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Comparison of Ca^{2+} and CaMKII responses in IVF and ICSI in the mouse

Styliani Markoulaki[1,†,‡], Manabu Kurokawa[2,†,¶], Sook-Young Yoon[2,†], Sara Matson[3], Tom Ducibella[1,3,4] and Rafael Fissore[2]

[1]Sackler School of Biomedical Sciences, Program in Cell, Molecular, and Developmental Biology, Tufts University School of Medicine, Boston, MA, USA, [2]Department of Veterinary and Animal Sciences, University of MA, Amherst, MA, USA and [3]Department of Obstetrics and Gynecology, Tufts-New England Medical Center, Boston, MA, USA

[4]To whom Correspondence should be addressed at: Department of Obstetrics and Gynecology, Box 36, Tufts-New England Medical Center, 750 Washington St., Boston, MA 02111, USA. E-mail: tducibella@tufts-nemc.org

Novel methods of egg activation in human assisted reproductive technologies and animal somatic cell nuclear transfer are likely to alter the signalling process that occurs during normal fertilization. Intracytoplasmic sperm injection (ICSI) bypasses the normal processes of the acrosome reaction, sperm–egg fusion, and processing of the sperm plasma membrane, as well as alters some parameters of intracellular calcium ($[Ca^{2+}]_i$) dynamics (reported previously by Kurokawa and Fissore (2003)). Herein, we extend these studies to determine if ICSI alters the activity of the Ca^{2+}-dependent protein, Ca^{2+}/calmodulin-dependent kinase II (CaMKII), which is responsible for the completion of meiosis in vertebrate eggs. After ICSI or *in vitro* fertilization (IVF), individual mouse eggs were monitored for their relative changes in both $[Ca^{2+}]_i$ and CaMKII activity during the first $[Ca^{2+}]_i$ rise and a subsequent rise associated with second polar body extrusion. The duration of the first $[Ca^{2+}]_i$ rise was greater in ICSI than in IVF, but the amplitude of the rise was transiently higher for IVF than ICSI. However, a similar mean CaMKII activity was observed in both procedures. During polar body extrusion, the amplitude and duration of the Ca^{2+} rises were increased by a small amount in ICSI compared with IVF, whereas the CaMKII activities were similar. Thus, compared with IVF, ICSI is not associated with decreased or delayed CaMKII activity in response to these Ca^{2+} signals in the mouse.

Key words: calcium/CaMKII/fertilization/ICSI/IVF

Introduction

Like many cellular processes (Berridge *et al.*, 2000), fertilization-induced events rely on a rise in intracellular calcium $[Ca^{2+}]_i$ in all species studied (Stricker, 1999; Runft *et al.*, 2002). In mammals, the fertilization-associated $[Ca^{2+}]_i$ oscillations induce egg activation, a multi-step process, which entails the release of cortical granules (CG), exit from the metaphase II (MII) stage arrest and completion of meiosis, recruitment of maternal mRNAs and cell-cycle progression into interphase (Schultz and Kopf, 1995).

Although egg activation and embryo development in mammals have been accomplished using a wide range of $[Ca^{2+}]_i$ patterns, consensus is emerging that a physiological pattern of oscillations, like those induced by sperm, results in greater embryo survival (Ozil and Huneau, 2001; Ozil *et al.*, 2005; Ducibella *et al.*, 2006). Consistent with this evidence, distinct events of egg activation are initiated and completed by different number of $[Ca^{2+}]_i$ rises; i.e. initiation of CG exocytosis seems to require less than four rises whereas recruitment of mRNAs and progression into interphase necessitate more than eight rises (Ducibella *et al.*, 2002). Furthermore, the sum of the rises in the $[Ca^{2+}]_i$ over time dictates the extent of egg activation (Toth *et al.*, 2006).

The rise in the $[Ca^{2+}]_i$ is transduced by Ca^{2+}-dependent proteins, such as protein kinase C (PKC) (Eliyahu and Shalgi, 2002; Halet *et al.*, 2004) and Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) (Winston and Maro, 1995; Johnson *et al.*, 1998; Tatone *et al.*, 1999, 2002; Markoulaki *et al.*, 2004). Fertilization-induced MII exit in mammals is caused by CaMKII-mediated activation of the anaphase-promoting complex/cyclosome (APC) (Madgwick *et al.*, 2005), most likely by phosphorylating specific proteins that regulate the APC as shown in *Xenopus* eggs (Liu and Maller, 2005; Rauh *et al.*, 2005). The fertilized mammalian egg exhibits simultaneous long-lasting oscillations in the levels of both $[Ca^{2+}]_i$ and CaMKII activity (Markoulaki *et al.*, 2004), whereas prolonged elevation of $[Ca^{2+}]_i$ causes down-regulation of CaMKII activity (Ozil *et al.*, 2005). In addition, the expression of CaMKII protein increases during meiotic maturation in mouse oocytes (Abbott *et al.*, 1999).

The introduction of novel methods of fertilization and egg activation in human assisted reproductive technologies (ART) and animal somatic cell nuclear transfer raise questions about how these methods impact the changes in $[Ca^{2+}]_i$ and CaMKII activity. Intracytoplasmic sperm injection (ICSI) is a commonly used form of ART

[†]These authors contributed equally to this work.
[‡]Current address: Whitehead Institute for Biomedical Research, Cambridge, MA 02142, USA.
[¶]Current address: Department of Pharmacology and Cancer Biology, Duke University Medical Center, Durham, NC 27710, USA.

whereby fertilization is accomplished by directly injecting a sperm into the ooplasm. Fertilization by ICSI has resulted in live births in numerous species and is frequently used in human clinics in cases of male infertility (Yanagimachi, 2005). It is widely considered to be a successful clinical procedure.

