescence of fura-2 was observed beyond that associated with a gradual decline in

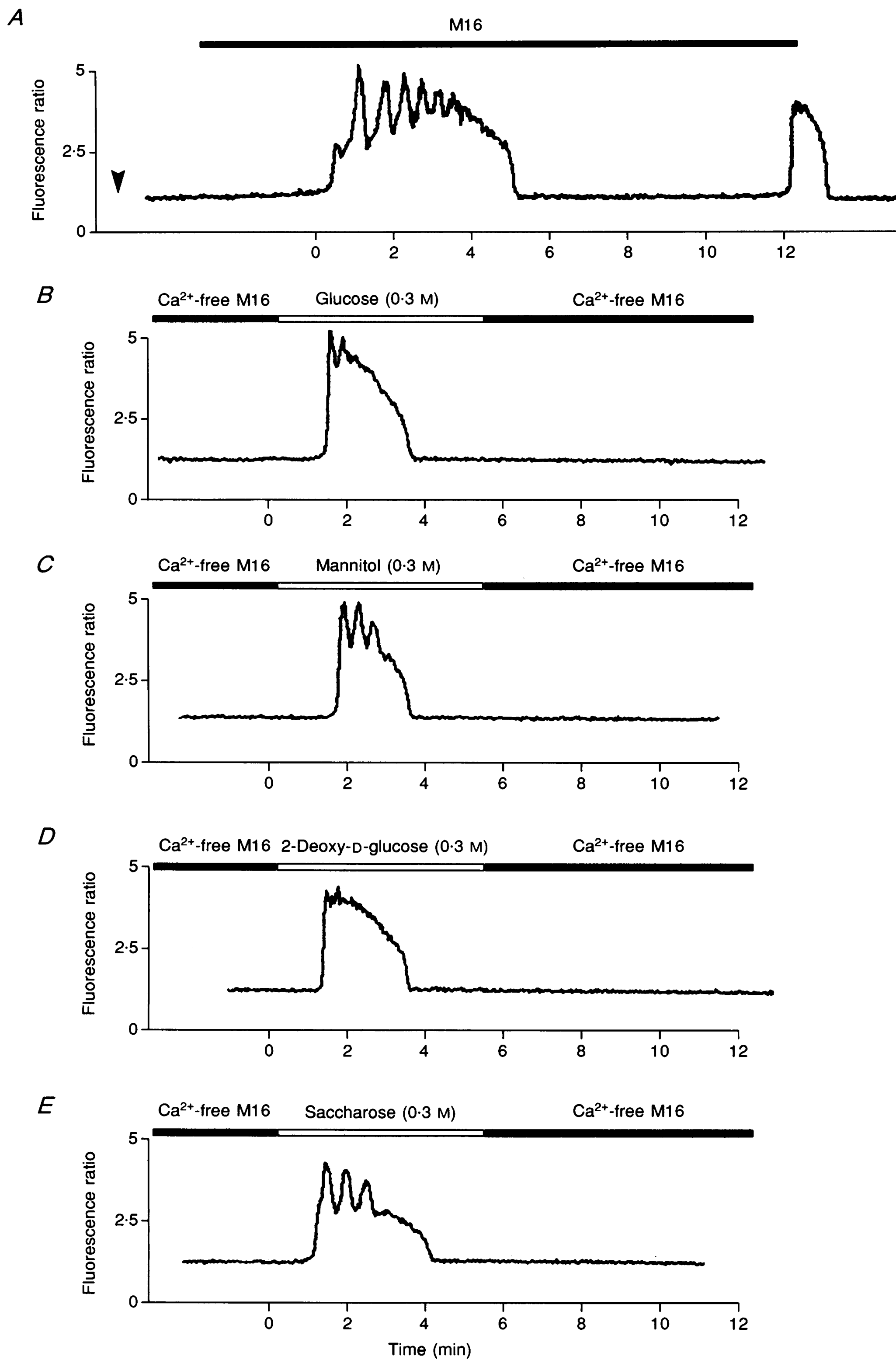

Figure 2. The $[Ca^{2+}]_i$ levels in individual mouse eggs as measured by the fluorescence ratio of intracellular indo-1

A shows the first two $[Ca^{2+}]_i$ increases recorded from an egg in the same chamber a few minutes after insemination (arrowhead) in Ca^{2+}-containing M16 medium. The perfusion protocols are indicated by the bars under each indo-1 record. In *B*, the egg was rapidly perfused with Ca^{2+}-free M16 medium and then for 3 min with a solution of 0·3 M glucose in water (18 MΩ cm), followed by reperfusion with Ca^{2+}-free medium. Similar conditions apply in *C–E* except glucose was replaced by mannitol, 2-deoxy-D-glucose and saccharose, respectively.

