Relocalization of STIM1 in mouse oocytes at fertilization: early involvement of store-operated calcium entry

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Abstract

Calcium waves represent one of the most important intracellular signaling events in oocytes at fertilization required for the exit from metaphase arrest and the resumption of the cell cycle. The molecular mechanism ruling this signaling has been described in terms of the contribution of intracellular calcium stores to calcium spikes. In this work, we considered the possible contribution of store-operated calcium entry (SOCE) to this signaling, by studying the localization of the protein STIM1 in oocytes. STIM1 has been suggested to play a key role in the recruitment and activation of plasma membrane calcium channels, and we show here that mature mouse oocytes express this protein distributed in discrete clusters throughout their periphery in resting cells, colocalizing with the endoplasmic reticulum marker calreticulin. However, immunolocalization of the endogenous STIM1 showed considerable redistribution over larger areas or patches covering the entire periphery of the oocyte during Ca²⁺ store depletion induced with thapsigargin or ionomycin. Furthermore, pharmacological activation of endogenous phospholipase C induced a similar pattern of redistribution of STIM1 in the oocyte. Finally, fertilization of mouse oocytes revealed a significant and rapid relocalization of STIM1, similar to that found after pharmacological Ca²⁺ store depletion. This particular relocalization supports a role for STIM1 and SOCE in the calcium signaling during early stages of fertilization.

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Introduction

At fertilization, transient and repetitive increases of the cytosolic Ca²⁺ concentration ([Ca²⁺]ᵢ) drive the exit from metaphase arrest of oocytes in meiosis II and trigger cortical granule exocytosis (Cuthbertson & Cobbold 1985, Cran et al. 1988). It is widely accepted that this calcium signaling is initiated by the activity of the sperm-specific phospholipase Cζ (PLCζ; Saunders et al. 2002), with the production of inositol 1,4,5-trisphosphate (InsP₃) that binds to its receptor (InsP₃R) at the endoplasmic reticulum (ER), leading to the release of Ca²⁺ from this intracellular store (reviewed in Malcuit et al. (2006)). Restoration of calcium concentration to basal levels is achieved by the activation of calcium pumps, i.e. plasma membrane (PM) Ca²⁺-ATPase that mediates Ca²⁺ extrusion, as well as the sarco(endo)-plasmic reticulum Ca²⁺-ATPase (SERCA) that catalyzes Ca²⁺ uptake into ER.

In other cell types, it has been demonstrated that long-lasting signaling based on repetitive calcium waves is accompanied by a Ca²⁺ influx pathway to refill calcium intracellular stores in order to ensure the durability of the signaling. This pathway is established by the store-operated calcium (SOC) channels located in the PM, and it is one of the most important pathways for Ca²⁺ entry in non-excitable cells (Putney 1986, 2007). This SOC entry (SOCE) is a Ca²⁺ influx pathway tightly regulated by the status of the [Ca²⁺] within the ER (Lewis 2007, Putney 2007).

In oocytes, it is known that a continuous Ca²⁺ influx is required to preserve Ca²⁺ spiking (Igusa & Miyazaki 1983), which otherwise ceases in Ca²⁺-free extracellular medium (Kline & Kline 1992). However, the pathway for this Ca²⁺ entry in mammalian oocytes at fertilization remains unknown. SOCE could be one of the mechanisms regulating Ca²⁺ entry at fertilization, since this pathway has been observed in Xenopus oocytes (Machaca & Haun 2000), porcine oocytes (Machaty et al. 2002), mouse oocytes (McGuinness et al. 1996, Halet et al. 2004), and recently in human oocytes (Martin-Romero et al. 2008) in resting...
conditions. Despite the importance of this mechanism in Ca\textsuperscript{2+} signaling, the actual function of SOCE at fertilization has yet to be addressed. Most of the problems that researchers have faced since SOCE was described for the first time (Putney 1986), were due to the lack of knowledge of the molecular nature of the Ca\textsuperscript{2+} channels and of the molecular basis of the retrograde signaling that links Ca\textsuperscript{2+} levels within the ER to the Ca\textsuperscript{2+} influx at the PM. However, in 2005 two independent groups reported that the protein stromal interaction molecule 1 (STIM1) is an essential component of the molecular machinery governing SOCE (Liou et al. 2005, Roos et al. 2005). STIM1 is a single transmembrane protein with an EF-hand domain close to the N-terminus (Williams et al. 2002). This EF-hand domain has been predicted to be located in the luminal space of the ER and to function as the Ca\textsuperscript{2+} domain has been predicted to be located in the luminal space of the ER and to function as the Ca\textsuperscript{2+} sensor in the signaling pathway connecting Ca\textsuperscript{2+} store depletion to Ca\textsuperscript{2+} influx (Liou et al. 2005, Zhang et al. 2005). Consequently, knockdown of STIM1 expression reduced SOCE in a variety of cell lines, including HEK293, SH-SY5Y, Jurkat T, and HeLa cells (Liou et al. 2005, Roos et al. 2005), and overexpression of this protein enhanced SOCE in HEK293 cells (Roos et al. 2005).