However, it is often noted that the ICSI procedure bypasses most of the natural selection processes encountered by sperm during its normal transit to the egg membrane, adds risk of mechanical injury to the egg, may affect sperm head decondensation and introduces additional sperm components as well as the injection solution into the egg cytoplasm (reviewed in Schultz and Williams, 2002; Winston and Hardy, 2002). In addition, blastocyst formation is more successful when using *in vitro* fertilization (IVF) than ICSI in the human (Dumoulin *et al.*, 2000), and ICSI does not result in successful egg activation in the bovine and equine (Bedford *et al.*, 2003; Malcuit *et al.*, 2006).

In the mouse and human, ICSI appears to induce normal egg activation, although, upon closer examination, subtle differences have been observed in most events of egg activation between natural fertilization and ICSI (Hewitson *et al.*, 1999; Ajduk *et al.*, 2006). Regarding $[Ca^{2+}]_i$ responses, besides inducing an artifactual initial rise, the spatio-temporal characteristics of the first sperm-induced rise and the persistence of oscillations are altered in ICSI eggs (Nakano *et al.*, 1997; Sato *et al.*, 1999; Kurokawa and Fissore, 2003). Moreover, the lag time to the first sperm-induced $[Ca^{2+}]_i$ rise and the persistence of oscillations after ICSI can be further modified according to how the sperm is handled prior to injection (Yanagida *et al.*, 2001; Kurokawa and Fissore, 2003). Lastly, a recent report shows that events of egg activation and preimplantation development can be induced by expression of a constitutively active form of CaMKII in the absence of $[Ca^{2+}]_i$ increases (Knott *et al.*, 2006). Therefore, because activation of CaMKII depends on $[Ca^{2+}]_i$ rises and given that the latter are influenced by the type of fertilization, here we investigate whether CaMKII responses are similar in eggs fertilized by IVF or ICSI.

Materials and methods

Gamete collection

MII eggs were obtained from 6 to 12 week old B6D2F1 (C57BL/6J x DBA/2J) female mice superovulated by injection of 5 IU of pregnant mare's serum gonadotrophin (PMSG; Sigma, St. Louis, MO, USA) followed 48 h later by 5 IU of human chorionic gonadotrophin (hCG; Sigma). Eggs were recovered 14 h post-hCG into a HEPES-buffered Tyrode-lactate solution supplemented with 5% heat-treated calf serum (Invitrogen, Carlsbad, CA, USA). Cumulus cells were removed by brief treatment with 0.1% bovine testes hyaluronidase (Sigma). When required, zona pellucida (ZP) were removed by brief treatment with acid tyrode's solution (pH 2.7), and eggs were allowed to recover in human tubal fluid (HTF) medium (Quinn *et al.*, 1985) for 30 to 60 min.

Mouse sperm were collected from cauda epididymides of 12–24 week old B6D2F1 male mice. All procedures involving live animal handling and euthanasia were performed according to standard animal protocols approved by the University of Massachusetts Animal Care Committee.

In vitro *fertilization*

IVF was conducted using HTF medium. Sperm were directly collected into HTF medium previously equilibrated at 36°C under a 5.5% CO_2 atmosphere and were further incubated for 1–2 h prior to insemination to induce sperm capacitation. Capacitated sperm were added into HTF medium containing eggs to final concentrations of $2–5 \times 10^4$ and $1–3 \times 10^5$ sperm ml^{-1} for ZP-free and ZP-intact eggs, respectively. For IVF with ZP-intact eggs, cumulus complexes were used. In order to synchronize fertilization time, the ZP-intact eggs were recovered, washed and transferred to sperm-free HTF medium within 30 min of the addition of sperm.

Intracytoplasmic sperm injection

ICSI was carried out as previously described (Kurokawa and Fissore, 2003) at room temperature in HCZB medium (HEPES-CZB medium, Chatot *et al.*, 1989). Sperm were washed twice with injection buffer (75 mM KCl and 20 mM HEPES, pH 7.0). One part sperm suspension was mixed with one part injection buffer containing 12% polyvinyl pyrrolidone (PVP, MW 360 kDa; Sigma). Immediately prior to injection into the ZP-intact egg, sperm heads were individually separated from their tails by applying a few piezo-pulses at the live sperm. Thereafter, the sperm heads were delivered into the ooplasm using a piezo micropipette-driving unit (Piezodrill; Burleigh Instruments Inc., Rochester, NY, USA) as described elsewhere (Kimura and Yanagimachi, 1995); a few piezo-pulses were applied to puncture the egg plasma membrane following penetration of the ZP. Second polar body (PB2) protrusion was photographed using a Zeiss Axiovert 200M microscope and Hamamatsu Orca AG cooled CCD camera controlled through Openlab software (Improvision, Lexington, MA, USA).