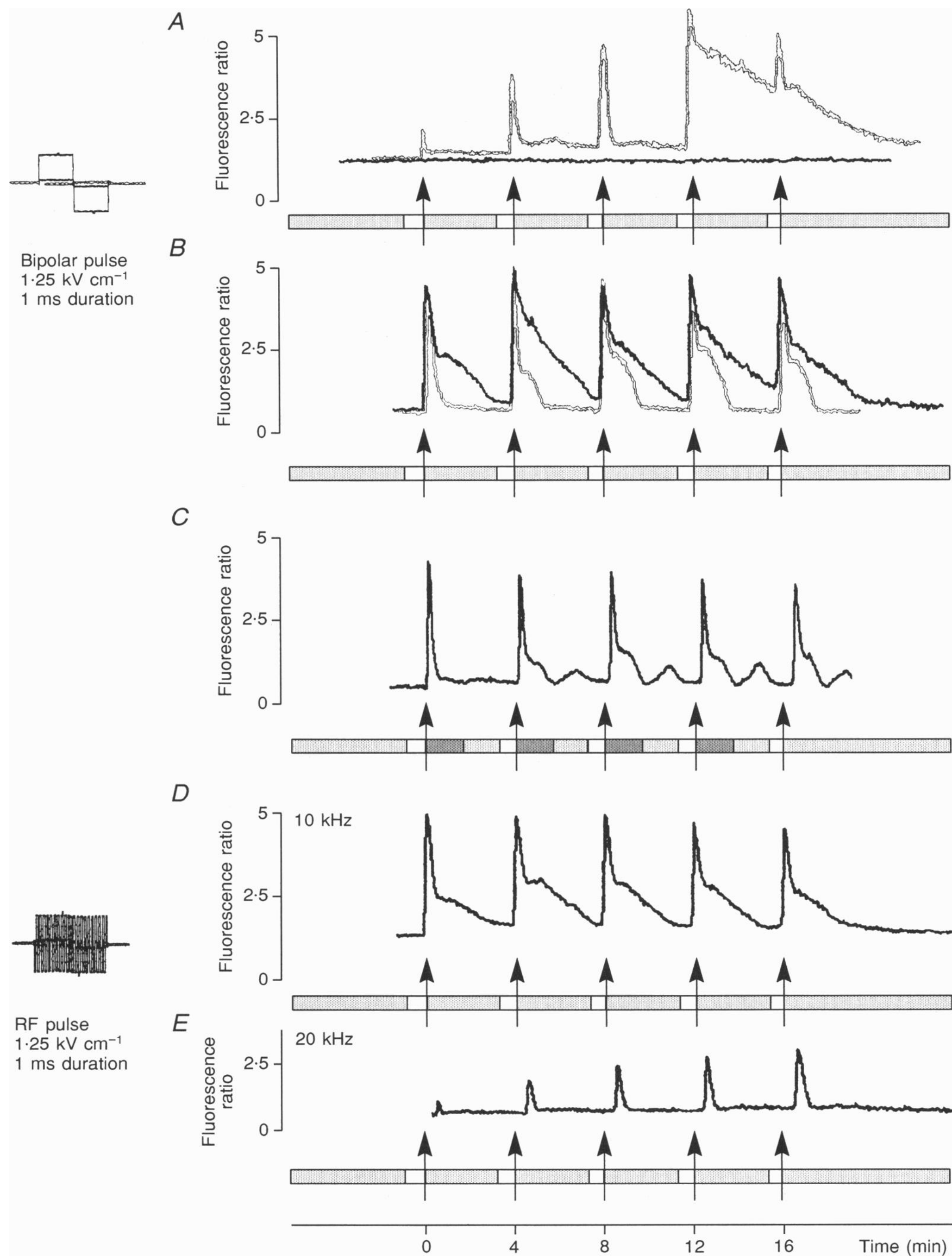

Figure 3. Indo-1 based records of $[Ca^{2+}]_i$ from eggs stimulated with EF pulses

As in Fig. 2 the bar sections below each trace indicate the wash protocol with each medium represented by a different shade: light grey for culture medium, dark grey for M16 medium enriched with 4 mM ATP and white for the glucose-containing medium. The arrows indicate the times of the EF pulses which were always 1·25 kV cm^{-1} and 1 ms duration. In *A*, bipolar pulses were applied in glucose medium with no added Ca^{2+}; the dark trace is from an egg in which the washing protocol was efficient (as judged by the change in conductivity); the light trace is typical for an egg where the washing was not always efficient due to turbulence in the chamber. In cases where the return of $[Ca^{2+}]_i$ to basal levels was incomplete, eggs eventually lysed. In *B*, the conditions are the same as in *A* except that 50 μM Ca^{2+} was added to the glucose medium. The dark trace is from an egg where normal M16 was perfused back into the chamber and the light trace is from an egg where a Ca^{2+}-free M16 medium was used to perfuse the eggs after EF stimulation. In *C* the same protocol was used as in *B* except that medium perfused immediately after the EF pulse (dark grey bar) contained 4 mM ATP. In *D* and *E* conditions were identical to those in *B* except the EF pulse was modulated at 10 and 20 kHz respectively.

fluorescence seen throughout the recording period. This suggests that no leakage of dye occurs after pulsation. However when an egg was pulsed in the presence of 40 μM Mn^{2+}, a decrease in fura-2 fluorescence of approximately 10% occurs with each EF pulse. Figure 4*B* shows that the measured decrease in fluorescence caused by Mn^{2+} influx was similar in a fertilized egg to that seen in an unfertilized egg (see later). These data suggest that Mn^{2+} influx, and by implication Ca^{2+} influx, occurs rapidly either during or shortly after each EF pulse in the glucose-containing medium and that cation influx is similar with each pulse in unfertilized and fertilized eggs.