The localization of STIM1 during store depletion is currently a point of debate. Some earlier reports proved that a considerable fraction of STIM1 is present in the PM (Manji et al. 2000, Williams et al. 2002, Zhang et al. 2005, Spassova et al. 2006), and an insertional model of action, i.e. the translocation of STIM1 from ER to PM in response to store depletion, has been suggested for the function of STIM1 in SOC activation (Zhang et al. 2005). In this sense, additional roles have been attributed to STIM1, including the direct control of the Ca\textsuperscript{2+} entry channel by a pool of STIM1 located in the PM rather than in the ER (Spassova et al. 2006). However, further reports suggested that STIM1 protein does not translocate to the PM, and that STIM1 in the ER approaches and interacts with the components in the PM upon depletion of calcium stores (Wu et al. 2006). Recently, there has been growing evidence associating STIM1 with different Ca\textsuperscript{2+} channels, including the family of transient receptor potential canonical (TRPC) channels (Worley et al. 2007, Yuan et al. 2007) and ORAI1 (Xu et al. 2006, Li et al. 2007).

Most studies targeted at unraveling the molecular mechanisms underlying SOCE have used transfected cell lines (HEK293, SH-SY5Y, Jurkat T, HeLa, Drosophila S2, HSG cells, etc.) and the results obtained with the overexpression of these proteins are often assumed to be extrapolatable to all types of cells. Really, however, the endogenous level of these proteins in cells is still unclear, and needs to be addressed to validate or exclude the recent reported conclusions. There is no information regarding endogenous STIM1 levels in oocytes, and there is a major gap in knowledge of the molecular components of SOCE in oocytes, and of the contribution of this Ca\textsuperscript{2+} influx pathway in the cell signaling at fertilization. The aim of the present work was to study STIM1 expression and localization in mammalian oocytes, using mouse mature oocytes, in order to define the role of SOCE at fertilization.

**Results**

**SOCE in mouse oocytes**

First, we examined the occurrence of SOCE in mouse metaphase II (MII) oocytes as we reported recently for human oocytes (Martin-Romero et al. 2008). SOCE was activated in mouse MII oocytes with the addition of thapsigargin (TG), a potent and specific inhibitor of the SERCA (Thastrup et al. 1990). Figure 1 (panel A) shows a transient F340/F380 peak after addition of TG, which proves the increase of cytosolic free calcium concentration ([Ca\textsuperscript{2+}]) due to the Ca\textsuperscript{2+} leak from intracellular Ca\textsuperscript{2+} stores (mainly ER). The subsequent decrease of [Ca\textsuperscript{2+}], due to Ca\textsuperscript{2+} extrusion leads to the emptying of ER, thus activating Ca\textsuperscript{2+} entry through SOC channels that remain unidentified in mammalian oocytes. This opening of SOC channels can be followed by employing calcium or barium addition to the extracellular medium. Barium (Ba\textsuperscript{2+}) has been shown to enter the cell via SOC channels in other cell types (Hoth 1995, Fischer et al. 1998), as well as in oocytes (Martin-Romero et al. 2008), but cannot be extruded by PM calcium pumps. Since fura-2 is also sensitive to Ba\textsuperscript{2+}, this offers a useful tool with which to reveal the occurrence of SOCE.

In addition, we show here that the quenching of fura-2 fluorescence produced by the increase in Mn\textsuperscript{2+} permeability after incubation of oocytes with TG (Fig. 1, panel B). Intracellular Ca\textsuperscript{2+} store depletion induced by TG leads to the opening of Ca\textsuperscript{2+} channels at the PM, which is controlled by the filling state of these Ca\textsuperscript{2+} stores. Since Mn\textsuperscript{2+} is transported through SOC channels, this technique is a very convenient method to describe the existence of SOCE, as we have reported previously (Gutierrez-Martin et al. 2005, Martin-Romero et al. 2008).