$[Ca^{2+}]_i$ measurements

$[Ca^{2+}]_i$ measurements were carried out as previously described (Kurokawa and Fissore, 2003). Briefly, eggs were loaded with 1 μM Fura-2 acetoxymethylester (Fura-2 AM; Molecular Probes, Eugene, OR, USA) supplemented with 0.02% pluronic acid (Molecular Probes) for 20 min at room temperature. Oocytes were monitored simultaneously using a 20X objective on a Nikon Diaphot inverted microscope (Nikon Corp., Tokyo, Japan) fitted for fluorescence measurements. Excitation light was provided by a 75 W Xenon lamp. The excitation wavelength was alternated between 340 and 380 nm by a filter wheel (Ludl Electronic Products, Hawthorne, NY, USA), and emitted light was passed through a 510 nm barrier filter and collected with either a cooled Photometrics SenSys CCD camera or a cool SNAP ES digital camera (Roper Scientific, Tucson, AZ, USA). SimplePCI software (Compix Imaging Inc., Cranberry, PA, USA) was used to monitor $[Ca^{2+}]_i$ and synchronize filter wheel rotation. $[Ca^{2+}]_i$ values were reported as the ratio of 340/380 nm fluorescence. Fluorescence ratios were obtained every 10 or 20 s. All $[Ca^{2+}]_i$ measurements were conducted on a warming stage (36°C) using HTF medium, which had been previously equilibrated in 5.5% CO_2.

A timeline diagram of the steps involved in IVF and ICSI to monitor the two chosen rises of $[Ca^{2+}]_i$ is shown in Figure 1. In order to monitor the first Ca^{2+} rise in IVF, ZP-free eggs preloaded with Fura-2 AM were adhered onto the bottom of a Ca^{2+} dish that had been treated with CellTak® (Mehlmann and Kline, 1994). While $[Ca^{2+}]_i$ was being measured, sperm were added to the medium containing the eggs. The first Ca^{2+} rise was usually seen 5–15 min after sperm addition. For the first $[Ca^{2+}]_i$ rise in ICSI fertilization, $[Ca^{2+}]_i$ measurements were started within 3 min after sperm injection and subsequent ZP removal. Typically, the first $[Ca^{2+}]_i$ rise was observed 5–20 min after ICSI (also see Kurokawa and Fissore, 2003).

To monitor $[Ca^{2+}]_i$ at the time of PB2 protrusion, IVF was carried out with ZP-intact eggs (Figure 1B). After insemination (30 min), the eggs were thoroughly washed and further cultured in sperm-free HTF medium for 60 min. Thereafter, eggs were loaded with Fura-2 AM and were returned to HTF medium. They were monitored every 15 min for signs of PB2 protrusion, at which time the ZP was removed and the egg immediately subjected to $[Ca^{2+}]_i$ imaging. At this stage, $[Ca^{2+}]_i$ monitoring for ICSI-fertilized eggs was as described for IVF-fertilized eggs, except that eggs were cultured for 70–80 min after sperm injection (Figure 1B).

CaMKII activity

CaMKII activity was measured on a single-egg basis, as described previously (Markoulaki *et al.*, 2004). As in that previous study, once the desired $[Ca^{2+}]_i$ level had been reached during a rise or a subsequent decrease to baseline, eggs were removed from $[Ca^{2+}]_i$ monitoring and frozen within 1 min (to freeze while $[Ca^{2+}]_i$ was elevated, experiments were carried out by two people). For peak I, eggs were removed 1 min after the first $[Ca^{2+}]_i$ rise had reached the maximum level. For peak PB2-associated $[Ca^{2+}]_i$ rise, eggs were removed as soon as an unequivocal rise in $[Ca^{2+}]_i$ was detected. For baseline I and baseline PB2 determinations, eggs were removed 2 min after $[Ca^{2+}]_i$ levels had returned to baseline values for peak I and peak PB2, respectively. Immediately after eggs were removed from Ca^{2+} monitoring, they were rapidly washed in Ca^{2+}/Mg^{2+}-free Earle's balanced salt solution (Sigma),

