Phospholipase Cδ4 is required for Ca²⁺ mobilization essential for acrosome reaction in sperm

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Zona pellucida (ZP)-induced acrosome reaction in sperm is a required step for mammalian fertilization. However, the precise mechanism of the acrosome reaction remains unclear. We previously reported that PLCδ4 is involved in the ZP-induced acrosome reaction in mouse sperm. Here we have monitored Ca²⁺ responses in single sperm, and we report that the [Ca²⁺]i increase in response to ZP, which is essential for driving the acrosome reaction in vivo, is absent in PLCδ4−/− sperm. Progestrone, another physiological inducer of the acrosome reaction, failed to induce sustained [Ca²⁺]i increases in PLCδ4−/− sperm, and consequently the acrosome reaction was partly inhibited. In addition, we observed oscillatory [Ca²⁺]i increases in wild-type sperm in response to these acrosome inducers. Calcium imaging studies revealed that the [Ca²⁺]i increases induced by exposure to ZP and progesterone started at different sites within the sperm head, indicating that these agonists induce the acrosome reaction via different Ca²⁺ mechanisms. Furthermore, store-operated channel (SOC) activity was severely impaired in PLCδ4−/− sperm. These results indicate that PLCδ4 is an important enzyme for intracellular [Ca²⁺]i mobilization in the ZP-induced acrosome reaction and for sustained [Ca²⁺]i increases through SOC induced by ZP and progesterone in sperm.

Introduction

Phosphoinositide metabolism is an important intracellular signaling system involved in a variety of cell functions, such as secretion of hormones, transduction of neurotransmitters and growth factor signaling, membrane traffic, ion channel activity, and regulation of the cytoskeleton (Fukami et al., 1992; Takenawa et al., 1999; Martin, 2001). PLC is one of the key enzymes in this system and acts by hydrolyzing phosphatidylinositol 4,5-bisphosphate (PIP2) to generate two second messengers, inositol 1,4,5-trisphosphate (IP3)* and DAG. DAG mediates the activation of PKC, and IP3 releases Ca²⁺ from intracellular stores. Among PLC family members, PLCδ is considered to be the most basal isoform because its structure is the simplest, comprising a pleckstrin homology domain, an EF hand domain, X and Y domains, and a C2 domain, and it is evolutionarily conserved even in plants and yeast (Rebecchi and Pentyala, 2000; Rhee, 2001). The activation mechanism of PLCδ remains unclear, although it is the most sensitive to Ca²⁺. PLCδ4, one of the δ isoforms, was first identified from a cDNA library from regenerating liver (Liu et al., 1996), and therefore it is thought to be involved in cell growth. In addition, based on the fact that PLCδ4 is abundantly expressed in brain and testis (Lee and Rhee, 1996; Nagano et al., 1999), we proposed that PLCδ4 plays a specific role in reproductive functions. In fact, through the generation of PLCδ4-deficient mice, we have recently reported that PLCδ4 is involved in the acrosome reaction in sperm (Fukami et al., 2001).

The acrosome reaction, which entails exocytosis of the acrosomal vesicles, is an essential step for fertilization. In mammalian sperm, the acrosome reaction is thought to be initiated in vivo by binding to the zona pellucida (ZP), the extracellular matrix of the egg, and only sperm that have
completed the acrosome reaction can penetrate the ZP and fuse with the egg plasma membrane (Wassarman, 1999; Wassarman et al., 2001; Primakoff and Myles, 2002). In general, it is thought that sperm binding to ZP3, one of the glycoprotein components of the ZP, induces a transient $\text{Ca}^{2+}$ influx into the sperm through voltage-dependent non-selective cation channels, which in turn leads to activation of a pertussis toxin–sensitive trimeric G/o protein–coupled PLC (Patrat et al., 2000; Darszon et al., 2001). A tyrosine kinase–regulated PLC may also be activated during ZP3 binding (Patrat et al., 2000). Activation of PLCs generates IP3, thereby mobilizing $[\text{Ca}^{2+}]_i$ from the sperm's intracellular $\text{Ca}^{2+}$ store, the acrosome, although the $\text{Ca}^{2+}$ storing capacity of this organelle seems very limited (Rossato et al., 2001). Nonetheless, these early responses appear to promote a subsequent sustained $\text{Ca}^{2+}$ influx signal via store-operated channels (SOCs) that results in the acrosome reaction (Florman, 1994; O’Toole et al., 2000; Breitbart, 2002). Recent studies have provided evidence for the expression in sperm of transient receptor potential protein channels 1, 3, and 6 (Trp1, Trp3, and Trp6), all putative $\text{Ca}^{2+}$-permeant SOCs (Trevino et al., 2001), and Trp2 has been described to play a role in the ZP3-induced acrosome reaction in mouse sperm (Jungnickel et al., 2001). However, the precise molecular mechanism by which the acrosome reaction occurs has remained unclear.

