Strontium-induced rat egg activation

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Abstract

Parthenogenetic agents that evoke cytosolic calcium concentration ($[Ca^{2+}]_i$) oscillations similar to those evoked by sperm, mimic fertilization more faithfully than agents that trigger a single $[Ca^{2+}]_i$ transient. Strontium chloride ($SrCl_2$) binds to and activates the Ca^{2+} -binding site on the inositol 1,4,5-trisphosphate receptor and evokes $[Ca^{2+}]_i$ oscillations. Although $SrCl_2$ has been reported to activate mouse eggs, little is known regarding the pattern of the $[Ca^{2+}]_i$ oscillations it evokes in rat eggs and their effect on the early events of egg activation: cortical granule exocytosis (CGE) and completion of meiosis (CM). In the current study we investigated the effect of various concentrations of $SrCl_2$ (2, 4 or 6 mM) on $[Ca^{2+}]_i$ by monitoring $[Ca^{2+}]_i$ oscillations in fura-2-loaded rat eggs. Treatment with 2 mM $SrCl_2$ was optimal for inducing the first $[Ca^{2+}]_i$ transient, which was similar in duration to that triggered by sperm. However, the frequency and duration of the subsequent $[Ca^{2+}]_i$ oscillations were lower and longer in $SrCl_2$ -activated than in sperm-activated eggs. The degree of CGE was identical in eggs activated by either sperm or $SrCl_2$, as assessed by semi-quantitative immunohistochemistry combined with confocal microscopy. Evoking 1, 2 or 10 $[Ca^{2+}]_i$ oscillations (8, 15 or 60 min in $SrCl_2$ respectively) had no effect on the intensity of fluorescent CGE reporter dyes, while 60-min exposure to $SrCl_2$ caused a delay in CM. Our results demonstrate that $SrCl_2$ is an effective parthenogenetic agent that mimics rat egg activation by sperm, as judged by the generation of $[Ca^{2+}]_i$ oscillations, CGE and CM.

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Introduction

The initial increase in cytosolic calcium concentration ($[Ca^{2+}]_i$) occurring immediately after sperm–egg interaction at fertilization is necessary for triggering two major events: cortical granule exocytosis (CGE) and stimulation of the cell cycle resumption to complete meiosis (CM), collectively known as egg activation (Whitaker & Larman 2001). This concept is supported by evidence derived from experiments performed with eggs of several species, including the rat (Raz & Shalgi 1998): (1) the actual increase in $[Ca^{2+}]_i$ within the fertilized egg cytoplasm, (2) the initiation of egg development by parthenogenetically activating a rise in $[Ca^{2+}]_i$, and (3) the prevention of egg development initiation by suppression of the $[Ca^{2+}]_i$ rise (Kline & Kline 1992). The signaling pathway responsible for initiating and mediating $[Ca^{2+}]_i$ increase has been studied by several research groups. It has been shown that mammalian eggs express phospholipase C (Dupont *et al.* 1996, Mehlmann *et al.* 1998), which, when activated, hydrolyzes phosphatidylinositol 4,5-bisphosphate to the second messenger inositol 1,4,5-trisphosphate ($InsP_3$). $InsP_3$ interacts with $InsP_3$ receptors ($InsP_3R$) at the

endoplasmic reticulum, thus inducing Ca^{2+} release (Patel *et al.* 1999). CM is mediated by a decrease in activity of both the cytostatic factor and the M-phase promoting factor, a heterodimer comprised of catalytic, CDK1, and regulatory, cyclin B1, components (Nixon *et al.* 2000, Carroll 2001). Recent studies have demonstrated that Ca^{2+} mediates the degradation of cyclin B1 by increasing the activity of an E3 ubiquitin ligase (Hyslop *et al.* 2004, Jones 2004).

Sperm–egg fusion initiates $[Ca^{2+}]_i$ oscillations within the egg that last several hours (Fissore *et al.* 1992). A fertilization-like $[Ca^{2+}]_i$ oscillation frequency dramatically increases the extent of parthenogenetic development of newly ovulated eggs, as compared with a single Ca^{2+} stimulus (Ozil & Huneau 2001), whereas an abnormally high frequency of oscillations results in cell death (Gordo *et al.* 2000). Some activators, such as ionomycin or ethanol, cause a single $[Ca^{2+}]_i$ transient, while others such as $InsP_3$, adenophostin A, thimerosal and strontium ($SrCl_2$) evoke repetitive $[Ca^{2+}]_i$ oscillations (Jellerette *et al.* 2000). $SrCl_2$ binds to and activates the Ca^{2+} -binding site on the $InsP_3R$ (Kline & Kline 1992, Marshall & Taylor 1994); moreover, $SrCl_2$ -mediated $[Ca^{2+}]_i$ oscillations are generated via