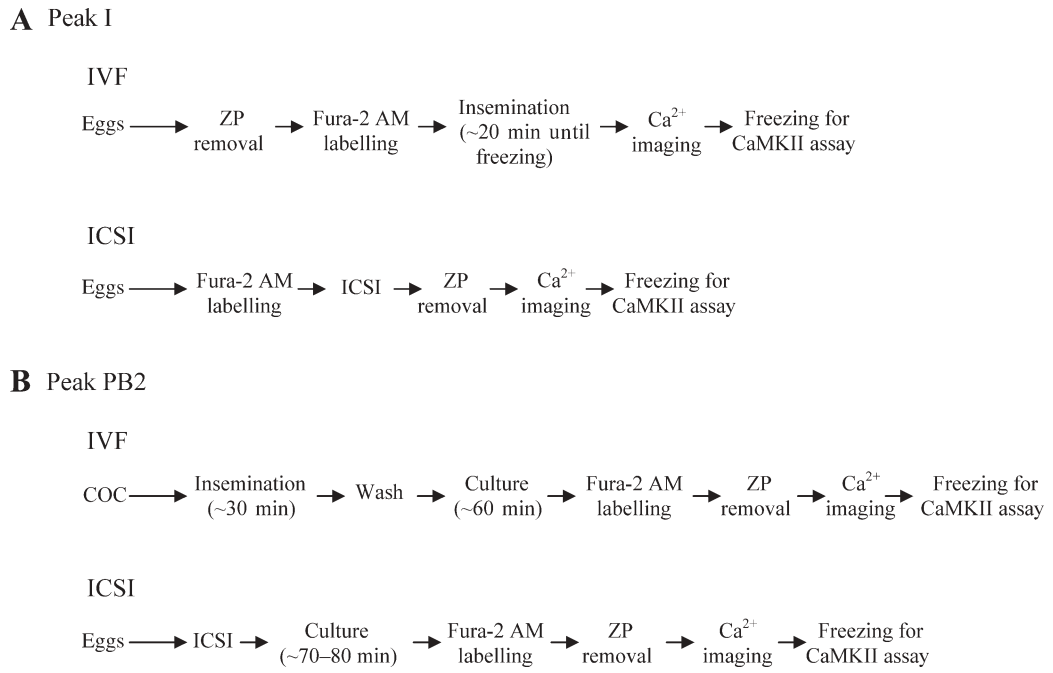

Figure 1. Protocols for preparing eggs for $[Ca^{2+}]_i$ and Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) activity measurements. During culture, eggs were monitored every 15 min for signs of second polar body (PB2) protrusion, at which time $[Ca^{2+}]_i$ monitoring was initiated. COC, cumulus-enclosed oocyte complexes.

and then placed into a microfuge tube containing 2 μl lysis buffer. The tube was immediately frozen and stored at −80°C. Autonomous enzyme activity, representing autophosphorylated enzyme that remains active in the absence of Ca^{2+}/CaM (see Discussion), was detected with the SignaTECT assay system (Promega, Madison, WI, USA), utilizing a biotinylated peptide substrate and 2.5 μCi [γ^{32}P]ATP (Perkin Elmer, Boston, MA, USA) (Markoulaki *et al.*, 2003).

Cell-cycle staining

Eggs were fixed in 3% paraformaldehyde and co-stained for DNA using 4,6-diamidino-2-phenyl indole (Polysciences, Warrington, PA, USA) and Hoechst 33258 (Sigma) to determine the cell-cycle status as previously described (Ducibella *et al.*, 1994) and examined with a 100X Plan-Apo objective on a Nikon Eclipse TE2000-U inverted fluorescence microscope (Nikon Corp.).

Statistics

Relative CaMKII activity data for ICSI and IVF was compared by the Student's unpaired *t*-test. For each egg, the relative activity is the ratio of the activity in that egg and the mean value of untreated control eggs on the same experimental date. Comparisons of $[Ca^{2+}]_i$ responses between IVF and ICSI were also performed by the Student's unpaired *t*-test using the Sigmaplot program (SPW 8.0, SPSS Inc., Chicago, IL, USA). Unless otherwise indicated, experiments were performed at least twice.

Results

Fertilization by IVF and ICSI initiates $[Ca^{2+}]_i$ oscillations in mouse eggs

Fertilization by IVF or ICSI in mouse eggs has been shown to initiate $[Ca^{2+}]_i$ oscillations (Nakano *et al.*, 1997; Kurokawa and Fissore, 2003). As the aim of our study was to compare CaMKII activity associated with specific rises in $[Ca^{2+}]_i$ in IVF and ICSI, fertilization conditions were required to make possible these comparisons while preserving the inherent differences in $[Ca^{2+}]_i$ responses that might be induced by IVF and ICSI. The main complications for IVF were to obtain rapid, synchronous and monospermic fertilization.

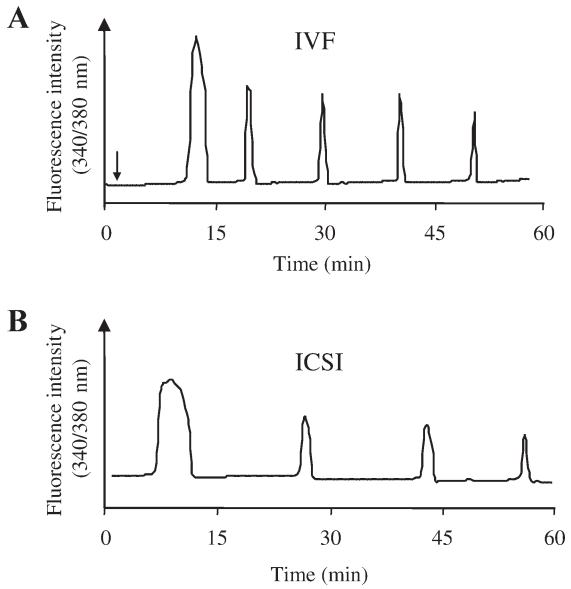

Figure 2. Representative $[Ca^{2+}]_i$ traces comparing $[Ca^{2+}]_i$ rises during the first hour of *in vitro* fertilization (IVF) (**A**) and intracytoplasmic sperm injection (ICSI) (**B**). The arrow represents time of insemination during IVF (**A**). Ca^{2+} monitoring was initiated within 3 min after ICSI.