**Expression and localization of STIM1 in mouse oocytes**

On the basis of using various cell lines (HeLa, HEK293, HSG, Jurkat, Drosophila S2, etc.) it has recently been suggested that the protein STIM1 mediates the molecular mechanism ruling SOCE (Liou et al. 2005, Roos et al. 2005, Zhang et al. 2005, Soboloff et al. 2006). However, the presence of this protein in other cells, including gametes, and its participation in calcium signaling at endogenous levels are still under study. Here, we show that STIM1 is expressed in mouse oocytes (Fig. 2), this expression being strongly dependent on the maturation stage. Our results were essentially the same when two different commercial antibodies were used to detect STIM1 by immunoblot, i.e. an antibody raised against
the amino terminus (from BD Biosciences, Franklin Lakes, NJ, USA) or an antibody raised against the carboxy terminus (from ProSci Inc., Poway, CA, USA). Both antibodies revealed a single band with an apparent molecular weight similar to the predicted size for this protein (77 kDa; the results shown in Fig. 2 were obtained with the antibody against the carboxy terminus domain). However, fully mature oocytes (MII oocytes) showed three to four times higher expression than germinal vesicles (GV). The expression level in immature oocytes (MI), i.e. oocytes obtained after in vitro culture of GV for a minimum of 8 h, was similar to that found in MII oocytes, indicating that the upregulation of the expression of STIM1 could be a process concomitant with germinal vesicle breakdown (GVBD) or shortly after this stage. Whether this marked difference of expression is due to transcriptional or translational regulation will require further investigation.

In resting MII oocytes, STIM1 showed a cortical distribution (Fig. 3). In the cortex of the oocyte, STIM1 colocalized with the ER marker calreticulin, a particular distribution that fits well with the predicted localization of the protein in the membrane of the ER, with the N-terminus in the luminal space of this compartment (Liou et al. 2005, Roos et al. 2005). Interestingly, STIM1 distribution in the ER is not scattered, but localized in restricted patches or clusters in unstimulated oocytes, in both human tubal fluid (HTF) medium and Hank's
balanced salt solution (HBSS). In addition, oocytes fixed directly as cumulus–oocytes complexes (COC) retrieved from the oviduct and denuded after fixation showed the same distribution, indicating that the handling of the oocytes during recovery did not affect the localization of STIM1 in resting oocytes.

We studied the specificity of the anti-STIM1 antibody by blocking the immunofluorescence signal with the specific peptide that the antibody was raised against. Figure 4 shows that the pre-incubation of the antibody with the blocking peptide notably decreased the immunofluorescence signal in our experimental conditions. Indeed, the remaining and low background was not specific, i.e. did not show the specific distribution observed in the experiments in the absence of the blocking peptide.

Concomitant with the emptying of intracellular Ca\(^{2+}\) stores induced by TG (see the time course of Ca\(^{2+}\) store depletion in Fig. 1), we observed a significant relocalization of STIM1 in larger areas close to the surface of the oocyte. This relocalization was clearly detectable under maximal projection of 1 μm sections with confocal microscopy (see Fig. 5). Relocalization of STIM1 in larger areas expanded over the entire periphery of the oocyte and was also detectable after addition of the Ca\(^{2+}\) ionophore ionomycin, in EGTA-buffered medium, supporting the finding that STIM1 can act as a Ca\(^{2+}\) sensor within the ER, and that this mechanism of relocalization is fully active in mouse mature oocytes.

**Activation of PLC relocalizes STIM1 in oocytes**

Ca\(^{2+}\) signaling in fertilized oocytes is triggered by sperm-specific PLCZ (Saunders *et al.* 2002) that releases InsP\(_3\) and 1,2-diacylglycerol via the hydrolysis of phosphatidylinositol 4,5-bisphosphate. InsP\(_3\) is assumed to activate a downstream pathway through the binding to its receptor in the ER, leading to the initial Ca\(^{2+}\) release from this intracellular store. Thus, PLC-mediated signaling could have a direct influence on Ca\(^{2+}\) store filling state in oocytes at fertilization. This hypothesis was studied following the effect of the activation of endogenous PLC activity by m-3M3FBS on the [Ca\(^{2+}\)]\(_i\) in oocytes. At concentrations similar to those previously used for other cell types to activate PLC (Bae *et al.* 2003, Jung *et al.* 2008), m-3M3FBS induced a transient increase of the [Ca\(^{2+}\)]\(_i\) in both Ca\(^{2+}\)-containing and Ca\(^{2+}\)-free media (Fig. 6), with the same kinetics in both conditions. This result suggests that m-3M3FBS triggered Ca\(^{2+}\) release from ER, as expected. Indeed, m-3M3FBS
induced a partial emptying of intracellular stores in oocytes and therefore the activation of SOCE, as revealed by the increase in Ba\(^{2+}\) influx when this cation was added to the medium (data not shown). The increase of the [Ca\(^{2+}\)], induced by m-3M3FBS was prevented by U73122, a well-characterized inhibitor of PLC (Smallridge et al. 1992, Muto et al. 1997) further confirming the involvement of PLC activation by m-3M3FBS.

