Phospholipase C ζ causes Ca²⁺ oscillations and parthenogenetic activation of human oocytes

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Abstract

At fertilization in mammals the sperm activates development of the oocyte by inducing a prolonged series of oscillations in the cytosolic free Ca²⁺ concentration. One theory of signal transduction at fertilization suggests that the sperm cause the Ca²⁺ oscillations by introducing a protein factor into the oocyte after gamete membrane fusion. We recently identified this sperm-specific protein as phospholipase C ζ (PLC ζ), and we showed that PLC ζ triggers Ca²⁺ oscillations in unfertilized mouse oocytes. Here we report that microinjection of the complementary RNA for human PLC ζ causes prolonged Ca²⁺ oscillations in aged human oocytes that had failed to fertilize during *in vitro* fertilization or intracytoplasmic sperm injection. The frequency of Ca²⁺ oscillations was related to the concentration of complementary RNA injected. At low concentrations, PLC ζ stimulated parthenogenetic activation of oocytes. These embryos underwent cleavage divisions and some formed blastocysts. These data show that PLC ζ is a novel parthenogenetic stimulus for human oocytes and that it is unique in its ability to mimic the repetitive nature of the Ca²⁺ stimulus provided by the sperm during human fertilization.

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Introduction

Mammalian oocytes are ovulated in a state of arrest at metaphase of the second meiotic division. During fertilization the sperm releases the oocyte from metaphase arrest and initiates development by inducing a series of repeated rises in the cytosolic free Ca²⁺ concentration (Whittingham 1980, Cuthbertson & Cobbold 1985, Stricker 1999). Such repeated rises in Ca²⁺, or Ca²⁺ oscillations, occur at intervals of several minutes for the first few hours after sperm–oocyte interaction. These Ca²⁺ oscillations have been observed during *in vitro* fertilization (IVF) in a range of mammalian species including oocytes from humans (Taylor *et al.* 1993). In addition, in human and mouse oocytes prolonged Ca²⁺ oscillations have also been demonstrated following intracytoplasmic sperm injection (ICSI; Tesarik & Sousa 1994, Nakano *et al.* 1997). The Ca²⁺ oscillations seen in mammals appear to be both necessary and sufficient for the activation of development. In the mouse, for example, it has been shown that the Ca²⁺ oscillations are necessary for second-polar-body emission and pronuclear formation (Kline & Kline 1992). The importance of Ca²⁺ is also underlined by

the finding that most of the treatments that cause the parthenogenetic activation of development in mammals are effective because they cause a marked rise in the cytosolic Ca²⁺ concentration (Whittingham 1980, Swann & Ozil 1994). In mouse and domestic animal oocytes parthenogenetic activating agents such as ethanol, Ca²⁺ ionophores and electrical-field stimulation cause a single and prolonged rise in Ca²⁺ (Swann & Ozil 1994). In mouse, and some other species, parthenogenetic activating agents such as Sr²⁺ ions or thimerosal have been shown to cause Ca²⁺ oscillations that are similar, but not identical, to those seen at fertilization (Kline & Kline 1992, Cheek *et al.* 1993).

The mechanism by which the sperm stimulates the Ca²⁺ oscillations at fertilization is not fully resolved (Stricker 1999). However, one theory is that the sperm introduces a protein factor into the egg cytosol after gamete fusion, and that this sperm factor protein initiates Ca²⁺ release in the oocyte via the generation of inositol 1,4,5-trisphosphate (InsP₃; Swann *et al.* 2004). This theory is supported by the finding that injecting cytosolic extracts from sperm can cause Ca²⁺ oscillations in a range of different mammalian oocytes, including those from

humans (Swann 1990, Homa & Swann 1994, Wu *et al.* 1997). We recently demonstrated that mouse sperm possess a novel and specific isoform of phospholipase C (PLC) referred to as PLC ζ (Saunders *et al.* 2002). PLC ζ was shown to be the protein present in the sperm extracts that is responsible for generating Ca^{2+} release and InsP_3 production (Saunders *et al.* 2002). Most critically it was demonstrated that PLC ζ is an effective mimic of the sperm because microinjection of complementary RNA (cRNA) encoding for PLC ζ into mouse oocytes causes Ca^{2+} oscillations identical to those seen during fertilization. Injection of cRNA for PLC ζ also leads to egg activation and development to the blastocyst stage in the mouse (Saunders *et al.* 2002). As well as mice, both humans and monkeys have been shown to possess a sperm-specific PLC ζ (Cox *et al.* 2002). Previous work suggested that the sperm factor protein was not species specific and, consistent with this, we found that injection of cRNA for the human or monkey isoforms of PLC ζ is able to cause Ca^{2+} oscillations in mouse oocytes and stimulate embryo development up to the blastocyst stage at similar rates to those seen after *in vivo* fertilization (Cox *et al.* 2002). However, the effect of PLC ζ in human oocytes has not previously been reported.