the InsP_3R (Brind *et al.* 2000). There are three known isoforms of InsP_3R that are expressed, to different extents, in various cell types. In mouse eggs, type I InsP_3R is by far the most abundant isoform; however, mRNAs of all isoforms are present within the egg (Parrington *et al.* 1998). The InsP_3Rs form tetramers at the membrane of the endoplasmic reticulum. The tetramers consist of a large cytoplasmic domain that contains the InsP_3 binding site, a membrane-spanning region that contains the Ca^{2+} channel, a small cytoplasmic C-terminus, and a large N-terminal cytoplasmic region that provides the main region for cytoplasmic regulators to act (Brind *et al.* 2000). InsP_3Rs are regulated by small modulators such as InsP_3 , Ca^{2+} and ATP, as well as by protein–protein type interactions with large proteins like calmodulin and FKBP12 (Taylor 1998). Another mechanism for the long-term regulation of InsP_3R activity is its downregulation by proteolysis, probably by the proteasome (Brind *et al.* 2000).

SrCl_2 is a known activator of mouse eggs (O'Neill *et al.* 1991, Wakayama *et al.* 1998, Kishikawa *et al.* 1999, Otaegui *et al.* 1999), but little is known regarding its effectiveness in activating rat eggs. It has recently been reported that SrCl_2 effectively activated rat eggs and triggered development to the blastocyst stage (Krivokharchenko *et al.* 2003). However, no information was presented regarding the dynamics of $[\text{Ca}^{2+}]_i$ and its effect on the early events of egg activation (CGE, CM).

SrCl_2 provides a simple yet tightly-controlled technique of egg activation. It is added directly to the medium, without the need for microinjection and the possible damage to the egg membrane, whereas the extent of $[\text{Ca}^{2+}]_i$ signal and its duration are easily controlled by SrCl_2 concentration and time of exposure. In the current study, we monitored $[\text{Ca}^{2+}]_i$ changes during incubation with various SrCl_2 concentrations and established the optimal concentration for the occurrence of CGE and of CM. We also studied the effect of the number of SrCl_2 -induced $[\text{Ca}^{2+}]_i$ transients on CGE and CM. Although SrCl_2 is extensively used as a parthenogenetic activator of eggs in a number of species (Tateno & Kamiguchi 1997, Okada *et al.* 2003) and its action has been described in considerable detail in the mouse egg (Kline & Kline 1992, Bos-Mikich *et al.* 1993), the present report is the first comprehensive study relating SrCl_2 parthenogenetic activation of rat eggs to the early events of fertilization - $[\text{Ca}^{2+}]_i$ dynamics, CGE and CM.

Materials and Methods

Animals

Wistar-derived rats were housed in air-conditioned, light-controlled rooms, with food and water available *ad libitum*. Twenty-three- to twenty-five-day-old female rats were primed with a subcutaneous injection of 10 IU pregnant mare's serum gonadotropin (PMSG; Syncro-Part, Sanofi, Paris, France). An intraperitoneal injection of 10 IU human

chorionic gonadotropin (hCG; Sigma Chemical Co., St Louis, MO, USA) was administered 48–54 h after PMSG.

Collection of eggs

Ovulated eggs at metaphase II (MII) were isolated, 14 h after hCG injection, from the oviductal ampullae into regular or Ca^{2+} - and Mg^{2+} -free culture medium (Toyoda HEPES (TH or TH – /–) supplemented with 0.4% BSA; Talmor *et al.* 1998). Cumulus cells were removed by hyaluronidase (400 IU/ml, Sigma). For *in vitro* fertilization, eggs were treated with α -chemotrypsin (50 $\mu\text{g}/\text{ml}$, Sigma) to remove the zona pellucida (ZP) prior to insemination. All manipulations were performed on a warm plate (37 °C).

Parthenogenetic activation

Eggs were parthenogenetically activated by either calcium ionophore (ionomycin 407950; Calbiochem, San Diego, CA, USA) or SrCl_2 . Aliquots of 4 mM ionomycin in dimethylsulfoxide (DMSO) were kept at –70 °C and diluted to the final concentration of 2 μM just before use in TH – /– medium. Freshly prepared SrCl_2 (2, 4 or 6 mM in TH – /– medium) was used. MII eggs were incubated for 5 min in the presence of ionomycin and then for 0, 10, 25, 40, 55 or 70 min in fresh TH medium without ionomycin. Other batches of MII eggs were incubated for 8, 15 or 60 min in the presence of various concentrations of SrCl_2 followed by additional incubation in SrCl_2 -free TH medium, up to a total incubation period of 15, 30, 45, 60 or 75 min. For details, see the Results section.