Thereafter, we proceeded to study the distribution of STIM1 in unfertilized oocytes in the same conditions, i.e. during the treatment with m-3M3FBS in Ca\(^{2+}\)-containing medium (HBSS). Pharmacological activation of PLC induced a marked modification in the localization of STIM1 in mouse oocytes, following a similar pattern to that found for TG or ionomycin (Fig. 6, panel B). This result strongly suggests a role for SOCE in oocytes when the phosphoinositide pathway is activated, and for this reason we proceeded to study its involvement at fertilization.

**Relocalization of STIM1 at fertilization**

To investigate further the contribution of this Ca\(^{2+}\) influx pathway at fertilization, we performed a time-course detection of STIM1 during the initial phase of IVF of mouse oocytes. Fertilization performed with COC that were incubated with capacitated sperm, also induced the relocalization of STIM1 in a TG/ionomycin-induced like profile (Fig. 7, panel A), supporting a role for STIM1 in Ca\(^{2+}\)-signaling at fertilization. The relocalization was observed in a time-window (2–6 h after addition of sperm), which is compatible with a role for STIM1, and therefore for SOCE, in the preservation of the long-term calcium waves at fertilization. Relocalization of STIM1 was found in 45–50% of the oocytes after 1 h, and in more than 90% of oocytes after 2–6 h. Shorter times were also studied (<30 min) but did not reveal any significant alteration in STIM1 localization. No difference was found in the sperm-triggered relocalization of STIM1 compared with the TG/ionomycin-induced relocalization (shown above).

Since the presence of follicular cells could be masking an earlier participation of STIM1 in this signaling, we proceeded to perform the immunolocalization of this protein during fertilization of zona pellucida (ZP)-free oocytes to investigate further the time-course of STIM1 relocalization induced by sperm. Panel B in Fig. 7, shows that STIM1 relocated in oocytes during early fertilization, since it was found 15 min after sperm addition in ~70–75% of oocytes and in more than 90% after 30–60 min. This time course of STIM1 relocalization develops in parallel to the beginning of calcium spikes induced by fertilization, since the initial Ca\(^{2+}\) spike was detectable in fura-2 loaded oocytes 7–10 min after addition of sperm (Fig. 7, panel C).

These data represent the first evidence of a role for STIM1, and consequently for the SOCE, in the early Ca\(^{2+}\) signaling at fertilization of mammalian oocytes, and increase the number of potential elements that may contribute to this signaling pathway together with the

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**Figure 5** Store depletion induced by TG or ionomycin triggers STIM1 relocalization in mature MII oocytes. Oocytes were incubated in HBSS (panel A) or EGTA-buffered Ca\(^{2+}\)-free HBSS (panels B and C) for 15 min at 37 °C, in the presence of 5 μM TG (panel B) or 10 μM ionomycin (panel C). Fixed oocytes were incubated with anti-STIM1 antibody and with anti-IgG conjugated with Alexa-Fluor 488 as described for Fig. 3. Images correspond to maximal projection of 1 μm sections obtained by confocal microscopy. The number of oocytes used for each experimental condition was at least 40 per condition, from five independent experiments.
recent members that have been shown to be involved in this Ca\textsuperscript{2+} influx pathway, e.g. CRACM1 (also called ORAI1) and members of the TRPC family.

**Discussion**

STIM1 has been suggested to be the protein that acts as the sensor of Ca\textsuperscript{2+} in the ER and is recruited to ER–PM junctions after partial store depletion. At these ER–PM junctions, STIM1 binds and activates Ca\textsuperscript{2+} channels, such as ORAI1, described as an essential subunit of the highly sensitive Ca\textsuperscript{2+} release-activated Ca\textsuperscript{2+} channel (Zhang *et al.* 2005, Feske *et al.* 2006, Vig *et al.* 2006). More recently, a ternary complex of TRPC1–STIM1–ORAI1 was found to be essential for the activation of Ca\textsuperscript{2+} influx in response to Ca\textsuperscript{2+} store depletion in human salivary gland cells (Ong *et al.* 2007). In addition, an excellent set of recent reports has described how the activation of STIM1 can be used to differentiate SOCE, which is STIM1-dependent, from another closely related Ca\textsuperscript{2+} influx, the receptor-operated calcium entry, that shows no dependence on the activation of STIM1 (Ambudkar *et al.* 2007, Ong *et al.* 2007, Pani *et al.* 2008). Therefore, STIM1 can be used as a selective marker of SOCE activation, and we have here taken advantage of this fact to show the involvement of this Ca\textsuperscript{2+} entry pathway during fertilization of mouse oocytes, to help in the better understanding of Ca\textsuperscript{2+} signaling in oocytes.