Like other mammals human oocytes can also be parthenogenetically activated by stimuli that elevate Ca^{2+} levels. Calcium ionophores such as A23187 have been shown to cause both fresh and aged oocytes to undergo pronuclear formation and begin early cleavage divisions (Winston *et al.* 1991). However, some studies have found that Ca^{2+} ionophore alone does not reliably activate human oocytes (Balakier & Casper 1993, Rinaudo *et al.* 1997). So the most common current activation protocols combine Ca^{2+} ionophore with protein synthesis or protein kinase inhibitors such as 6-dimethyl aminopurine (6-DMAP) or puromycin (Cibelli *et al.* 2001, Nakagawa *et al.* 2001, Lin *et al.* 2003). These combination protocols have proved effective in stimulating human oocytes to form pronuclei, but the success rates of subsequent preimplantation development are still poor compared with embryo development after fertilization. In order to try and improve activation and development after parthenogenesis, attempts have been made to use stimuli that mimic IVF in causing repetitive Ca^{2+} increases. Data in mouse and rabbit oocytes that were exposed to repeated electrical-field pulses have suggested that repeated rises in Ca^{2+} can improve activation rates, and subsequent development, compared with stimuli that cause a single Ca^{2+} increase (Ozil 1990, Ozil & Huneau 2001). Limited application of this technology has suggested that the repetitive stimuli may also be useful in activating human oocytes (Zhang *et al.* 1999). In this study we demonstrate that microinjection of cRNA encoding human PLC ζ protein can cause a prolonged series of Ca^{2+} oscillations in aged human oocytes that have failed to fertilize during IVF or ICSI. The induction of Ca^{2+} oscillations by PLC ζ can also lead to parthenogenetic activation up to the blastocyst stage.

Materials and Methods

Obtaining and handling of human oocytes

Human oocytes were obtained from patients whose gametes had failed to fertilize following conventional IVF or ICSI. Ethical approval for the project was obtained from St Thomas's Hospital Local Research Ethics Committee and from the Human Fertilization and Embryology Authority who issued a licence for the work (R0147). Consent for the use of unfertilized oocytes was obtained from patients before starting their treatment. For treatment the patients underwent pituitary downregulation and controlled ovarian hyperstimulation using gonadotrophins. Ovarian stimulation was achieved using a daily dose of 150–450 IU of recombinant follicle-stimulating hormone (Gonal F; Serono Laboratories, Welwyn Garden City, Herts, UK; or Puregon; Organon, Cambridge, UK). Human chorionic gonadotrophin, 10 000 IU (Profasi from Serono Laboratories or Pregnyl from Organon), was administered when at least three follicles had reached a mean diameter of 18 mm or more. Transvaginal follicular aspiration was performed 34–36 h after human chorionic gonadotrophin injection and 3–6 h later oocytes were prepared for IVF or ICSI dependent upon earlier semen analysis. Following IVF insemination or ICSI oocytes were cultured overnight and checked for signs of fertilization 19–20 h later. Only those oocytes that appeared to be at metaphase II or I and that showed no signs of fertilization were used for the project. Such unfertilized oocytes were transferred from the Assisted Conception Unit at Guy's Hospital to laboratories at University College London and microinjected within the next 1–2 h. Unless otherwise stated, all manipulations in the laboratory were carried out on oocytes in Hepes/KSOM (HKSOM) medium (Saunders *et al.* 2002).

In experiments where Ca^{2+} was monitored oocytes were injected with solutions containing various concentrations of PLC ζ cRNA and 1 mM Oregon Green BAPTA dextran (Molecular Probes, Eugene, OR, USA) in a buffered salt solution (120 mM KCl/20 mM Hepes, pH 7.4) that had been treated with Chelex 100 beads (Sigma) to remove divalent cations. In developmental experiments where Ca^{2+} was not monitored the Oregon Green BAPTA dextran was omitted from the injection buffer. cRNA encoding the 608-amino-acid sequence of the human form of PLC ζ was prepared as described previously (Saunders *et al.* 2002). The cRNA was stored in aliquots at -80°C until being thawed immediately prior to injection. For injection the oocytes were placed on a Nikon Diaphot stage and microinjected by application of brief pressure pulses as described previously (Swann 1990). The injection was between 3 and 5% of the oocyte's total volume. For Ca^{2+} measurements oocytes were immediately transferred to a chamber of approximately 1 ml containing HKSOM medium and fluorescence monitored as described below. For separate developmental studies the oocytes