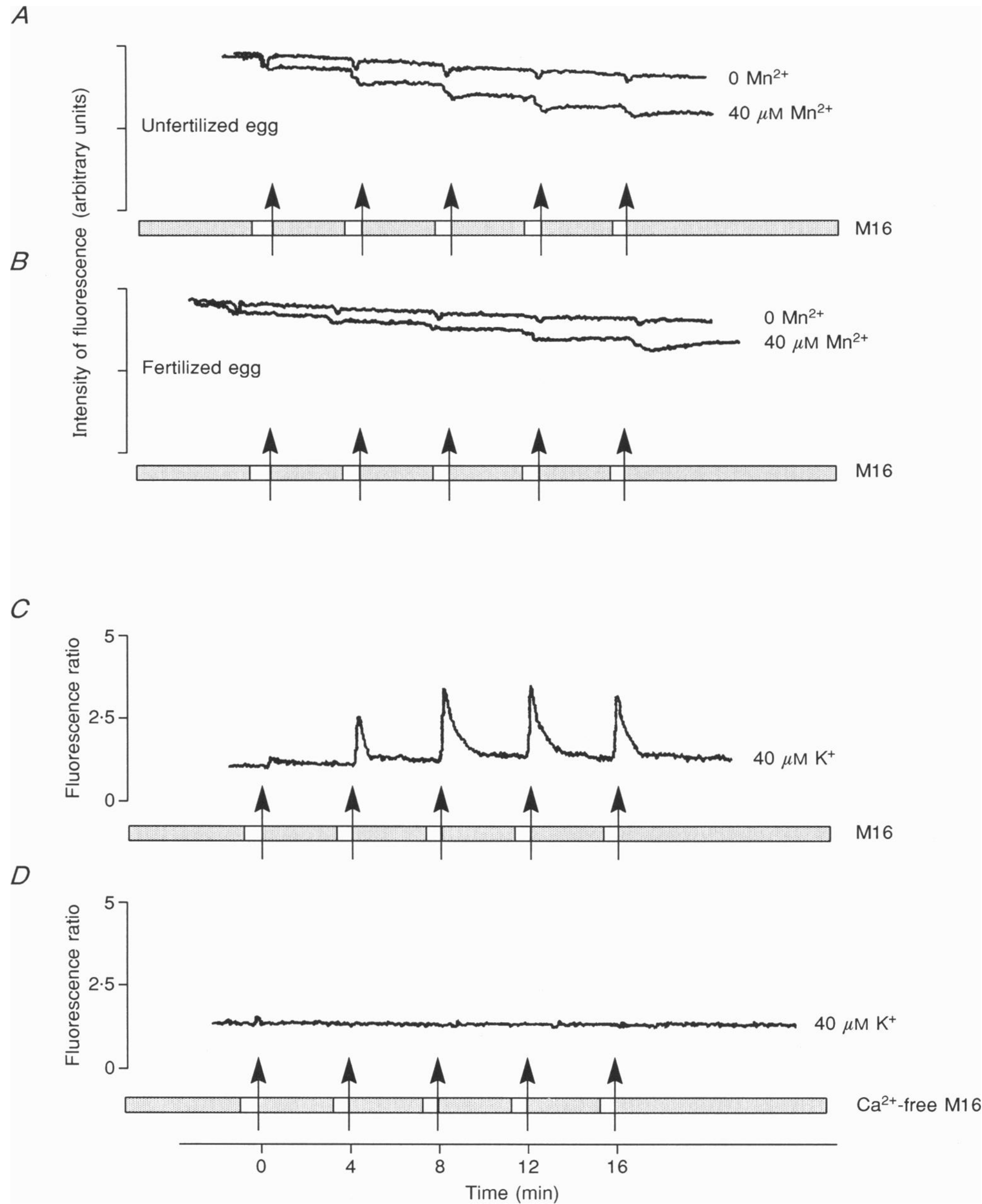

Figure 4. Ca^{2+} influx occurs rapidly either during or shortly after each EF pulse

In *A* and *B* individual mouse eggs loaded with fura-2 were perfused with M16 medium. The glucose media were with or without 40 μM Mn^{2+}. The fluorescence was monitored with 360 nm wavelength excitation. The arrows indicate the times of radio frequency EF pulses (1·25 kV cm^{-1}, 1 ms). The upper traces in *A* and *B* were obtained from control eggs in the absence of Mn^{2+} and the lower traces from eggs pulsed in the presence of Mn^{2+}. The greater decrease in fluorescence indicates the influx component of Mn^{2+} causing quenching of fura-2 fluorescence in an unfertilized egg (*A*) and a fertilized egg (*B*). In *C* and *D* $[Ca^{2+}]_i$ levels in eggs were monitored with indo-1 and again the arrows indicate the time of EF pulses, as in *A* and *B*. In *C* the $[Ca^{2+}]_i$ in the glucose medium was replaced by K^+ and rapid reperfusion carried out with normal M16 medium, whereas in *D* the reperfusion medium contained Ca^{2+}.

As well as Ca^{2+} influx occurring during the EF pulse we also considered the possibility that some Ca^{2+} influx occurs in our experiments during reperfusion with the M16 culture medium which contains 1·7 mM Ca^{2+}. In order to investigate this we pulsed eggs in the presence of glucose medium containing 40 μM K^+ in place of Ca^{2+} and washed eggs either with Ca^{2+}-free or Ca^{2+}-containing culture medium. Figure 4*C* shows clearly that when eggs were exposed to an EF pulse in the presence of 40 μM K^+ and washed immediately afterwards with a Ca^{2+}-free M16 medium there was no apparent $[Ca^{2+}]_i$ increase. In contrast, EF stimulation under the same conditions with reperfusion of normal Ca^{2+}-containing M16 medium caused a progressive $[Ca^{2+}]_i$ increase after each pulse. These results demonstrate that the EF pulse-induced Ca^{2+} influx probably occurs both during and immediately after each pulse.

The above experiments suggest that we can cause a $[Ca^{2+}]_i$ increase by controlling the amount of EF-stimulated Ca^{2+} influx by rapid perfusion in different media containing Ca^{2+}. We can then encourage a rapid recovery phase by perfusion in Ca^{2+}-free medium containing ATP. With this strategy, using different post-pulse perfusion, it was possible to mimic more closely the increases at fertilization. Figure 5*A* shows records of the $[Ca^{2+}]_i$ changes generated in eggs after exposing them to radiofrequency EF pulses followed by different perfusion protocols. When the duration of the post-pulse perfusion with a Ca^{2+}-containing medium increased in steps from 0 to 15 s, the $[Ca^{2+}]_i$ change was prolonged in a stepwise proportion. In each case the recovery of the $[Ca^{2+}]_i$ increase was similar. We considered that the pattern of $[Ca^{2+}]_i$ change obtained in Fig. 5*B* was one which mimics $[Ca^{2+}]_i$ transients seen after fertilization and we therefore arbitrarily adopted this protocol for the experiments on egg activation described below.

EF stimulation in the presence of inositol 1,4,5-trisphosphate

In order to trigger Ca^{2+} release from the intracellular stores, we have examined another strategy and exposed eggs to EF pulses in the presence of inositol 1,4,5-trisphosphate ($InsP_3$) (Rickords & White, 1993). We used the sequences of washes described in Fig. 6 where eggs were washed in a glucose solution for 10 s followed by application of a radio frequency pulse of 1·25 kV cm^{-1} and 1 ms duration. Eggs were maintained in $InsP_3$-containing glucose solution for 5 s after the pulse to allow $InsP_3$ influx. They were then washed in Ca^{2+}-free medium for 2 min and then perfused in Ca^{2+}-containing medium in order to allow internal Ca^{2+} stores to replenish.

Figure 6 shows that when eggs were pulsed in glucose solution contain 5 μM $InsP_3$, pulsation did not cause any significant $[Ca^{2+}]_i$ change (Fig. 6*A*). The prolonged exposure to glucose + $InsP_3$ after the pulse did not cause any $[Ca^{2+}]_i$ changes. However when 40 μM K^+ was added to the glucose medium, EF pulse stimulation did cause $[Ca^{2+}]_i$ to increase (Fig. 6*B*). This change is not caused by Ca^{2+} influx since eggs were washed after the pulse in Ca^{2+}-free medium and the data, therefore, demonstrate that $InsP_3$ was triggering $[Ca^{2+}]_i$ increases from internal stores in our eggs. Applying such EF pulses repetitively in the presence of $InsP_3$ caused further $[Ca^{2+}]_i$ increases that were slightly smaller in amplitude than the initial response (Fig. 6*B* and *C*). Since eggs did not desensitize to EF pulses in the presence of Ca^{2+} or Mn^{2+} these data further suggest that mouse eggs may slightly desensitize to the effects of $InsP_3$ with time.