For studying the first large $[Ca^{2+}]_i$ elevation, this was accomplished by utilizing ZP-free eggs and low number of sperm per insemination (Figure 1; see Materials and methods), which resulted in a first $[Ca^{2+}]_i$ rise within 20 min (Figure 2). The subsequent rises were slightly more frequent than anticipated, and this is possibly due to the fact that monitoring was performed in ZP-free eggs, which may slightly alter the organization of the egg cortex. In order to determine if an injection-mediated artifactual rise in $[Ca^{2+}]_i$ took place, which could modify the first endogenous rise (Nakano *et al.*, 1997), ICSI-fertilized eggs were monitored at ~3 min from the conclusion of the

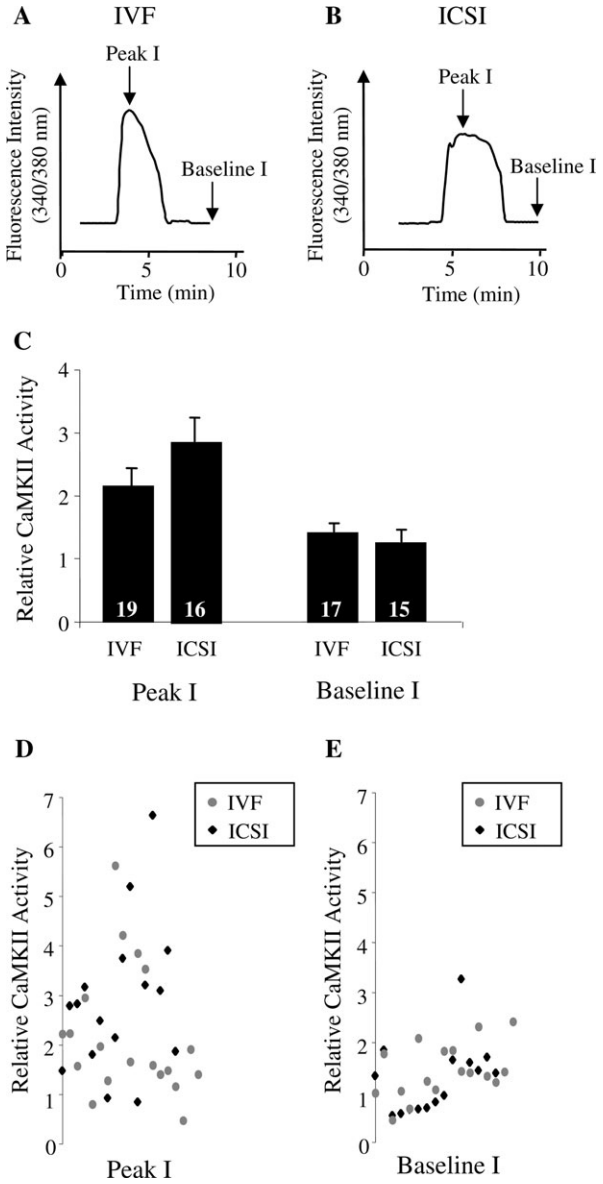

Figure 3. Comparison of $[Ca^{2+}]_i$ and CaMKII responses during the first $[Ca^{2+}]_i$ rise (peak I) of IVF (**A**) and ICSI (**B**). The CaMKII response is expressed as the mean relative activity, which is the ratio of the activity in an egg from IVF or ICSI relative to the mean activity of untreated control metaphase II eggs. Arrows indicate the time points at which eggs were removed for freezing for subsequent CaMKII activity assays (see Materials and methods). (**C**) The mean relative CaMKII activity during and following peak I of $[Ca^{2+}]_i$ in IVF or ICSI. Values represent the mean ± SEM (standard error of the mean). The number of determinations (1 egg/determination) is shown at the base of each bar. For peak I, the mean relative CaMKII activities for IVF and ICSI were not significantly different ($P = 0.19$). (**D, E**) Scattergram plots of the CaMKII activity data in (**C**).

procedure (Figure 2). An injection-induced rise was not detected, and the subsequent $[Ca^{2+}]_i$ rises occurred at 20 min intervals as observed previously (Kurokawa and Fissore, 2003).

ICSI results in normal increases in CaMKII activity, for two distinct $[Ca^{2+}]_i$ rises

To determine whether IVF and ICSI-initiated $[Ca^{2+}]_i$ rises equally stimulated CaMKII activity, the enzyme activity was examined during two different $[Ca^{2+}]_i$ rises: the first rise (peak I) and the rise associated with extrusion of the PB2 (peak PB2). Peak I was selected because it is the largest in terms of both amplitude and duration and results in the initial activation of CaMKII activity during fertilization (Lorca *et al.*, 1993; Markoulaki *et al.*, 2003) and induces recruitment of PKC to the egg's plasma membrane (Halet *et al.*, 2004). In addition, the spatio-temporal organization of the first $[Ca^{2+}]_i$ rise in ICSI-fertilized eggs is different from that in IVF (Sato *et al.*, 1999). The ZP-free eggs used in peak I studies were monospermic (data not shown).

Differences were observed in the $[Ca^{2+}]_i$ for peak I of IVF and ICSI. During the first 2 min of peak I, the $[Ca^{2+}]_i$ induced by IVF had a transiently higher maximal amplitude than that for ICSI. However, during the 3rd and 4th min, the level of $[Ca^{2+}]_i$ in the ICSI group was higher than that in IVF, because of the longer duration of maximal $[Ca^{2+}]_i$ elevation for ICSI (Figure 3 and Table I). For IVF and ICSI, the mean relative CaMKII activities were similar (Figure 3) for eggs with elevated $[Ca^{2+}]_i$ (Peak I; see Materials and methods). Although the somewhat lower mean CaMKII activity in IVF may be due to the relatively more rapid decrease in $[Ca^{2+}]_i$ after maximal elevation than in ICSI, the mean relative CaMKII activity values are not significantly different. The scatter plots show values from single eggs (Figure 3D). Baseline I values, from eggs whose $[Ca^{2+}]_i$ had returned to baseline, were similar (Figure 3C and E).