First, we show that endogenous STIM1 is expressed in oocytes, and that this expression is remarkably dependent on the maturation stage, being upregulated after GVBD. This developmentally upregulated profile of protein expression is similar to that found for other Ca\textsuperscript{2+} transport systems that have been shown to be involved in the generation of calcium waves at fertilization in oocytes, such as InsP\textsubscript{3}R and RyR (He *et al.* 1997, Parrington *et al.* 1998, Wang *et al.* 2005). Therefore, the SOCE, a STIM1-dependent pathway, could be considered to be a probable contributor in Ca\textsuperscript{2+} signaling in oocytes. If this is the case, STIM1 would act to regulate the pathway for the required Ca\textsuperscript{2+} entry at fertilization. On the contrary, other PM Ca\textsuperscript{2+} transport systems, such as PM L-type Ca\textsuperscript{2+} currents, decrease during maturation in bovine oocytes (Tosti *et al.* 2002), weakening the possibilities of a direct participation of L-type voltage-operated Ca\textsuperscript{2+} channels (VOCC) in calcium influx at fertilization. However, VOCC were found to have a potential role during the maturation process, since the exposure to verapamil affects the in vitro maturation efficiency (Tosti *et al.* 2002).

Because STIM1 has been described to be an ER-resident protein in other cell types, we studied the distribution profile of calreticulin as marker of the distribution of ER. Calreticulin showed that ER clusters accumulate underneath the PM in MII oocytes, as has been described with other markers (FitzHarris *et al.* 2007). STIM1 remains localized in small and discrete areas or clusters throughout the cortex in resting oocytes, with a high level of colocalization with calreticulin, indicating that in MII oocytes STIM1 is located in the ER. Also, it is shown here that simultaneously with Ca\textsuperscript{2+} store depletion, STIM1 is distributed over larger areas covering the entire periphery of the oocyte (i.e. near the PM) without any preferential point of accumulation. The fact that MII oocytes respond to variations in the filling state of Ca\textsuperscript{2+} stores with the concomitant relocation of STIM1 and the opening of SOC channels leads one to

**Figure 6** Relocalization of STIM1 induced by the PLC activator m-3M3FBS. (Panel A) Ca\textsuperscript{2+}-imaging was performed in fura-2 loaded oocytes in Ca\textsuperscript{2+}-containing medium (HBSS, black line) or in Ca\textsuperscript{2+}-free medium (EGTA-buffered Ca\textsuperscript{2+}-free HBSS, red line) in the presence of 10 μM m-3M3FBS. In a parallel series of experiments, oocytes were pre-incubated with 2 μM U73122 for 10 min in HBSS, and then F\textsubscript{140}/F\textsubscript{380} was monitored in the presence of 10 μM m-3M3FBS in HBSS (green line). (Panel B) Oocytes in Ca\textsuperscript{2+}-containing medium were incubated with 10 μM m-3M3FBS for 15 min at 37 °C and then fixed, permeabilized and incubated with anti-STIM1 antibody. Other experimental procedures were as in Fig. 3. Images are representative of results obtained with 48 oocytes from three different preparations.
conclude that this significant change in STIM1 distribution is a marker of the activation of SOCE in oocytes. It is remarkable that the extent of STIM1 aggregation exceeds that found in HEK293 cells stably transfected with STIM1-GFP (F J Martin-Romero & D R Alessi, unpublished observations; Supplementary Figure 3, which can be viewed online at www.reproduction-online.org/supplemental/). This raises a new question regarding the specific molecular basis of these large domains of STIM1 accumulation at ER–PM junctions in oocytes during TG/ionomycin-induced Ca\(^{2+}\) store depletion.

In this regard, the association of STIM1 with TRPC1 favors the insertion of TRPC1 into lipid rafts, where it acts as a SOC channel, in contrast with the STIM1-independent activity of TRPC1 as a receptor-operated calcium channel that shows a regular distribution in the PM (Alicia et al. 2008, Pani et al. 2008). Whether this particular localization of STIM1 in oocytes under ER depletion stimuli is due to the segregation of STIM1 to PM domains enriched in lipid rafts is beyond the scope of this work, although this plausible hypothesis opens up a new window of studies on Ca\(^{2+}\) signaling at fertilization. However, the extent of the areas enriched in STIM1 during ER depletion in oocytes requires additional explanation. In HEK293, Jurkat and RBL mast cells, there has been demonstrated to be an absolute requirement of multimerization or clustering of STIM1 for the activation of SOC channels (Wu et al. 2006, Calloway et al. 2009, Park et al. 2009). The intraluminal EF-hand domain of STIM1 senses [Ca\(^{2+}\)]\(_{\text{ER}}\) within ER, and a decrease of this value triggers STIM1 multimerization via EF-SAM (sterile α motif) domains (Stathopulos et al. 2006, Liou et al. 2007). Nevertheless, our immunolocalization experiments cannot monitor the oligomerization of STIM1 described in other cells, because in resting MII oocytes STIM1 already shows a high level of aggregation due to the specific distribution of the ER in fully mature oocytes.