The response of parthenogenetically activated and fertilized eggs to EF stimulation

As a final prelude to examining the effects of repetitive $[Ca^{2+}]_i$ increases on activation of unfertilized eggs we studied the effects of EF stimulation on eggs that had already been parthenogenetically activated or else fertilized.

Freshly fertilized mouse eggs were collected at the time of pronuclei formation (about 3–4 h after sperm penetration). They were loaded with indo-1 and submitted to one electrical pulse (1·25 kV cm^{-1}, 1 ms, 10 kHz, in the presence of 50 μM Ca^{2+}). Eggs at this stage have stopped undergoing the normal sequence of $[Ca^{2+}]_i$ oscillations that occur at fertilization. Figure 7 shows that fertilized eggs show a very different pattern of $[Ca^{2+}]_i$ response to EF stimulation from that usually observed in non-fertilized eggs. The first EF-induced $[Ca^{2+}]_i$ transient in fertilized eggs was of long duration and had a rapid decrease phase at the end, and this response was sometimes followed by a few extra $[Ca^{2+}]_i$ oscillations. In some eggs a series of spontaneous $[Ca^{2+}]_i$ oscillations occurred at a high frequency (Fig. 7*A*). This pattern of response resembles that seen after fertilization (Kline & Kline, 1992; Swann & Ozil, 1994). The pattern of response did not depend upon the stage of the cell cycle since parthenogenetically activated eggs that were also at the pronuclear stage responded differently. Figure 7*B* shows that a parthenogenetically activated egg exposed to the same treatment as the eggs in Fig. 7*A* responded with a single Ca^{2+} transient that was essentially the same as that described in the above sections on unfertilized eggs.

Initial observations of the effect of repetitive calcium transients on egg activation

All records of $[Ca^{2+}]_i$ changes at fertilization in mouse eggs show a single large $[Ca^{2+}]_i$ increase, followed by a series of smaller $[Ca^{2+}]_i$ transients. It is still not clear if the initial $[Ca^{2+}]_i$ increase is sufficient for egg activation, as judged by pronuclear formation, or whether repetitive $[Ca^{2+}]_i$ increases are required for efficient cell cycle resumption. We approached this problem by exposing eggs to conditions that generated a single $[Ca^{2+}]_i$ increase and compared the activation rate with that obtained from eggs exposed to repetitive $[Ca^{2+}]_i$ transients.

In the first case we have used the fact that eggs generate a single $[Ca^{2+}]_i$ increase, very similar to that initial response at fertilization, after simple perfusion in non-ionic solutions. When freshly ovulated eggs (12–13 h post-hCG injection) were washed in 0·3 M glucose solution for 4 min at 37 °C, thirty-one eggs out of 230 showed initial signs of activation by extruding a second polar, but none of them entered interphase and formed a pronucleus. This suggests that the single $[Ca^{2+}]_i$ increase was only able to cause an abortive form of activation. It was notable that this kind of result was only clearly obtained with freshly ovulated eggs. With eggs that were aged with respect to ovulation different results were obtained. When ninety-nine aged eggs (18–20 h post-hCG injection) were submitted to a glucose wash, as described above, fifty-eight eggs (66%) showed a single pronucleus 6 h after treatment.

In order to examine the role of different numbers and frequencies of repetitive $[Ca^{2+}]_i$ increase, we also submitted groups of freshly ovulated mouse eggs (12–13 h post-hCG injection) to three different treatments using repetitive EF stimulation. The protocol for EF stimulations and perfusion was that described in Fig. 5*B*: a 10 s wash in glucose solution containing 50 μM Ca^{2+}, a 10 s wash with M16 medium containing Ca^{2+}, a 1 min wash with Ca^{2+}-free medium enriched with 4 mM ATP (see Methods) and then a perfusion in Ca^{2+}-free medium. Figure 8 shows the activation rates, as monitored by the time course of pronuclear formation, of batches of eggs exposed to the

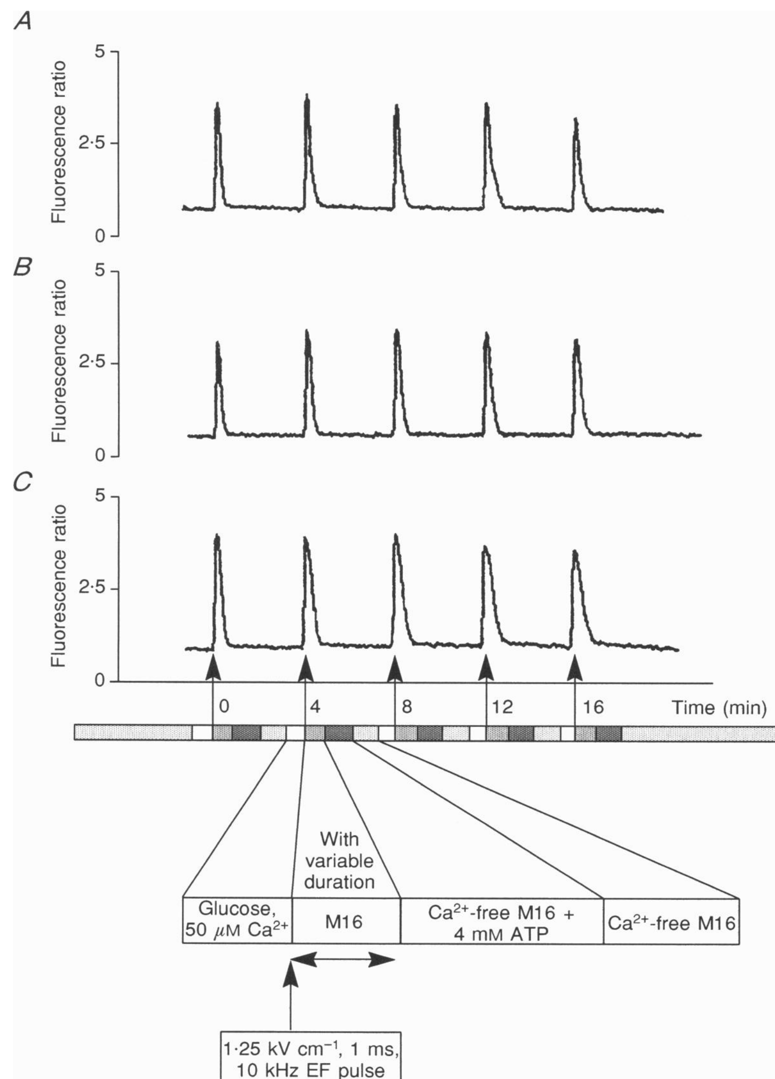

Figure 5. The final protocol adopted for obtaining $[Ca^{2+}]_i$ spikes that most closely resemble the $[Ca^{2+}]_i$ increases at fertilization