The $[Ca^{2+}]_i$ for peak PB2 was selected because previous studies have observed the elevation of CaMKII activity in mouse eggs between 45 and 60 min of fertilization (Johnson *et al.*, 1998; Tatone *et al.*, 2002), a time during which contractile ring formation and subsequent PB2 extrusion takes place. In addition, there is recent evidence that PB2 protrusion requires active calmodulin (CaM) (Matson *et al.*, 2006), which could also result in CaMKII stimulation. Lastly, the CaMKII activity described in one of the aforementioned studies seemed unusually persistent at this stage (Tatone *et al.*, 2002). To synchronize the time at which eggs were collected to evaluate enzyme activity, the appearance of the PB2 protrusion was used as a reference. The PB2 protrusion adopted several different morphologies (Figure 4), and the majority of these eggs from both IVF and ICSI were at similar cell-cycle stages and were monospermic based on chromatin staining (Table II).

Surprisingly, the $[Ca^{2+}]_i$ rise associated with the PB2 protrusion differed according to the fertilization method with ICSI-fertilized eggs exhibiting higher and longer $[Ca^{2+}]_i$ rises than those observed

Table I. Ca^{2+} parameters of *in vitro* fertilization (IVF) and intracytoplasmic sperm injection (ICSI)

	Peak I				PB2 peak			
	Duration (min)		Amplitude (RF 340/380 nm)		Duration (min)		Amplitude (RF 340/380 nm)	
	n	Average (SD)	*n*	Average (SD)	*n*	Average (SD)	*n*	Average (SD)
IVF	13	3.6 (± 0.2)	16	3.4 (± 0.2)	21	1.1 (± 0.1)	7	2.0 (± 0.06)
ICSI	8	5.5 (± 0.4)	15	2.8 (± 0.1)	17	1.6 (± 0.1)	9	2.5 (± 0.08)
P-value		0.0006		0.007		0.04		0.001

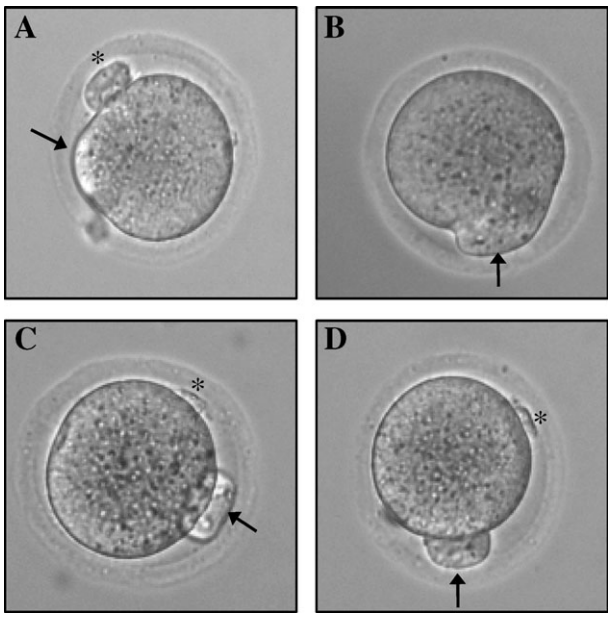

Figure 4. Representative photographs of eggs at stages of protrusion of the PB2 (**A–D**) after ICSI. Although eggs used for peak PB2 analysis were at various stages of protrusion of the PB2, the vast majority of eggs were at stages **B–D**. Arrows indicate the protrusion of the PB2 and asterisks show the first polar body.

after IVF (Figure 5 and Table I). However, at this stage, the mean relative CaMKII activity when $[Ca^{2+}]_i$ was either elevated or at baseline levels was not found to be different in IVF and ICSI (Figure 5).

Discussion

$[Ca^{2+}]_i$ responses are often modified in different egg activation procedures and alterations in $[Ca^{2+}]_i$ patterns can have long range developmental consequences (Ozil, 1990; Ozil and Huneau, 2001). Therefore, monitoring Ca^{2+}-dependent biochemical pathways is a logical strategy to compare routine IVF with other artificial procedures, such as ICSI. In addition, egg/embryo morphology is an insufficient predictor of developmental potential (Hammitt *et al.*, 1993; Graham *et al.*, 2000). This report is the first biochemical comparison of the activity of a developmentally crucial Ca^{2+}-dependent enzyme in mammalian IVF and ICSI and demonstrates similar CaMKII responses for the time points investigated.

The rationale for this study is also supported by observations that indeed there are subtle differences in the Ca^{2+} responses in ICSI compared with IVF (Introduction). Moreover, ICSI is not successful in all mammals (discussed later) and other ART methods have a

Table II. Cell cycle comparison associated with the Ca^{2+} rise during second polar body protrusion

	n	MII	Cell cycle[a]		Abnormal
			Anaphase	Post-anaphase	
Uninseminated controls	52	52 (100%)			
IVF[b]	23			22 (96%)	1 (4%)
ICSI[b]	28		4 (14%)	23 (79%)	1 (4%)

[a]Only fertilized eggs from IVF and ICSI treatments were analysed for cell-cycle status.
[b]Chromatin staining revealed 96% and 100% monospermy in IVF and ICSI treatments, respectively.