In vivo/in vitro fertilization

PMSG- and hCG-primed female rats were allowed to mate overnight with males of proven fertility. *In vivo* fertilized eggs were isolated from the oviductal ampullae of animals, 15.5 h after hCG administration, into TH medium, and their cumulus cells were removed as described earlier for MII eggs.

For *in vitro* fertilization, spermatozoa were collected from the uteri shortly after mating, and diluted in rat fertilization medium (Ben-Yosef *et al.* 1993) to a final concentration of 0.7 to 1.3×10^6 spermatozoa/ml. Insemination of ZP-free eggs by capacitated sperm was performed in a thermostatic chamber, suitable for $[\text{Ca}^{2+}]_i$ measurements, as previously described (Ben-Yosef *et al.* 1996).

Measurement of $[\text{Ca}^{2+}]_i$

To follow $[\text{Ca}^{2+}]_i$ changes, MII eggs, either ZP-enclosed or ZP-free, were collected as described and loaded with the Ca^{2+} -sensitive dye, fura-2-AM (3 μM ; Molecular Probes, Eugene, OR, USA), for 30 min in TH medium at 37 °C. Eggs were washed free of the dye and allowed to attach to a poly-L-lysine coated coverslip, in TH – /– medium, under mineral oil. The coverslip was placed in a thermostatic

chamber adjusted to $37 \pm 1^\circ\text{C}$. Free $[\text{Ca}^{2+}]_i$ was determined by monitoring the fluorescence ratio at 340/380 nm using an inverted microscope (Nikon TMD; Nikon Corp, Tokyo, Japan), attached to an imaging workstation, controlled by Metamorph and Metafluor software (Universal Imaging, Downingston, PA, USA).

DNA staining and CGE quantification

Eggs were fixed in 3% paraformaldehyde, stained with *Lens culinaris* agglutinin (LCA; Vector, Burlingame, CA, USA) and costained with Texas-red streptavidin (Vector) for detection of CGE (Eliyahu & Shalgi 2002) and labeled with Hoechst 33342 (Sigma) for determining cell cycle and fertilization status (Shalgi & Phillips 1988). Labeled eggs were visualized and photographed with a Zeiss confocal laser-scanning microscope (LSM). The Zeiss LSM 410 (Oberkochen, Germany) is equipped with a UV laser (Coherent Inc. Santa Clara, CA, USA) and with a 25 mW krypton-argon laser and a 10 mW helium-neon laser (488, 543, and 633 maximum lines). A $40\times$ NA/1.2 planapochromat water-immersion lens (Axiovert 135 M, Zeiss) was used for all imaging. Confocal micrographs of 3–4 eggs from each experimental group were analyzed by densitometry. The staining intensity was calculated using the corrected mean density values obtained by the LSM software (Abbott *et al.* 1999).

Statistical analysis

Data were evaluated by one-way ANOVA, and differences between treatment groups were determined by using a Chi-square test; $P < 0.01$ was considered significant.

Results

The effect of SrCl_2 concentration on $[\text{Ca}^{2+}]_i$ oscillations

A very early observable event occurring after sperm–egg interaction or after parthenogenetic activation is an increase in $[\text{Ca}^{2+}]_i$, which leads to the early events of egg activation (i.e. CGE and CM). We examined the amplitude and the duration of $[\text{Ca}^{2+}]_i$ oscillations in MII triggered by 2, 4 or 6 mM SrCl_2 and compared them to those caused by fertilization or by ionomycin (Table 1). All three

concentrations of SrCl_2 triggered an initial $[\text{Ca}^{2+}]_i$ transient that was followed by a series of $[\text{Ca}^{2+}]_i$ oscillations of shorter duration and lower amplitude. The oscillations in individual eggs were repetitive with regular peak-to-peak intervals. Eggs treated with either 2 or 4 mM SrCl_2 exhibited no significant differences in the duration or frequency of Ca^{2+} oscillations, which were similar to those observed during *in vitro* fertilization. The amplitude of the initial $[\text{Ca}^{2+}]_i$ transient induced by SrCl_2 (regardless of concentration) was higher than that of the following $[\text{Ca}^{2+}]_i$ oscillations (Fig. 1B–D), whereas the amplitude of the first sperm-induced transient was lower than the following $[\text{Ca}^{2+}]_i$ oscillations ($P < 0.01$; Fig. 1A). The oscillations induced by SrCl_2 were of longer duration (i.e. they had longer peak-to-peak intervals) than those induced by sperm (Fig. 1A–D). The duration and amplitude of the first $[\text{Ca}^{2+}]_i$ transient induced by 6 mM SrCl_2 were higher and significantly longer than those induced by either 2 or 4 mM SrCl_2 or those exhibited during fertilization ($P < 0.01$; Table 1, Fig. 1B–D). As expected, repetitive oscillations continued as long as SrCl_2 was present in the culture medium (data not shown).