were placed in 2 μ M cytochalasin D (Sigma) for approximately 2 h to prevent second-polar-body extrusion and then cultured in 20 μ l drops of Sydney IVF cleavage medium (COOK) under mineral oil (Sigma) at 37°C in a 6% CO₂ incubator from days 1 to 3 (up to the eight-cell stage). Embryos were transferred to Sydney IVF blastocyst medium (COOK) for the remaining culture time. Each day embryos were removed from the incubator briefly and their developmental stage was noted.

Measurements of intracellular Ca²⁺

Fluorescence measurements were carried out on oocytes in drops of media in a heated chamber on the stage of a Zeiss Axiovert microscope equipped with epifluorescence optics and a 20 $\times$ 0.75NA objective lens. Low-level fluorescence excitation was used to minimize oocyte damage. The light from a halogen lamp passed through a 490 nm bandpass filter and emission collected with a 510 nm long-pass filter. Fluorescence light (100–1000 photons/s) was measured from each oocyte with an imaging photon detector (Photek Ltd, East Sussex, UK) using software and a system designed by Science Wares (Falmouth, MA, USA).

Results

Microinjection of human PLC ζ cRNA into aged human oocytes caused a series of Ca²⁺ oscillations as indicated by the repetitive increases in the fluorescence of Oregon Green BAPTA dextran. Fig. 1 shows examples of the pattern of Ca²⁺ oscillations triggered in different oocytes by injecting different concentrations of PLC ζ cRNA. The Ca²⁺ oscillations consisted of series of sharp rises in Ca²⁺, followed by a fairly abrupt return to baseline Ca²⁺ levels. There was then a very gradual increase in Ca²⁺ before the next Ca²⁺ rise suggesting the existence of a pacemaker that leads to another Ca²⁺ increase every 10 min to 2 h (Fig. 1a). This general pattern of Ca²⁺ oscillations is similar to those reported after IVF or ICSI in human oocytes (Tesarik & Sousa 1994), and broadly similar to the pattern of Ca²⁺ oscillations seen in other mammals during fertilization (Swann & Ozil 1994). The main difference between the patterns of oscillation was in regard to the frequency of Ca²⁺ oscillations. In particular high concentrations of PLC ζ caused the highest-frequency oscillations. In addition we found that pipette concentrations of 1 or 10 μ g/ml cRNA lead to oscillations that started with a relatively low frequency, but oscillations tended to show an increase in frequency with time such that the final frequency tended to match that seen with higher concentrations of PLC ζ cRNA (Fig. 1b). A sustained low frequency of Ca²⁺ oscillations was only seen when we injected pipette concentrations of approximately 0.1 μ g/ml PLC ζ cRNA (Fig. 1c). This concentration of PLC ζ cRNA also appeared to be the minimally effective concentration because only six out of 13 oocytes showed Ca²⁺ responses. With pipette concentrations of 10 μ g/ml

Figure 1 Injection of different concentrations of human PLC ζ cRNA causes Ca²⁺ oscillations in aged human oocytes. Shown are three sample traces of the intracellular Ca²⁺ concentration, as measured by Oregon Green BAPTA dextran fluorescence (FI.; in arbitrary units). Recordings were made from aged human oocytes after injection of PLC ζ cRNA. In each panel the oocytes were injected 10–20 min before the start of the recording. The concentrations to the right of the traces refer to those in the injection pipette (2–5% oocyte volume injected). The repetitive rises in Ca²⁺ are seen as distinct spike-like increases in fluorescence.

or greater all the oocytes we injected underwent Ca²⁺ oscillations. Table 1 shows the intervals of Ca²⁺ oscillations seen after injecting different amounts of PLC ζ into human oocytes. Despite some changes in frequency with time at intermediate concentrations, there is a trend towards greater intervals between Ca²⁺ increases with lower concentrations of injected PLC ζ cRNA. Since the amount of PLC ζ protein synthesized in mouse oocytes was shown to be proportional to the concentration of PLC ζ cRNA injected (Saunders *et al.* 2002), these data suggest that the concentration of PLC ζ protein affects the frequency of Ca²⁺ oscillations.