$[Ca^{2+}]_i$ was measured in unfertilized mouse eggs as in previous figures. The bar section at the bottom of the figure shows the common serial perfusion protocol with different media for before, after and between EF pulses (at the arrows). In all three traces the EF pulses were applied every 4 min. The variation in $[Ca^{2+}]_i$ was obtained by varying the post-pulse perfusion time in M16 medium from 5 s in *A* to 10 s in *B* and 15 s in *C*.

three different treatments (T_1, T_2, T_3). Each treatment was maintained for 4 h. In T_1, eggs were submitted to eighty pulses, one every 3 min; in T_2 there were forty pulses, one every 6 min; and in T_3 there were twenty-seven pulses, one every 9 min. It can be seen that the rate of egg activation is dependent upon the frequency or total number of EF pulses, with pronuclei forming most rapidly in eggs exposed to higher frequency stimulation. These data imply that repetitive $[Ca^{2+}]_i$ transients are a more effective trigger for entry into the cell cycle and suggest that the rate of activation and

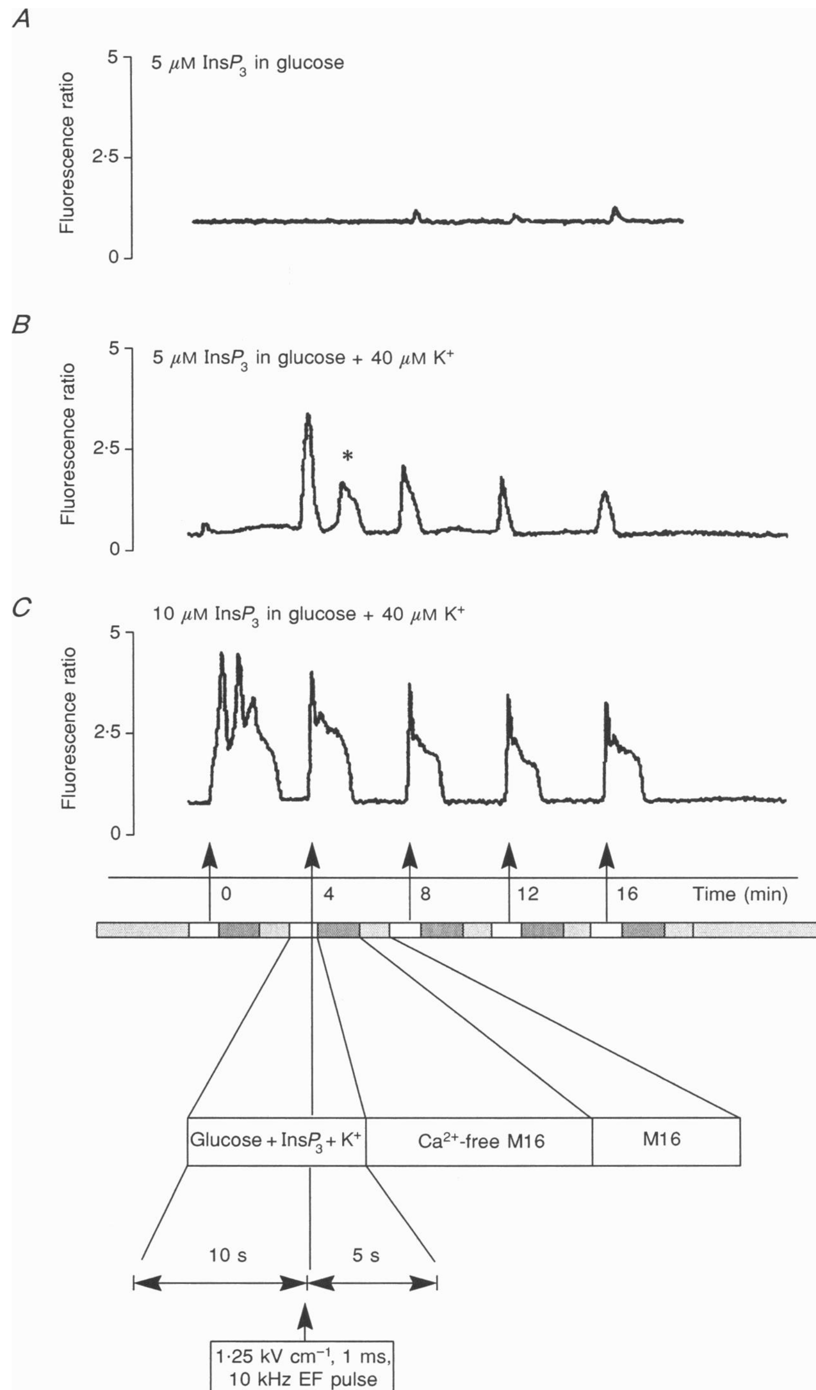

Figure 6. $[Ca^{2+}]_i$ levels in unfertilized eggs exposed to EF pulses as in previous figures except that no Ca^{2+} was added to the glucose medium, which instead contained InsP_3

As in Fig. 5 the common perfusion protocol and pulse application times (every 4 min) are indicated at the bottom of the figure. In *A*, 5 μM InsP_3 was added to the glucose medium that bathes the eggs during pulse application. In *B*, the glucose medium was supplemented with InsP_3 and 40 μM K^+ (* indicates a spontaneous $[Ca^{2+}]_i$ increase). In *C*, 10 μM InsP_3 + 40 μM K^+ was added to the glucose medium.