The duration of the single transient induced by ionomycin was longer than the duration of the first transient induced by 2 or 4 mM SrCl_2 or by sperm, but was shorter than the one induced by 6 mM SrCl_2 . Its amplitude was lower than any of the first peaks induced by either sperm or SrCl_2 (Table 1).

The effect on egg activation of a single $[\text{Ca}^{2+}]_i$ transient, induced by either SrCl_2 or ionomycin

We evaluated the effect on CM of a single $[\text{Ca}^{2+}]_i$ transient induced by SrCl_2 . As presented in Table 1, the second $[\text{Ca}^{2+}]_i$ transient, in SrCl_2 -activated eggs, occurs 12.2 ± 3.2 min post exposure to SrCl_2 . We thus exposed the eggs for 8 min to 2, 4 or 6 mM SrCl_2 in TH – / – medium followed by incubation in TH medium. After 30 min in culture (8 min in SrCl_2 and 22 additional min in TH), more than half of the eggs reached anaphase in a dose-dependent manner (53%, 78% or 88% induced by 2, 4 or 6 mM SrCl_2 respectively; $P < 0.01$), whereas 45 min were sufficient for 31–39% of the eggs to reach telophase at all SrCl_2 concentrations tested. After 60 min, 56% of the eggs induced by 4 mM SrCl_2 extruded polar body (PBII) while

Table 1 The effect of egg activators on Ca^{2+} oscillations. All values are presented as means $\pm$ S.E.M.

Conditions	Eggs (n)	First peak		Oscillations		
		Duration (min)	Amplitude (arbitrary units)	Duration (min)	Amplitude (arbitrary units)	Peak-to-peak interval (min)
2 mM SrCl_2	13	9.71 ± 2.61^a	7.28 ± 1.99	3.63 ± 1.38	4.50 ± 0.64	6.71 ± 2.05
4 mM SrCl_2	22	9.72 ± 3.20^a	5.90 ± 1.80	3.66 ± 1.36	4.39 ± 1.30	7.13 ± 1.98
6 mM SrCl_2	14	$14.75 \pm 2.38^{a*}$	9.03 ± 3.23	4.13 ± 0.90	5.51 ± 0.97	6.67 ± 2.54
Fertilization	3	9.00 ± 1.00	7.50 ± 1.87	2.50 ± 0.70	$8.50 \pm 2.88^*$	2.02 ± 0.40
Ionomycin	9	11.15 ± 1.93	3.82 ± 0.54	—	—	—

^a Measured as the time interval between the exposure to SrCl_2 until the $[\text{Ca}^{2+}]_i$ level returned to baseline.

* $P < 0.01$ statistically different.

Figure 1 Fertilization and SrCl_2 -induced Ca^{2+} oscillations. $[\text{Ca}^{2+}]_i$ in fura-2AM-loaded rat eggs that were either fertilized *in vitro* (A) or parthenogenetically activated by 2 mM (B), 4 mM (C) or 6 mM (D) SrCl_2 .

only 42% and 34% of the eggs were induced to do so by 6 or by 2 mM SrCl_2 respectively ($P < 0.01$; Fig. 2). The membrane of most of the eggs that were activated by 6 mM SrCl_2 was undulated (light microscopy; data not shown). CM induced by 1 mM SrCl_2 was very slow - only a small percentage of eggs extruded PBII after 75 min in culture (data not shown).

Exposure of eggs to $2 \mu\text{M}$ ionomycin for 5 min induced a single $[\text{Ca}^{2+}]_i$ transient which triggered both CGE and CM (Raz & Shalgi 1998). We compared the effect of ionomycin on egg activation with the effect of a single $[\text{Ca}^{2+}]_i$ transient induced by 2 mM SrCl_2 . We exposed eggs for 5 min to $2 \mu\text{M}$ ionomycin, or for 8 min to 2 mM SrCl_2 , in TH -/- medium. Eggs were then washed and cultured for additional time periods in TH to complete a total of 15, 30, 45, 60 or 75 min in culture. The differences in the degree of CM between eggs exposed to either activator were not statistically significant (Fig. 3). Thirty minutes after exposure to either SrCl_2 or ionomycin, more than 50% of the eggs reached anaphase, (54% and 58% respectively; $P < 0.01$). Fifteen minutes later, the eggs reached telophase (36% and 29% respectively; $P < 0.01$) and extruded PBII in another 30 min (70% and 71% respectively; $P < 0.01$; Fig. 3).