Regarding SOCE and oocyte maturation, it is important to bear in mind that SOCE has been described as being inactivated at the GVBD in Xenopus oocytes (Machaca & Haun 2000). This inactivation occurs in parallel with the increase in the levels of MAP kinase and...
maturation-promoting factor, and uncouples SOC channels activation in the PM from store depletion in frog oocytes (Machaca & Haun 2002). However, there cannot really be any surprise in the discrepancy between the reported SOC inactivation in Xenopus eggs and the present data, since mouse and frog oocytes activate in a different manner, and Xenopus eggs produce a single calcium wave that lasts for several minutes, in contrast with the repetitive Ca\(^{2+}\) spiking in mammals (Stricker 1999). This divergence in Ca\(^{2+}\) signaling at fertilization could be based on a different molecular choreography in the oocytes of these species. Our data indicate that SOCE is active in mouse mature oocytes, being sensitive to the store depletion induced by TG or ionomycin, in contrast to the case found in frog eggs.

The upregulation of STIM1 expression during the maturation of oocytes, monitored with two different commercial antibodies, together with the fact that STIM1 responds to Ca\(^{2+}\) store depletion, raises the question of the participation of this mechanism in Ca\(^{2+}\) signaling at fertilization. The localization of STIM1 in oocytes was used as a probe to test this hypothesis. In this regard, the pharmacological activation of endogenous PLC in oocytes yielded similar results to those obtained after treatment with TG or ionomycin in a Ca\(^{2+}\)-free medium, i.e. activation of PLC triggers relocalization of STIM1 in larger domains near the PM. Similarly, STIM1 relocalizes in HEK293 cells transfected with STIM1-GFP when they are treated with the same pharmacological activator of PLC in Ca\(^{2+}\)-containing medium (F J Martin-Romero & D R Alesi, unpublished observations; see Supplementary Figure 3). Therefore, it is concluded that the activation of the phosphoinositide pathway develops in parallel with the activation of SOCE, supporting the involvement of this Ca\(^{2+}\)-influx pathway at fertilization.

Even more noteworthy were the results obtained during IVF of oocytes. These experiments demonstrated a marked relocalization of STIM1, showing a pattern of aggregation similar to that found during TG/ionomycin treatment. These results were obtained with denuded oocytes as well as with COC. Additionally, shorter times of exposure of ZP-free oocytes to sperm demonstrated that STIM1 relocalization is a very early event in fertilization. This particular pattern of TG induced-like localization of STIM1 in fertilized oocytes was found 15 min after addition of sperm. It is interesting that at short times of exposure to sperm, the number of areas for STIM1 distribution in the cortex of the oocyte was much lower than that observed for longer times after fertilization (>2 h). This could be explained in terms of a more restricted stimulus initiated by the sperm during the initial stages of fertilization. On the other hand, at longer times, the Ca\(^{2+}\)-spiking could be inducing partial emptying in the whole ER all over the periphery of the oocyte. This overall stimulus, similar to that induced by the different drugs used throughout this study, could lead to a relocalization of STIM1 in a more dispersed manner compared with what is found at shorter times after fertilization.

Taking into consideration that STIM1 is a selective marker for SOCE activation, our findings strongly suggest a role for the Ca\(^{2+}\) influx mediated by SOC channels at the early phase of fertilization. Indeed, this initial relocalization of STIM1 in oocytes matches well with the first Ca\(^{2+}\) spike found at fertilization in mouse ZP-free oocytes (i.e. 7–15 min after the exposure to sperm) suggesting that SOCE could be involved not only in the support of the long-lasting Ca\(^{2+}\) waves, but also in the generation of the earliest Ca\(^{2+}\) spikes.

Our results offer new explanations for a long unsolved question, i.e. the pathway for the Ca\(^{2+}\) entry required to preserve Ca\(^{2+}\) spiking at fertilization. The relocalization of STIM1 suggests a direct participation of SOC in the Ca\(^{2+}\) influx in mammalian oocytes early during fertilization. Although we still do not have information regarding the molecular nature of the SOC channels involved in this signaling, unpublished observations from our laboratory indicate the presence of two Ca\(^{2+}\) channels in mouse oocytes, ORAI1 and TRPC1, which have been shown to act as SOC channels in transfected cell lines (see Introduction). Nevertheless, the consequences of this expression in oocytes are still under study.