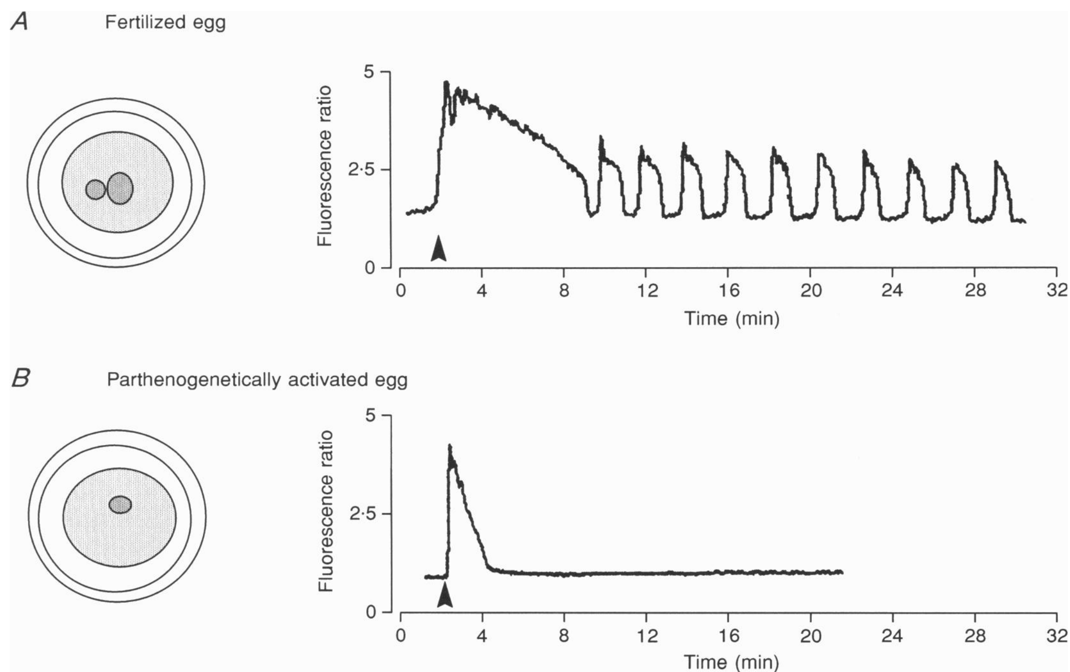

Figure 7. $[Ca^{2+}]_i$ measurements in single mouse eggs as in previous figures except the eggs were only exposed to a single EF pulse in the presence of 50 μM Ca_i^{2+}

A fertilized egg (*A*) and a parthenogenetically activated egg at the same developmental stage (*B*) were stimulated with single EF pulses in the presence of 50 μM Ca_i^{2+} at the times indicated by the arrowheads.

entry into the cell cycle is to some extent dependent upon both the total number and frequency of $[Ca^{2+}]_i$ transients.

DISCUSSION

The idea that repetitive $[Ca^{2+}]_i$ transients, or $[Ca^{2+}]_i$ oscillations, are somehow digitally encoded by cells has been discussed by a number of reviewers (Berridge & Galione, 1988; Tsien & Tsien, 1990; Meyer & Stryer, 1991). Mammalian fertilization represents a useful test of ideas about $[Ca^{2+}]_i$ oscillations since there is a prolonged series of $[Ca^{2+}]_i$ oscillations with a clear overall role (Miyazaki, 1991; Swann & Ozil, 1994). While it is well established that some form of $[Ca^{2+}]_i$ increase is essential for mammalian egg activation there have been few attempts to examine the specific function of $[Ca^{2+}]_i$ oscillations, as opposed to monotonic changes in $[Ca^{2+}]_i$. This study extends and builds upon previous findings and supports the general hypothesis that mammalian egg development is most effectively triggered by a repetitive $[Ca^{2+}]_i$ signal (Ozil, 1990; Vitullo & Ozil, 1992; Collas *et al.* 1993*a*; Collas *et al.* 1993*b*; Swann & Ozil, 1994).

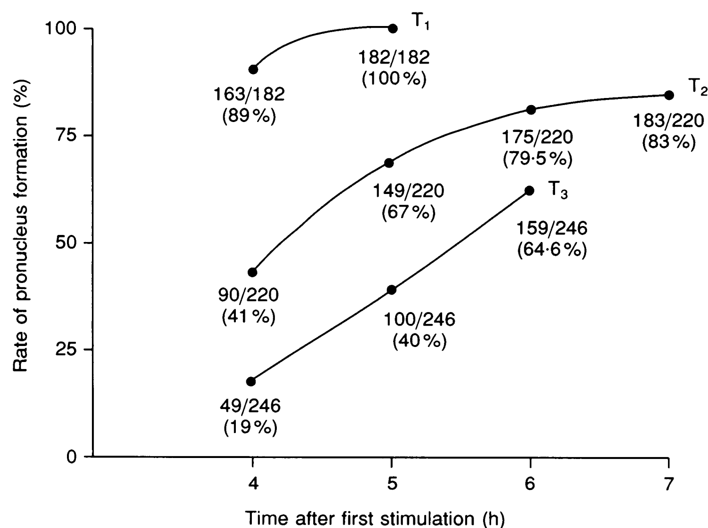

Figure 8. The rate of egg activation is dependent on the number of EF pulses

The EF pulses stimulation protocol was adopted as shown in Fig. 5*B*. Groups of unfertilized eggs were exposed to EF pulses in a chamber similar to that used for the previous $[Ca^{2+}]_i$ measurement experiments. The activation that was induced was monitored by examining the time course of pronuclear formation. For each time point the proportion of eggs activated is noted with percentages in parentheses. The three different treatments (T_1, T_2, T_3) varied the frequency and number of EF pulses as described in the text.

In order to demonstrate that mammalian eggs are responding to a given pattern of $[Ca^{2+}]_i$ oscillations one needs a method of imposing $[Ca^{2+}]_i$ transients on the cytoplasm. Very few methods currently lie to hand for manipulating $[Ca^{2+}]_i$ in eggs, or indeed in other cell types. One possibility would be to use caged Ca^{2+}-containing compounds to photorelease Ca^{2+} at defined times (Tsien, 1992). We have not adopted this technique here largely for technical reasons, but we consider that the need to expose eggs to intense UV irradiation and to load cells with a caged compound might, in any case, compromise later embryonic development. The aim of this study has therefore been to establish the experimental conditions that allow precise control of $[Ca^{2+}]_i$ transients through reversible electropermeabilization by EF stimulation. The EF stimulation technique was adopted because it has already been successfully applied to different types of eggs in its simplest form, i.e. when causing a single $[Ca^{2+}]_i$ transient (Knight, 1981; Rossignol *et al.* 1983; Onodera & Tsunoda, 1989; Sun *et al.* 1992; Collas *et al.* 1993*b*). It is also established that the exposure of early rabbit embryos to repeated EF pulses alone does not interfere with development to term (Ozil, 1990). In addition, EF pulses can be readily applied simultaneously to large numbers of eggs, which is an essential requirement for generating sufficient data on developmental rates of embryos.