We also compared the effects of SrCl_2 and ionomycin on CGE intensity. Eggs were incubated in TH -/- medium for 8 min in the presence of 2 mM SrCl_2 , or for 5 min in the presence of $2 \mu\text{M}$ ionomycin. Eggs were then transferred for additional time periods to TH medium to complete 45 min in culture, then fixed and labeled for CGE and for DNA. The degree of CGE was calculated by the LSM program. The values representing CGE in SrCl_2 -activated eggs were expressed relative to those obtained for ionomycin-activated eggs, which were arbitrarily set at 1.0. The single $[\text{Ca}^{2+}]_i$ transient induced by both activators caused CGE in all examined eggs. However, the intensity of the reaction differed between the two activators. The CGE intensity induced by 2 mM SrCl_2 was almost double (1.89 ± 0.19) that induced by $2 \mu\text{M}$ ionomycin, but was similar to sperm-induced CGE (1.81 ± 0.08 ; Table 2, Fig. 4).

The effect of $[\text{Ca}^{2+}]_i$ oscillations induced by SrCl_2 on egg activation

The observation that CGE is more sensitive to $[\text{Ca}^{2+}]_i$ increase than CM (Raz *et al.* 1998) led us to investigate whether CGE and CM require a different number of $[\text{Ca}^{2+}]_i$ oscillations.

Figure 2 Completion of meiosis induced by 2, 4 or 6 mM SrCl_2 . Eggs were collected 14 h after hCG administration and activated by exposure to 2, 4 or 6 mM SrCl_2 . MII eggs were exposed for 8 min to 2 mM (A), 4 mM (B) or 6 mM (C) SrCl_2 in TH $-/-$ medium, washed and cultured in TH medium for additional time periods to complete a total of 15, 30, 45 or 60 min in culture. Each graph represents 9 experiments, performed on different days. At least 25 eggs were examined at each time point on each experimental day. Time (min) indicates total time in culture. Eggs (%) indicates the percent of eggs at MII ($\diamond$), anaphase ($\blacksquare$), telophase ($\blacktriangle$) and PBII (X).

SrCl_2 (2 mM) caused $[\text{Ca}^{2+}]_i$ oscillations with a 6.6 min peak-to-peak interval (Table 1). We exposed the eggs to 2 mM SrCl_2 for 8, 15 or 60 min, which was expected to induce 1, 2 or 10 oscillations respectively, and then transferred them into TH medium to complete a period of 60 min in culture. The eggs were fixed and labeled for

Figure 3 Completion of meiosis induced by either ionomycin or SrCl_2 . Eggs were collected 14 h after hCG administration and activated by exposure to 2 mM SrCl_2 for 8 min (A) or to 2 μM ionomycin for 5 min (B) in TH $-/-$ medium, washed and cultured for additional time periods in TH medium to complete 15, 30, 45, 60 or 75 min in culture. Each graph represents 5 experiments performed on different days. At least 25 eggs were examined at each time point on each experimental day. Time (minutes) indicates total time in culture. Eggs (%) indicates the percent of eggs at MII ($\diamond$), anaphase ($\blacksquare$), telophase ($\blacktriangle$) and PBII (X).

CGE and for DNA. Longer exposure to SrCl_2 (15 vs 8 min) correlated with a higher rate of PBII extrusion (44% vs 34% respectively; $P < 0.01$). However, eggs exposed to SrCl_2 for 60 min proceeded from MII to anaphase (34%) and telophase (36%), but only 18% extruded PBII ($P < 0.01$; Fig. 5).

Table 2 Intensity of CGE after activation by SrCl_2 , ionomycin or fertilization. Data were obtained from three experiments. CGE values are means $\pm$ S.E.M.

Treatment	Estimated number of Ca^{2+} oscillations	Eggs (n)	CGE relative intensity
2 mM SrCl_2 for 8 min	1	40	$1.89 \pm 0.19^*$
2 mM SrCl_2 for 15 min	2	40	$2.11 \pm 0.18^*$
2 mM SrCl_2 for 60 min	10	30	$2.07 \pm 0.17^*$
ionomycin	1	40	1.00 ± 0.00
Fertilization <i>in vivo</i>		30	$1.81 \pm 0.08^*$

The values obtained for ionomycin-activated eggs were arbitrarily set at 1.0.

* $P < 0.01$ compared with ionomycin-activated eggs.