In summary, the results presented in this work indicate that SOCE is a very early event in the signaling during fertilization of the mammalian oocyte, and therefore could be directly involved in the origin of the Ca\(^{2+}\) spiking after sperm fusion, as well as in the long-term maintenance of the calcium waves thereafter. Most importantly, these results and the recent reports showing the interaction of STIM1 with SOC channels, offer an exceptional opportunity to unravel the molecular pathway for Ca\(^{2+}\) entry early at fertilization.

Materials and Methods

Animals

B6D2F1 hybrid mice were purchased from Harlan Sprague–Dawley (Indianapolis, IN, USA) and housed in the Animalarium of the University of Extremadura under a regular 12 h light:12 h darkness cycle. Approval of the study was granted by the University of Extremadura, Spain.

Chemicals

Human chorionic gonadotropin, equine chorionic gonadotropin, hyaluronidase, collagenase, Tyrode’s solution and U73122 (1-[6-[[1(7B)-3-methoxyestra-1,3,5(10)-trien-17-ylaminoo]hexyl]-1H-pyrole-2,5-dione) were from Sigma Chemical Co. HTF medium from Millipore (Billerica, MA, USA). Fura-2-AM, TG, ionomycin and m-3M3FBS (2,4,6-trimethyl-N-(m-3-trifluoromethylphenyl)benzenesulfonylamine) were from Calbiochem (brand of Merck Chemicals Ltd). Anti-rabbit IgG antibody labeled with Alexa-Fluor 488, anti-sheep IgG antibody labeled with Alexa-Fluor 594, and

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Oocyte collection and IVF

Oocytes arrested in MII were collected from 8–12 weeks old B6D2F1 mice. Mice were stimulated with i.p. injection of 7.5 IU equine chorionic gonadotropin and 49 h later with 10 IU human chorionic gonadotropin. After 14 h, COC were collected from the ampulla of the oviduct and denuded mechanically in HTF medium supplemented with hyaluronidase (150 U/ml). Oocytes with a clear extruded polar body were collected for the experiments as MII oocytes.

Oocytes arrested in prophase I, or GV, were collected 46 h after i.p. injection of 7.5 IU equine chorionic gonadotropin. Ovaries from these mice were treated with hyaluronidase (150 U/ml) and collagenase (0.5 mg/ml) in HTF medium. GV were directly collected from the plate and treated with an additional step with hyaluronidase before performing the experiments. MI oocytes were obtained after in vitro culture of GV in HTF medium for 8–10 h in a 95% air/5% CO2 controlled atmosphere at 37°C.

In vitro fertilization was performed by adding 5x10^7/ml capacitated spermatozoa to 8–10 COC in a final volume of 0.5 ml HTF medium, and kept in a 95% air/5% CO2 controlled atmosphere at 37°C. At the indicated times, oocytes were fixed in freshly made 4% paraformaldehyde for 10 min at room temperature, washed thoroughly in PBS-PVP (PBS supplemented with 0.1% polyvinylpyrrolidone) and processed immediately for the immunolocalization experiments.

When ZP-free oocytes were used for IVF, oocytes were treated with acidified Tyrode’s solution to remove the ZP. ZP-free oocytes were incubated with 5x10^7/ml capacitated spermatozoa in HTF medium for the indicated times and processed immediately for the immunolocalization experiments as described for intact oocytes.

[Ca^{2+}]_i measurement and activation of SOCE

Intracellular free calcium concentration ([Ca^{2+}]_i) was measured basically as indicated previously (Martin-Romero et al. 2008). Briefly, ZP-intact MII oocytes were incubated with 2 μM fura-2-acetoxymethyl ester (fura-2-AM) and 0.025% Pluronic-F127 in HTF medium for 45 min, and rinsed thoroughly with HBSS containing Ca^{2+} and Mg^{2+} BBSS. Then, the [Ca^{2+}]_i was measured with an inverted microscope equipped with micro-incubation platform DH-40i (Warner Instruments, Inc., Hamden, CT, USA). All measurements were performed at 37°C (heater controller TC-324B from Warner Instruments, Inc.). Ratio fluorescence images were obtained with excitation filters of 340 and 380 nm, a 510 nm dichroic mirror and a 520 nm emission filter (Semrock, Rochester, NY, USA). Digital images were taken with a Hamamatsu C9100-02 electron multiplier charge-coupled device (CCD) camera. [Ca^{2+}]_i was calculated according to the equation [Ca^{2+}]_i = (K_q (R - R_{min})/(R_{max} - R)) + R_{min}, where R is the measured fluorescence ratio (F340/F380), and R_{max} and R_{min} are the ratio values for Ca^{2+}-bound and Ca^{2+}-free dye in fura-2 loaded oocytes.