During the course of our study we identified several phenomena associated with electropermeabilization that have complicated the use of an otherwise apparently simple method for raising $[Ca^{2+}]_i$ in cells. The first finding was that perfusing eggs with low ionic strength medium by itself triggered a $[Ca^{2+}]_i$ increase similar to the initial response at fertilization. A wash into low ionic strength medium, supplemented with sugars like glucose, has been used by nearly all workers in the field (Onodera & Tsunoda, 1989; Sun *et al.* 1992; Collas *et al.* 1993*b*). It is used in order to reduce the number of ionic components that pass into the egg during permeabilization (Tsong, 1991), and because a lower conducting medium will also reduce deleterious Joule heating that occurs during EF pulse application (Knight, 1981; Tsong, 1991). However, it is clear that the wash in glucose and mannitol by itself has the ability to release internal stores of Ca^{2+} in mouse eggs (the Ca^{2+} is likely to be from internal stores since we added no calcium to the external medium). The use of such nominally Ca^{2+}-free medium is sufficient to reduce influx enough to stop $[Ca^{2+}]_i$ oscillations at fertilization (Igusa & Miyazaki, 1983). The glucose or mannitol in the medium did not cause Ca^{2+} release via their metabolism since the non-metabolizable form of the glucose, 2-deoxy-D-glucose, and the non-permeant saccharose, induce the same phenomena. This type of response is also not peculiar to mouse eggs because it was also observed in bovine oocytes washed in 0·3 M mannitol (Collas *et al.* 1993*b*). The phenomenon is useful in that it can be used to cause a $[Ca^{2+}]_i$ increase that mimics the initial response at fertilization, but since it is observed only once in any one egg it cannot be employed to cause repetitive $[Ca^{2+}]_i$ oscillations. However, with fertilized eggs at the pronuclear stage, repetitive glucose washes caused repetitive $[Ca^{2+}]_i$ transients (data not shown). It is fortuitous that the $[Ca^{2+}]_i$ increase caused by a wash in glucose medium is unlikely to have confounded previous studies of activation because a single $[Ca^{2+}]_i$ increase does not appear to be a sufficient stimulus for activation in mouse eggs.

An important finding of the current study is that a rapid perfusion protocol is required in order to allow for repeated EF stimulations. Several groups have already shown that single EF pulses can cause $[Ca^{2+}]_i$ increases in eggs and lead to a degree of activation of eggs that are aged with respect to time of ovulation (Onodera & Tsunoda, 1989; Sun *et al.* 1992; Collas *et al.* 1993*b*). These studies all used medium exchanges performed manually and did not report the effect of prolonged series of EF pulses. Our results suggest that it is difficult, if not impossible, to stimulate eggs repetitively unless they are perfused rapidly. The problem appears to be that the $[Ca^{2+}]_i$ transient fails to recover rapidly unless a culture medium is reperfused onto the egg. Any protocol that fails to allow the $[Ca^{2+}]_i$ increases to return to baseline values is liable to lyse the eggs.

The reasons for needing to rapidly replace culture medium around the eggs probably lies in the behaviour of pores formed by the EF. Several studies have indicated that EF pulses of the magnitude used in this study cause pores to form in the plasma membrane (Knight, 1981; Tsong, 1991). It is through these pores that the Ca^{2+} influx is liable to occur (Rossignol *et al.* 1983). This hypothesis is supported by the findings that both Mn^{2+} and InsP_3 also appear to permeate the egg during, or shortly after, EF pulse application. If pores form, then the resealing of pores is likely to be an important determinant of the survival of the cell (Tsong, 1991). Previous studies have shown that when cells are maintained in low ionic strength medium there is often a progressive expansion of the pore sizes and cell lysis occurs (Tsong, 1991). It seems likely that the rapid wash into culture medium accelerated the spontaneous closure of pores. This is important for two reasons. Firstly, the rapid closure of pores will prevent leakage of cytoplasmic components. Secondly, the longer the pores stay open the more Ca^{2+} influx occurs. Our Mn^{2+} quench experiments demonstrate that cation influx may occur for several seconds after each EF pulse. The pores evidently stay open long enough for some Ca^{2+} influx to occur from the reperfused culture medium itself. This is actually useful in that it allows us to maintain high $[Ca^{2+}]_i$ for longer periods by washing with a medium containing extra Ca^{2+}. It also allows us to eletroporate InsP_3 into the cell (see Fig. 6). However, prolonged Ca^{2+} influx is clearly liable to overload the cell with Ca^{2+} and delay recovery from $[Ca^{2+}]_i$ transients. These data all suggest that attention has to be paid to the composition of the perfusates and the sequence

of perfusions immediately following EF pulses if this system is to be used to successfully manipulate $[Ca^{2+}]_i$.

Our data suggest that, as well as a rapid wash protocol, the type of EF pulse used can affect the recovery from individual $[Ca^{2+}]_i$ transients. Chang (1989) has suggested that superimposing radio frequency oscillations on top of EF pulses causes pores to form in the plasma membrane at the same time as causing less damage to membrane proteins. Our data in mouse eggs support this idea and suggest that radio frequency pulses affect pore formation with less disruption of Ca^{2+} pumps. The importance of a Ca^{2+}-ATPase in the recovery phase of the $[Ca^{2+}]_i$ transients is emphasized by the fact that 4 mM ATP post-pulse also significantly improved the rate of $[Ca^{2+}]_i$ decrease. It is likely that there is a significant loss of ATP during the permeabilization of the plasma membrane (Swezey & Epel, 1989).

The combination of radio frequency EF pulses and rapid washing allowed us to generate $[Ca^{2+}]_i$ increases of comparable magnitude to those seen at fertilization, while still allowing for a fairly rapid recovery of each transient. We note, however, that the recovery of $[Ca^{2+}]_i$ increases at fertilization is still different from that seen in our best perfusion pulse protocol (Fig. 5). The $[Ca^{2+}]_i$ increases at fertilization may also have a different spatial distribution from those seen after EF pulse stimulation (Sun *et al.* 1992). Consequently we cannot claim to be able to provide a perfect imitation of $[Ca^{2+}]_i$ oscillations at fertilization in mammalian eggs. However, we are able to cause defined patterns of $[Ca^{2+}]_i$ transients for prolonged periods at any frequency up to about one per 2 min.