Figure 4 CGE in eggs induced by SrCl_2 , ionomycin or sperm. A–D, light microscope; A'–D', fluorescence microscope. Representative confocal micrographs of eggs, colabeled for CGE (red) and DNA (blue) are shown. MII; metaphase II eggs were cultured for 8 min in TH $-/-$ medium devoid of activators followed by 37 min in TH medium (control; A, A'). SrCl_2 : eggs were cultured for 8 min in 2 mM SrCl_2 in TH $-/-$ medium followed by 37 min in TH medium (B, B'). Ionomycin: eggs were cultured for 5 min in 2 μM ionomycin in TH $-/-$ medium followed by 40 min in TH medium (C, C'). Fertilization: *in vivo* fertilized egg (D, D'), arrow indicates the sperm head. Quantification of CGE intensity is presented in Table 2. Scale bar = 10 μm .

CGE is a rapid process, occurring immediately after sperm–egg interaction. To study whether repetitive oscillations enhance the intensity of CGE induced by the first $[\text{Ca}^{2+}]_i$ transient, we monitored the intensity of CGE after exposure to SrCl_2 for 8, 15 or 60 min or after fertilization *in vivo*. A single $[\text{Ca}^{2+}]_i$ transient caused CGE of the same intensity regardless of the stimulus— SrCl_2 or sperm. Additional oscillations did not enhance the intensity of CGE (Table 2).

Discussion

SrCl_2 is a commonly employed agent for induction of parthenogenetic activation of mouse eggs. Some

researchers reported that SrCl_2 triggers a series of $[\text{Ca}^{2+}]_i$ oscillations in mature MII mouse (Kline & Kline 1992, Bos-Mikich *et al.* 1993), pig (Okada *et al.* 2003) and hamster (Tateno & Kamiguchi 1997) eggs and is able to cause egg activation. In a recent study, the efficiency of 2 mM SrCl_2 for parthenogenetic development of rat eggs was tested, but no details were reported regarding its ability to cause $[\text{Ca}^{2+}]_i$ oscillations, CGE or PBII extrusion (Krivokharchenko *et al.* 2003). The present study examines the effect of SrCl_2 on the early temporal correlation among these three parameters of egg activation.

SrCl_2 , as well as many other parthenogenetic activators (InsP₃, sperm factor, thimerosal, adenophostin A) induces repetitive and regular $[\text{Ca}^{2+}]_i$ oscillations and mimics

Figure 5 The effect on the progression of CM incubation in SrCl₂. Eggs were cultured in the presence of 2 mM SrCl₂ in TH – / – medium for 8, 15 or 60 min and were then transferred to TH medium to complete a period of 60 min in culture. Each column represents at least 3 experiments performed on different days. At least 25 eggs were examined at each time point at each experimental day ($P < 0.01$). Time (minutes) indicates the duration of exposure to SrCl₂. Eggs (%) indicates the percent of eggs at MI (white section), anaphase (black section), telophase (dark gray section), and PBII (light gray section) after 60 min culture period.

sperm-induced calcium dynamics. Despite the superficial similarity of the overall phenomena, there are marked differences in the pattern of $[Ca^{2+}]_i$ changes among the different parthenogenetic stimuli (e.g. Jellerette *et al.* 2000). Thus, for example, in the rat egg SrCl₂ induces a greater first calcium transient, followed by oscillations that are lower in amplitude, more prolonged and of lower frequency than those induced by sperm. By comparison, ionomycin induces only a single calcium transient. Nevertheless, all these agents can cause egg activation up to PBII extrusion. Krivokharchenko *et al.* (2003) recently reported that ethanol or SrCl₂ treatments produced similar success rates of rat embryo implantation. In view of these reports and our own data presented here, what is the importance of the orderly pattern of calcium oscillations induced by sperm and mimicked to a varying degree by other parthenogenetic agents? Are they necessary for the efficient block of polyspermy? Do they support better success rates of activation progression and, ultimately, better rates of implantation?

The only definitive experimental evidence, correlating electroporation-induced calcium oscillations in mouse eggs with 50% CGE (4 transients) or pronuclei formation (8–24 transients), was reported by Ducibella *et al.* (2002). Varying the duration of SrCl₂ exposure, we could control the number of transients (1, 2 or 10), yet found there was little difference in the intensity of CGE or the progression to PBII extrusion; these parameters were comparable to those induced by sperm. Actually, 1 or 2 transients exhibited a better rate of PBII extrusion than 10 oscillations, possibly reflecting the toxic effects of prolonged exposure to SrCl₂. The differences between the results of Ducibella *et al.* (2002) and those reported here may be attributed to differences between methods causing a different pattern of $[Ca^{2+}]_i$ oscillations or differences in species studied.