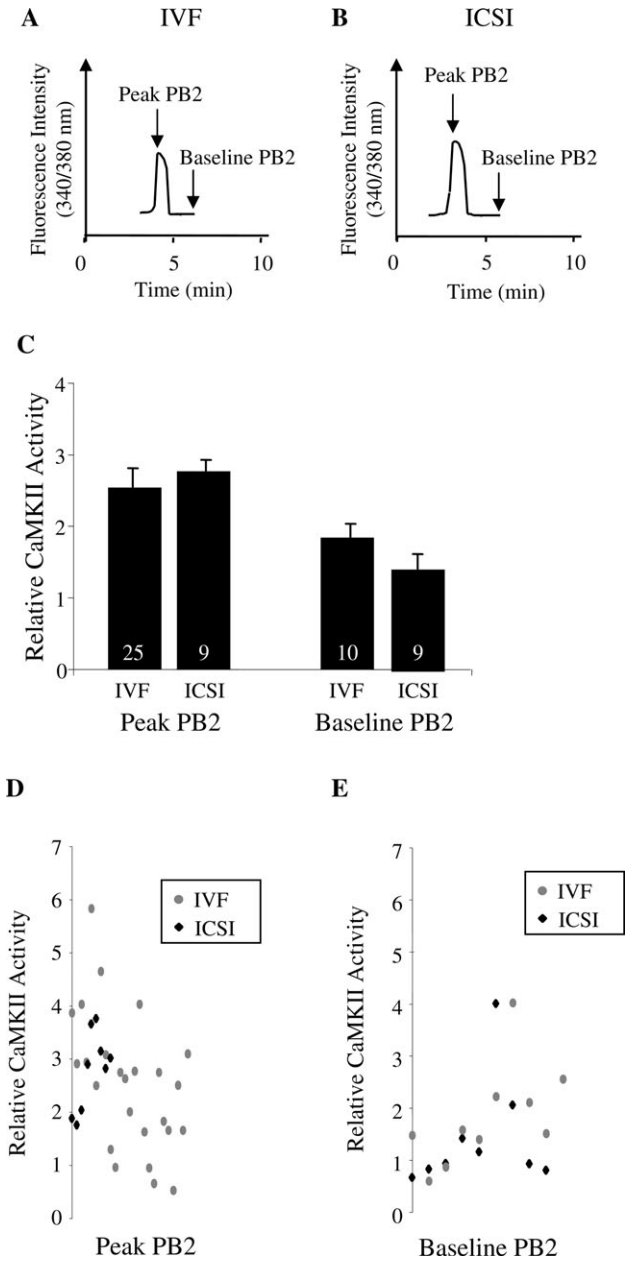

Figure 5. Comparison of the $[Ca^{2+}]_i$ and CaMKII responses during protrusion of the PB2 in IVF and ICSI. Representative Ca^{2+} traces of the rise directly following protrusion of the PB2 in IVF (**A**) and ICSI (**B**). Arrows indicate the time points at which eggs were removed for freezing for subsequent CaMKII activity assays (see Materials and methods). (**C**) The mean relative CaMKII activity (see Figure 3 legend) during and following the change in $[Ca^{2+}]_i$ associated with peak PB2 in IVF or ICSI. Values represent the mean ± SEM. The number of determinations (1 egg/determination) is shown at the base of each bar. (**D**, **E**) Scattergram plots of the CaMKII activity data in (**C**).

significant impact on the zygote and offspring, e.g. different IVF culture conditions cause changes in embryonic gene expression, post-implantation development and behaviour after birth (reviewed in Schultz and Williams, 2002; Winston and Hardy, 2002; also see Ecker *et al.*, 2004; Fernandez-Gonzalez *et al.*, 2004; Mann *et al.*, 2004; Rinaudo and Schultz, 2004), consistent with the Barker hypothesis (Hales and Barker, 2001).

It is important to note that the similar mean CaMKII activity responses observed in IVF and ICSI are consistent with other observations. For example, some major features of the $[Ca^{2+}]_i$ responses

are similar in IVF and ICSI; e.g. in both cases, the first rise in $[Ca^{2+}]_i$ is of longer duration and higher amplitude than subsequent ones. Also, the egg, and not the sperm, is the major source of CaMKII activity during mouse egg activation (Markoulaki *et al.*, 2004). This is relevant because the mechanism of incorporation and initial location of sperm components in the egg is different in ICSI and IVF (Sutovsky *et al.*, 1996). The clinical success of ICSI in humans coupled with the importance of CaMKII in activating early development (Knott *et al.*, 2006) also argues against abnormal responses of CaMKII after ICSI. In this regard, no major differences in gene expression patterns were observed between IVF and ICSI in mice (Wilson *et al.*, 2006).