Western blot

Total oocyte lysates were prepared by collecting 60–80 oocytes per sample. Oocytes were washed thoroughly in HBSS, and frozen in 5 μl HBSS at −80°C until use. Thawed samples were diluted with NuPAGE LDS-sample buffer, and NuPAGE sample reducing agent was added before heating at 90–95°C for 10 min. Samples were loaded onto NuPAGE 4–12% acrylamide Bis-Tris gels, and electrophoresed proteins were transferred to nitrocellulose membranes by electroblotting using NuPAGE transfer buffer. Membranes were blocked for 1 h at room temperature in TBS-T (Tris-buffered saline buffer, pH 7.5, 0.2% Tween-20) containing 10% (w/v) non-fat milk. Membranes were incubated overnight at 4°C with the primary anti-STIM1 antibody. Two antibodies were used: 1) mouse anti-STIM1 antibody from BD Biosciences, diluted 1:250 in TBS-T (0.05% Tween-20) with 1% non-fat milk, and 2) rabbit anti-STIM1 antibody from Prosci, diluted 1:1000 in TBS-T with 5% BSA. After washing in TBS-T, anti-mouse or rabbit IgG HRP-conjugated secondary antibody was added at 1:2500 dilution and incubation continued for 1 h at room temperature. Finally, luminol substrate was added and membranes were exposed to Kodak BioMax Light Films for 5–10 min. Developed films were scanned and the signal was quantified by volumetric integration using the Quantity One software package (Bio-Rad). As a control to test the specificity of the primary antibody, a membrane containing a single lane with 60 MII oocytes was incubated with a non-specific rabbit IgG. After incubation with the secondary antibody and subsequent developing step, the membrane was stripped briefly with Restore Western Blot Stripping buffer (from Pierce), washed thoroughly, blocked again, and incubated with the rabbit polyclonal anti-STIM1 antibody under the same conditions as used for the non-specific IgG. The absolute
absence of chemiluminescence signal when the non-specific IgG was used (data not shown) ensures the specificity of the anti-STIM1 antibody in the experimental conditions described in this study (see Supplementary Figure 1, which can be viewed online at www.reproduction-online.org-supplemental/).

In parallel, loading controls were performed for the determination of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) protein levels using an anti-GAPDH antibody at 1:3000 dilution in TBS-T with 2% (w/v) non-fat milk for 1 h at room temperature, followed by incubation with anti-mouse IgG HRP-conjugated antibody for 1 h at room temperature. Determination of GAPDH levels was used as loading control since the expression of this protein remains constant between GV and MII stages in mouse oocytes (see Supplementary Figure 2, which can be viewed online at www.reproduction-online.org-supplemental/).

**Immunofluorescence staining**

Oocytes were incubated in the experimental conditions described in the Results section and fixed in freshly made 4% paraformaldehyde for 10 min at room temperature. They were then treated with Tyrode’s acidified solution to remove the ZP, permeabilized with 0.1% saponin for 10 min and incubated in blocking solution (0.1 M glycine in PBS, pH 7.4, supplemented with 5% BSA) for 1 h at room temperature. After washing in PBS +5% BSA, oocytes were incubated overnight with the anti-STIM1 antibody (ProSci Inc.) diluted 1:50 in blocking solution at 4°C. Thereafter, they were incubated for 20 min with anti-rabbit IgG antibody labeled with Alexa-Fluor 488 diluted 1:1000. The specificity of the antibody in our immunolocalization experiments was tested by incubating the antibody for 1 h at 37°C with the blocking peptide (ProSci Inc.) that the antibody was raised against, in 1:1 (v/v) ratio. Thereafter, oocytes were incubated with pre-blocked anti-STIM1 antibody at 1:50 dilution, as described above.

Immunolocalization of the ER-resident protein calreticulin was used as marker of intracellular distribution of the ER (Payne & Schatten 2003). Oocytes stained for endogenous STIM1 were incubated overnight with the anti-calreticulin antibody diluted 1:50 in blocking solution at 4°C. Thereafter, they were incubated for 20 min with anti-sheep IgG antibody labeled with Alexa-Fluor 594 diluted 1:1000. After immunostaining, samples were visualized by confocal microscopy with a Leica TCS SP5 system using a 40× oil immersion objective.

The treatment of oocytes with TG or ionomycin prior to immunostaining was performed in EGTA-buffered Ca2+-free HBSS, with the composition described above. Immunolocalization of STIM1 during treatment with the PLC activator m-3M3FBS was performed in Ca2+-containing HBSS buffer. TG, ionomycin, m-3M3FBS, and U73122 were dissolved in DMSO as concentrated solutions, and the percentage of this solvent was always lower than 0.1% in the assay medium.

**Declaration of interest**

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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