Although both 2 μ M ionomycin and 8-min exposure to 2 mM SrCl₂ cause a single $[Ca^{2+}]_i$ transient, the ionomycin-induced transient is of much lower amplitude. Interestingly, ionomycin evokes a much lower CGE response (see Table 2), but a similar degree of PBII extrusion (Fig. 2). This could be interpreted in terms of two independent processes being initiated by the first $[Ca^{2+}]_i$ transient: CGE and CM. The hypothesis that an increase in $[Ca^{2+}]_i$ is required for CGE is well established (Kline & Kline *et al.* 1992, Raz *et al.* 1998). It is possible that the high transient resulting from SrCl₂ exposure, but not from ionomycin, activates Ca^{2+} -dependent proteins such as Ca^{2+} -dependent protein kinase C, which, in turn, affect the degree of CGE (Bos-Mikich *et al.* 1995, Jellerette *et al.* 2000). It should be noted that Raz *et al.* (1998), using ionomycin and BAPTA-AM (to buffer cytosolic $[Ca^{2+}]_i$), reported that CGE was more sensitive than PBII extrusion to $[Ca^{2+}]_i$ concentrations. Their experiments, however, were performed in Ca^{2+} -containing medium and the influx of extracellular calcium through the ionophore could account for these differences.

In conclusion, the current study demonstrated that SrCl₂ can be used as an easily controlled parthenogenetic agent, which mimics fairly faithfully the early events of egg activation ($[Ca^{2+}]_i$ increase, CM and CGE). Our results, as well as those reported by others, suggest that a single large calcium transient is sufficient for the orderly progression of the egg through these early events. The role of the subsequent transients in the sperm-activated egg is less clear and merits further study.

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References

- Abbott A, Fissore R & Ducibella T 1999 Incompetence of pre-ovulatory mouse oocytes to undergo cortical granule exocytosis following induced calcium oscillations. *Developmental Biology* **207** 38–48.
- Ben-Yosef D, Oron Y & Shalgi R 1993 Prolonged, repetitive calcium transients in rat oocytes fertilized *in vitro* and *in vivo*. *FEBS Letters* **331** 239–242.
- Ben-Yosef D, Oron Y & Shalgi R 1996 Intracellular pH of rat eggs is not affected by fertilization and the resulting calcium oscillations. *Biology of Reproduction* **55** 461–468.
- Bos-Mikich AB, Swann K & Whittingham DG 1993 Sr²⁺-induced parthenogenetic activation of mouse oocytes is enhanced by cycloheximide. *Journal of Reproduction and Fertility Abstract Series* 12–18.
- Bos-Mikich AB, Swann K & Whittingham DG 1995 Calcium oscillations and protein synthesis inhibition synergistically activate mouse oocytes. *Molecular Reproduction and Development* **41** 84–90.