As well as studying Ca²⁺ oscillations we also examined groups of oocytes injected with PLC ζ for signs of activation. Since light exposure during fluorescence measurements can impair development, we carried out a separate set of experiments where PLC ζ cRNA was injected without monitoring Ca²⁺. In mice it is known that diploid parthenogenetic embryos have greater developmental potential than haploid embryos (Liu *et al.* 2002). Consequently we treated oocytes with cytochalasin D (to block second-polar-body emission) for the first 2 h following PLC ζ injection. When we injected oocytes with a pipette

Table 1 The pattern of Ca²⁺ oscillations induced by PLCζ in aged human oocytes. The mean interval of Ca²⁺ transients was only scored for oocytes that showed Ca²⁺ oscillations. For concentrations of 10 μg/ml PLCζ cRNA and less the data are taken from oocytes where prolonged recording were made (at least 10 h). The final frequency of Ca²⁺ transients was scored in the last 4 h of a 12 h (*) or 10 h (†) recording. PLCζ cRNA concentration refers to the concentration in the pipette.

Concentration of PLCζ cRNA	Number of oocytes	Mean interval between Ca ²⁺ spikes (min)
1 mg/ml	3	18
100 μg/ml	4	24
10 μg/ml	9	86
		20*
1 μg/ml	5	75
		23†
0.1 μg/ml	5	109

concentration of 10 μg/ml PLCζ cRNA 10 out of 14 of them formed pronuclei, but only two embryos reached the two-cell stage where they then arrested (see Table 2). Previous experiments injecting human PLCζ into mouse oocytes showed that high-frequency Ca²⁺ oscillations lead to cleavage stage arrest (Cox *et al.* 2002), so it was possible that relatively poor development was due to the development of the later high-frequency responses seen in Fig. 1*b*. Accordingly we tested the developmental potential further by injecting oocytes with the lowest concentration that caused low frequency Ca²⁺ oscillations in most oocytes. We injected oocytes with pipette concentrations of 0.1 μg/ml PLCζ cRNA and found that more embryos reached the two-cell stage. Furthermore, some of these embryos developed further and we obtained four parthenogenetic blastocysts from the 24 injected oocytes (Table 2). These data suggest that injecting low concentrations of PLCζ into aged human oocytes can activate development to the blastocyst stage.

Discussion

PLCζ is a sperm-specific isoform of the PLC family of enzymes (Saunders *et al.* 2002). Injection of PLCζ as cRNA, or as a recombinant protein, has been shown to cause Ca²⁺ oscillations and oocyte activation in the mouse (Saunders *et al.* 2002, Kouchi *et al.* 2004). PLCζ protein has also been shown to be present in mammalian sperm at concentrations that are effective at causing Ca²⁺ oscillations in mouse eggs. We have previously identified

Table 2 *In vitro* development of human oocytes following injection of PLCζ cRNA. PLCζ cRNA concentration refers to the concentration in the pipette.

Concentration of PLCζ cRNA	Number of oocytes injected	Number		Number of blastocysts
		at 2-cell stage	at 8-cell stage	
10 μg/ml	14	2	0	0
0.1 μg/ml	24	18	5	4

the human isoform of PLCζ and showed that it causes prolonged Ca²⁺ oscillations and the activation of development in mouse oocytes. The current data are the first to report that human PLCζ is also highly effective at causing Ca²⁺ oscillations in aged human oocytes. PLCζ generates the Ca²⁺-releasing second messenger InsP₃ which has been shown to cause Ca²⁺ release in human oocytes (Goud *et al.* 2002). Our data therefore suggest that PLCζ could be the protein from the sperm that is responsible for stimulating InsP₃ production and Ca²⁺ oscillations and the activation of development in humans.

Our work also represents the first clear demonstration of a method for generating prolonged repetitive Ca²⁺ signals in human oocytes. One study has reported the activation of aged human oocytes by applying three electrical-field pulses rather than one (Zhang *et al.* 1999). However, the fertilized human oocyte clearly displays more than three Ca²⁺ rises and extending this methodology requires rapid washing techniques that are technically demanding (Ozil 1990). Strontium-containing medium has been used to stimulate repetitive Ca²⁺ increases in mouse eggs and might offer another simpler means of stimulating oscillations in human oocytes. However, we failed to observe any Ca²⁺ transients when we incubated human oocytes in 10 mM Sr²⁺ media (N Rodgers & K Swann, unpublished observations), and it remains uncertain whether Sr²⁺ can be used for this purpose. Thimerosal is reported to cause Ca²⁺ oscillation in human oocytes (Homa & Swann 1994), but as a thiol oxidizing agent it also effects the cytoskeleton and may not be compatible with human embryo development (Cheek *et al.* 1993). Our current data with PLCζ, therefore, provide perhaps one of the only relatively simple means of stimulating multiple and long-lasting Ca²⁺ increases in human oocytes.