The two CaMKII responses investigated are associated with important events of egg activation. The first of these events is the relatively long duration and high amplitude of the initial sperm-induced $[Ca^{2+}]_i$ rise compared with subsequent $[Ca^{2+}]_i$ elevations during fertilization (Miyazaki *et al.*, 1993). Since the egg appears to sum its exposure to intracellular $[Ca^{2+}]_i$ (Ozil *et al.*, 2005; Toth *et al.*, 2006), the first rise is likely to have a larger impact on Ca^{2+}-dependent events by virtue of its longer duration, which should prolong CaMKII activity until soon after $[Ca^{2+}]_i$ falls to basal levels (Markoulaki *et al.*, 2004). The observed (herein) longer duration of the first rise in $[Ca^{2+}]_i$ in ICSI compared to IVF did not result in a significantly higher mean CaMKII activity. This is consistent with a previous study in which the level of CaMKII activity did not change during 10 min of prolonged continuous elevation of $[Ca^{2+}]_i$, although activity was down-regulated by 20 min (Ozil *et al.*, 2005).

The second event investigated was formation of the PB2. The meiotic cell cycle is driven to completion by eight to 24 $[Ca^{2+}]_i$ rises (Ducibella *et al.*, 2002) and an inhibitor of myosin light chain kinase, which is CaM-dependent, prevents PB2 formation (Matson *et al.*, 2006). CaMKII activity represents a means to track the activity of active CaM and availability of $[Ca^{2+}]_i$ to enzymes that are dependent upon it during the completion of meiosis, for which PB2 formation is an important landmark. Interestingly, even though the amplitude of the second rise in $[Ca^{2+}]_i$ is less than that of the first and the extent of autonomous CaMKII activity can be dependent upon the concentration of $[Ca^{2+}]_i$, significant differences in mean enzyme activity were not observed at the two times investigated herein for peaks I and PB2. CaMKII (activity) results consistent with these were obtained in another study in which the first longer duration $[Ca^{2+}]_i$ rise was compared to one at 60 min later in IVF with the CF-1 strain mouse (Markoulaki *et al.*, 2004).

Autonomous enzyme activity assays are often used to investigate cellular changes in the activity of CaMKII (Hudmon and Schulman, 2002) and have the advantages of using single eggs whose level of $[Ca^{2+}]_i$ is known (Markoulaki *et al.*, 2003, 2004) as well as capturing a 'snapshot' of relative activity in the living cell. This activity is a function of the extent to which individual subunits of this highly multimeric protein are autophosphorylated in the cell—the higher the percentage of phosphorylated subunits (which continue to be active in the assay performed in the absence of Ca^{2+} and CaM), the greater the overall activity. However, as is the case for many enzymes, the precise activity in the cell can be a function of many factors, including location, substrate availability and positive as well as negative effectors. In addition, since a low level of kinase activity can occur without autonomous activity (e.g. Ca^{2+} and CaM binding without autophosphorylation at low endogenous levels of $[Ca^{2+}]_i$), this activity would not be detected in an autonomous activity assay.

Thus, it cannot be ruled out that some differences are present in IVF and ICSI and, if so, related to the extent of non-autonomous activity or intracellular location. With regard to the latter, ICSI in the mouse is characterized by the absence of both the Ca^{2+} wave and heterogeneity between the cortex and interior (Nakano *et al.*, 1997). This is likely to be due to differences in the presentation of PLCzeta-release in IVF and ICSI, which in turn regulates inositol triphosphate (IP_3) production and opening of IP_3 receptor Ca^{2+} channels in the egg cytoplasm (Jellerette *et al.*, 2004; Larman *et al.*, 2004; Kurokawa *et al.*, 2005). In addition, unlike IVF, mouse ICSI is sometimes (although not detected in our study) associated with an additional initial large artificially-induced rise in $[Ca^{2+}]_i$, resulting in a total of two large early rises (Tesarik *et al.*, 1994; Nakano *et al.*, 1997). However, the number of subsequent smaller oscillatory rises in $[Ca^{2+}]_i$ is somewhat reduced in ICSI compared with IVF in mice (Kurokawa and Fissore, 2003). To what extent these are offsetting factors or provide functionally relevant differences in enzyme activity over time is unknown.

In a more extreme case, ICSI in the bovine and equine has not been successful, apparently due to a substantial reduction in the number of oscillatory rises in $[Ca^{2+}]_i$ (Bedford *et al.*, 2003; Malcuit *et al.*, 2006). Since CaMKII activity rises and falls with each transient change in $[Ca^{2+}]_i$ (Markoulaki *et al.*, 2004) and the total exposure to $[Ca^{2+}]_i$ is related to the extent of egg activation in the mouse (Ducibella *et al.*, 2002, 2006; Ozil *et al.*, 2005; Toth *et al.*, 2006), the aborted oscillations in $[Ca^{2+}]_i$ in ICSI of these species may result in insufficient total CaMKII activity.

Monitoring multiple biochemical parameters in the same living egg, such as $[Ca^{2+}]_i$ and CaMKII activity, represents a major advancement over averaging the results from batches of eggs whose $[Ca^{2+}]_i$-related parameters are oscillating or changing asynchronously in different eggs. However, our study did not continuously monitor CaMKII activity in a single egg, which would require a suitable probe for fluorescence resonance energy transfer. Thus, it is possible that if differences exist between IVF and ICSI, they could have gone undetected. In this regard, the emerging field of using multiple reporters in the same living egg will be a further improvement to compare protein kinase responses with simultaneous measurements of $[Ca^{2+}]_i$ during activation and other developmentally important events. Recent studies using such probes in living eggs represent a step in that direction (Nixon *et al.*, 2002; Halet *et al.*, 2004; Shirakawa and Miyazaki, 2004).

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