- Brind S, Swann K & Carroll J 2000 Inositol 1,4,5-trisphosphate receptors are downregulated in mouse oocytes in response to sperm or adenophostin A but not to increases in intracellular Ca^{2+} or egg activation. *Developmental Biology* **223** 251–265.
- Carroll J 2001 The initiation and regulation of Ca^{2+} signaling at fertilization in the mammals. *Seminars in Cell and Developmental Biology* **12** 37–43.
- Ducibella T, Huneau D, Angelichio E, Xu Z, Schultz R, Kopf G, Fissore R, Madoux S & Ozil JP 2002 Egg to embryo transition is driven by differential responses to Ca^{2+} oscillation number. *Developmental Biology* **250** 280–291.
- Dupont G, McGuinness O, Johnson MH, Berridge MJ & Borgese F 1996 Phospholipase C in mouse oocytes: characterization of isoforms and their possible involvement in sperm-induced Ca^{2+} spiking. *Biochemistry Journal* **316** 583–591.
- Eliyahu E & Shalgi R 2002 A role for protein kinase C during rat egg activation. *Biology of Reproduction* **67** 189–195.
- Fissore RA, Dobrinsky JR, Balise JJ, Duby RT & Robl JM 1992 Patterns of intracellular Ca^{2+} concentration in fertilized bovine oocytes. *Biology of Reproduction* **47** 960–969.
- Gordo AC, Wu H, He CL & Fissore RA 2000 Injection of sperm cytosolic factor into mouse metaphase II oocyte induces different development fates according to the frequency of $[\text{Ca}^{2+}]_i$ oscillations and oocyte age. *Biology of Reproduction* **62** 1370–1379.
- Hyslop LA, Nixon VL, Levasseur M, Chapmann F, Chiba K, McDougall A, Venables JP, Elliott DJ & Jones KT 2004 Ca^{2+} -promoted cyclin B1 degradation in mouse oocytes requires the establishment of a metaphase arrest. *Developmental Biology* **269** 206–219.
- Jellerette T, He CL, Wu H, Parys JB & Fissore RA 2000 Down-regulation of the inositol 1,4,5-trisphosphate receptor in mouse eggs following fertilization or parthenogenetic activation. *Developmental Biology* **223** 238–250.
- Jones KT 2004 Turning it on and off: M-phase promoting factor during meiotic maturation and fertilization. *Molecular Human Reproduction* **10** 1–5.
- Kishikawa H, Wakayama T & Yanagimachi R 1999 Comparison of oocyte activating agents for mouse cloning. *Cloning* **1** 153–159.
- Kline D & Kline D 1992 Repetitive calcium transient and the role of calcium in exocytosis and cell cycle activation in the mouse egg. *Developmental Biology* **149** 80–89.
- Krivokharchenko A, Popova E, Zaitseva I, Vil'ianovich L, Ganten D & Bader M 2003 Development of parthenogenetic rat embryos. *Biology of Reproduction* **68** 829–836.
- Marshall IC & Taylor CW 1994 Two calcium-binding sites mediated the interconversion of liver inositol 1,4,5-trisphosphate receptors between three conformational states. *Biochemistry Journal* **301** 591–598.
- Mehlmann LM, Carpenter G, Rhee SG & Jaffe LA 1998 SH2 domain-mediated activation of phospholipase C is not required to initiate Ca^{2+} release at fertilization of mouse eggs. *Developmental Biology* **203** 221–232.
- Nixon VL, McDougall A & Jones KT 2000 Ca^{2+} oscillations and the cell cycle at fertilization of mammalian and ascidian eggs. *Biology of the Cell* **92** 187–196.
- Okada K, Miyano T & Miyake M 2003 Activation of pig oocytes by intracytoplasmic injection of strontium and barium. *Zygote* **11** 159–165.
- O'Neill GT, Rolfe LR & Kaufman MH 1991 Developmental potential and chromosome constitution of SrCl_2 -induced mouse parthenogenones. *Molecular Reproduction and Development* **30** 214–219.
- Otaegui PJ, O'Neill GT & Wilmut I 1999 Parthenogenetic activation of mouse oocyte by exposure to SrCl_2 as a source of cytotoblasts for nuclear transfer. *Cloning* **1** 111–117.
- Ozil JP & Huneau D 2001 Activation of rabbit oocyte: the impact of the Ca^{2+} signal regime on development. *Development* **128** 917–928.
- Parrington J, Brind S, De Smedt H, Gangeswaran R, Lai FA, Wojcikiewicz R & Carroll J 1998 Expression of inositol 1,4,5-trisphosphate receptors in mouse oocytes and early embryos: the type I isoform is upregulated in oocytes and down-regulated after fertilization. *Developmental Biology* **203** 451–461.
- Patel S, Joseph SK & Thomas AP 1999 Molecular properties of inositol 1,4,5-trisphosphate receptors. *Cell Calcium* **25** 247–264.
- Raz T & Shalgi R 1998 Early events in mammalian egg activation. *Human Reproduction* **13** 133–145.
- Raz T, Ben-Yosef D & Shalgi R 1998 Segregation of the pathways leading to cortical reaction and cell cycle activation in the rat egg. *Biology of Reproduction* **58** 94–102.
- Shalgi R & Phillips DM 1988 Motility of rat spermatozoa at the site of fertilization. *Biology of Reproduction* **39** 1207–1213.
- Talmor A, Kinsey WH & Shalgi R 1998 Expression and immunolocalization of $\text{p59}^{\text{c-Fyn}}$ tyrosine kinase in rat egg. *Developmental Biology* **194** 38–46.
- Tateno H & Kamiguchi Y 1997 Parthenogenetic activation of Chinese hamster oocytes by chemical stimuli and its cytogenetic evaluation. *Molecular Reproduction and Development* **47** 72–78.
- Taylor CW 1998 Inositol trisphosphate receptors: Ca^{2+} -modulated intracellular Ca^{2+} channels. *Biochimica et Biophysica Acta* **1436** 19–33.
- Wakayama T, Perry AC, Zuccotti M, Johnson KR & Yanagimachi R 1998 Full-term development of mice from enucleated oocytes injected with cumulus cell nuclei. *Nature* **394** 369–374.
- Whitaker M & Larman MG 2001 Calcium and mitosis. *Cell and Developmental Biology* **12** 53–58.

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