The $[Ca^{2+}]_i$ increase seen after application of each pulse seems to be mainly due to direct influx. There does not appear to be any Ca^{2+} influx-induced Ca^{2+} release (CICR) in mouse eggs. We base this conclusion on several points. Experiments injecting Ca^{2+} iontophoretically into hamster eggs have demonstrated CICR by showing a non-linearity in the the size of Ca^{2+} transients generated by stepwise increases in Ca^{2+} injection current (Igusa & Miyazaki, 1983). The CICR response in hamster eggs also shows a characteristic desensitization and latent period for recovery (Igusa & Miyazaki, 1983). The Ca^{2+} release response in mouse and other mammalian eggs after EF pulse stimulation does not desensitize, does not show a latent period and is graded in response to the amount of extracelluar Ca^{2+} in the glucose pulsating medium (see Figs 3 and 5, and Sun *et al.* 1992). Since our Mn^{2+} quench experiments suggest that each EF pulse causes a similar degree of Ca^{2+} influx, these results suggest that significant CICR is not demonstrable in unfertilized mouse eggs. Similar conclusions have been reached in mouse eggs in response to iontophoretic injection of Ca^{2+} (Swann, 1994).

In contrast to the CICR we were able to release Ca^{2+} from internal stores by electroporation in the presence of InsP_3. The conditions required to achieve electroporation of InsP_3 are not simple; a relatively long incubation is required after the pulse to allow for InsP_3 influx. Nevertheless, the InsP_3 experiments do support the idea that we are inducing reasonable sized pores in the plasma membrane that last for several seconds. Previously, periodic $[Ca^{2+}]_i$ oscillations were observed after electroporation of InsP_3 (Rickords & White, 1993). The reason why we did not see oscillations of $[Ca^{2+}]_i$ is probably because, unlike Rickords & White, we removed the InsP_3 from around the eggs for most of the interval between the EF pulses. It appears that future studies might use InsP_3 electroporation as a means of activating mammalian eggs. However, with pulsed, intermittent InsP_3 electroporation we observed a degree of desensitization. Such desensitization to the effects of injected InsP_3 is more marked in hamster eggs and may limit the usefulness of InsP_3 for this purpose in mammalian eggs (Swann, Igusa & Miyazaki, 1989; Galione, Swann, Georgiou & Whitaker, 1994).

In contrast to the lack of demonstrable CICR in unfertilized mouse eggs using the eletroporation technique, we were able to see evidence for CICR in fertilized mouse eggs. The same EF pulse conditions that led to a single monotonically decreasing $[Ca^{2+}]_i$ transient in unfertilized eggs triggered a longer-lasting $[Ca^{2+}]_i$ transient and, in some cases, a series of small oscillations in pronuclear stage fertilized mouse egg. However, similar responses were often seen when eggs were stimulated in between spontaneous $[Ca^{2+}]_i$ oscillations shortly after fertilization (data not shown). Since the degree of EF-induced cation influx is not appreciably altered after fertilization these data clearly suggest an enhancement of CICR after sperm penetration in mouse eggs. This is consistent with the reported tenfold increase in the gain of CICR after fertilization in hamster eggs (Igusa & Miyazaki, 1983). The change in gain of CICR does not depend upon the cell cycle stage and is, therefore, specifically associated with sperm penetration. Since injection of sperm cytosol is able to reproduce this effect in both mouse and hamster eggs, it is possible that the enhancement of CICR is due to the sperm introducing a factor into the egg after gamete membrane fusion (Swann, 1990, 1994).

Despite the observation that sustained $[Ca^{2+}]_i$ oscillations are a unique feature of mammalian fertilization there has been little investigation of the specific function of these oscillations. Experiments that block all the $[Ca^{2+}]_i$ transients at fertilization have shown that the oscillations as a whole are essential for mammalian egg activation by the sperm (Kline & Kline, 1992). However, since many parthenogenetic stimuli only generate a single Ca^{2+} increase and yet do cause egg activation, the precise function and consequences of these Ca^{2+} oscillations has remained unclear (Miyazaki, 1991; Sun *et al.* 1992; Swann & Ozil, 1994). However, it is often overlooked that the activation rates achieved by monotonic $[Ca^{2+}]_i$ stimuli are highly variable and very dependent upon the post ovulatory age of the eggs (Whittingham, 1980; Swann &

Ozil, 1994). Our current results echo several previous reports that mammalian egg activation is virtually impossible when a single $[Ca^{2+}]_i$ increase is induced in freshly ovulated eggs. Monotonic $[Ca^{2+}]_i$ stimuli may only be effective in activating 100% of 'younger' eggs when they are combined with non-physiological stimuli such as inhibition of protein synthesis (Bos-Mikich, Swann & Whittingham, 1993). The central point is that induction of $[Ca^{2+}]_i$ transients similar to the initial increase at fertilization is clearly insufficient to trigger development. In contrast both the current data and those obtained previously on mouse and rabbit eggs demonstrate that recently ovulated eggs can be activated at rates of 100% if a repetitive $[Ca^{2+}]_i$ stimulus is applied. Previous experiments applying repetitive EF pulses to mouse eggs demonstrated a modulation of the rate of cell cycle resumption (pronuclear formation) by the alteration of the amplitude of a fixed frequency of Ca^{2+} stimuli (Vitullo & Ozil, 1992). This study extends these findings and shows that the rate of pronuclear formation is also modulated by the frequency of fixed amplitude $[Ca^{2+}]_i$ transients. Since the number and frequency of $[Ca^{2+}]_i$ oscillations at fertilization show a degree of variability (Cuthbertson & Cobbold, 1985; Kline & Kline, 1992; Swann & Ozil, 1994), it is attractive to speculate that the variation in frequency response at fertilization determines the variation in developmental rates observed after *in vitro* fertilization. Development of mammalian eggs may represent a prime example of a signalling pathway that senses specific patterns of $[Ca^{2+}]_i$ transients. The technique of EF pulse stimulation should prove useful in establishing which biochemical changes within eggs are responding to the specified patterns of $[Ca^{2+}]_i$ oscillations.

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