Previous work on mouse oocytes demonstrated that injecting cRNA encoding the human, mouse or monkey isoforms of PLCζ could initiate Ca²⁺ oscillations and activation of mouse oocytes (Cox *et al.* 2002, Saunders *et al.* 2002). The injected cRNA is converted into PLCζ protein in proportion to the amount of cRNA injected (Saunders *et al.* 2002). The amount of human PLCζ that caused Ca²⁺ oscillations in aged human oocytes in this study is within a similar range to that which causes Ca²⁺ oscillations in mouse oocytes (Cox *et al.* 2002). However, mouse oocytes were induced to undergo Ca²⁺ oscillations by injection of final concentrations of approximately 1 ng/ml human PLCζ cRNA, which is about 10 times lower than the amount that we used to generate Ca²⁺ oscillations in aged human oocytes. This could mean that there are differences in sensitivity to PLCζ between mouse and human oocytes. Nevertheless, it is also likely that aged human oocytes do not translate the cRNA into protein as efficiently as the recently ovulated mouse oocytes. We used the method of injecting cRNA, rather than PLCζ protein, because of the unstable nature of the recombinant protein. Injecting the cRNA also provides a way of introducing PLCζ into an

oocyte without any contamination from other proteins. Nevertheless, injecting cRNA rather than recombinant protein leads to a gradual increase in PLC ζ with time. This probably accounts for the increase in frequency of Ca²⁺ oscillations seen in oocytes injected with intermediate concentrations of PLC ζ .

There appears to be a narrow concentration range of PLC ζ that can be used to activate oocytes. The lowest concentrations of PLC ζ cRNA are not effective in all oocytes, but too high a concentration can lead to high-frequency Ca²⁺ oscillations that appear to be detrimental to development beyond the two-cell stage (Cox *et al.* 2002). The low frequency of Ca²⁺ oscillations that was consistent with reasonable rates of implantation development is similar to the low frequency of oscillations that were first reported using aequorin to measure Ca²⁺ levels during human fertilization (Taylor *et al.* 1993). Such low-frequency Ca²⁺ oscillations during fertilization are also seen in the mouse when aequorin is used to measure Ca²⁺ (Cuthbertson & Cobbold 1985). In parallel experiments we found that a slightly greater proportion of oocytes activated (75%) than showed Ca²⁺ oscillations (approximately 50%) when 0.1 μ g/ml PLC ζ cRNA was injected. This could reflect the fact that Oregon Green BAPTA dextran buffers the Ca²⁺ levels to some extent, and so a higher proportion of oocytes may actually undergo oscillations when Ca²⁺ is not measured. In future it would be useful to measure Ca²⁺ with less-invasive probes so that the same oocytes can be observed for Ca²⁺ oscillations and development.

Whereas the overall numbers are small, our data are noteworthy in that development to the blastocyst stage was seen after artificial activation of aged human oocytes. Previous studies have reported the same parthenogenetic development to the blastocyst stage with freshly ovulated human oocytes (Cibelli *et al.* 2001, Lin *et al.* 2003). However, when using aged human oocytes the Ca²⁺-ionophore-based protocols have stimulated early cleavage stage development, but blastocyst formation has not been reported (Winston *et al.* 1991, Balakier & Casper 1993, Nakagawa *et al.* 2001). It is possible that we obtained blastocysts in some cases because oocytes were stimulated with a repetitive Ca²⁺ signal. However, more-extensive studies are required to make direct developmental comparisons between oocytes activated by PLC ζ compared with those stimulated by ionophore. Whatever the case our data do suggest that PLC ζ could be used to generate human parthenogenetic embryos and this in itself has clinical implications. First, there are clearly some cases where failed fertilization after ICSI is due to failed oocyte activation (Rawe *et al.* 2000). In some cases embryo development and live births have been achieved after sperm injection by providing an activation stimulus in the form of Ca²⁺ ionophore (Eldar-Geva *et al.* 2003, Murase *et al.* 2004). PLC ζ offers an alternative means by which failed activation may be restored with a more physiological stimulus than ionophore. Secondly, the

generation of parthenogenetic blastocysts from oocytes can provide a source of embryos for the creation of stem cells (Lin *et al.* 2003, Vrana *et al.* 2003). The use of such parthenogenetic embryos may be more ethically acceptable than using embryos from fertilized zygotes.

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