In addition to ZP, thapsigargin, a specific blocker of the sarco/endoplasmic reticulum $\text{Ca}^{2+}$-ATPase, which causes $\text{Ca}^{2+}$ depletion from internal stores and leads to capacitative $\text{Ca}^{2+}$ entry (Sabala et al., 1993), is also able to induce the acrosome reaction (Llanos, 1998). Furthermore, progesterone released from the cumulus cells, and thus one of the major components of follicular fluid, has also been shown to in-

![Image](80 The Journal of Cell Biology | Volume 161, Number 1, 2003)

Figure 1. PLC$\delta$4/ALT-II is expressed in sperm. (A) The molecular weight of PLC$\delta$4 in brain (B) and sperm (S) was compared by Western blot analysis. 30 $\mu$g of each homogenate was subjected to SDS-PAGE. Western blot analysis was performed using an antibody against PLC$\delta$4 raised in the laboratory. (B) The molecular weight of sperm PLC$\delta$4 was compared with PLC$\delta$4 and alternative splicing isoforms of PLC$\delta$4. PLC$\delta$4 (44), and splicing isoforms of PLC$\delta$4, PLC$\delta$4/ALT-I (44-I) and PLC$\delta$4/ALT-II (44-II), were overexpressed in COS-7 cells. 40 $\mu$g of lysates was subjected to SDS-PAGE, and then Western blotting was performed. The arrowhead denotes that the sperm protein closely resembles in size the product from the PLC$\delta$4/ALT-II splice variant. (C) The products of RT-PCR using testis RNA were compared in size with authentic products using PLC$\delta$4 (491 bp), PLC$\delta$4/ALT-I (44-I; 587 bp), PLC$\delta$4/ALT-II (44-II; 533 bp), and PLC$\delta$4/ALT-III (44-III; 398 bp) plasmids. The arrowhead denotes that the major testis PCR product coincides in size with that of the PLC$\delta$4/ALT-II splice variant.

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Figure 2. Requirement of PLC$\delta$4 in ZP- and progesterone-induced acrosome reaction. (A) Fluorescence and phase images of sperm treated with several acrosome reaction–inducing reagents. PLC$\delta$4$^{+/+}$ (+/+) and PLC$\delta$4$^{-/-}$ (-/-) sperm capacitated for 1 h were treated with 1.0% DMSO, 3 Zp/$\mu$l solubilized mouse ZP (ZP), 100 $\mu$M progesterone (PG), or 5 $\mu$M thapsigargin (TG) for 15 min at 32°C in the presence of 2 $\mu$M Alexa Fluor® 594–conjugated SBTI to monitor the occurrence of the acrosome reaction. The same microscopic fields were shown for acrosome reaction (AR) and bright field images. Bar, 10 $\mu$m. (B) Percent of sperm that underwent the acrosome reaction. The numbers above each column represent the number of sperm examined. Data were obtained from total amount of sperm by four independent experiments for progesterone and ZP and three independent experiments for thapsigargin.

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duce the acrosome reaction in a presumed physiological manner (Roldan et al., 1994; Kobori et al., 2000). Although the mechanism of action of progesterone on sperm is not yet fully understood, it is thought to induce Ca^{2+} influx by activating a GABAA-like progesterone receptor/Cl^{-} channel (Meizel et al., 1997).

On the basis of the consensus that the acrosome reaction requires Ca^{2+} influx, global patterns of [Ca^{2+}], changes in single sperm have been observed. However, only a few studies have reported detailed spatio-temporal analysis of [Ca^{2+}], rises at the single-sperm level because of difficulties of the measurement in these cells. Here we report, using Ca^{2+} imaging of single sperm, that sperm show Ca^{2+} waves in response to ZP, progesterone, and thapsigargin, although the site of initiation of the rises appears to differ with the agonist, and that PLC4 is an important protein in the regulation of the Ca^{2+} responses that drive the acrosome reaction.

**Results**

**Expression of PLC4/ATL-II in sperm**

Western blot analysis using a specific antibody against PLC4 (Liu et al., 1996) revealed that an 85-kD PLC4 protein is expressed in brain, whereas a 90-kD protein is expressed in sperm (Fig. 1A). There are three splicing variants, termed PLC4/ATL-I, PLC4/ATL-II, and PLC4/ATL-III in addition to PLC4 in testis (Nagano et al., 1999). PLC4/ATL-I and PLC4/ATL-II contain an additional 32 and 14 amino acids in their respective sequences in the linker region between the X and Y domains, and PLC4/ATL-III lacks PLC activity because the COOH-terminal region of the X domain and the linker region are substituted for 32 amino acids corresponding to the insert sequence of PLC4/ATL-I. To understand which splicing isoform exists in sperm and contributes to the acrosome reaction, we compared a band of molecular weight of ~90 kD in sperm with PLC4 splicing variants overexpressed in COS-7 cells (Fig. 1B). Western blot analysis showed that the 90-kD protein coincided with PLC4/ATL-II. There were no other PLC4 proteins in sperm. To confirm further the predominant role/expression of PLC4/ATL-II in sperm, we performed RT-PCR using total testis RNA and compared the molecular weight of the major PCR products using testis cDNA and plasmids coding for each of the splicing variants as templates. The major testis PCR product had a similar molecular weight to that produced from the PLC4/ATLII plasmid (Fig. 1C, arrowhead), although a faint band corresponding to PLC4/ATLI was also detected under this condition. Therefore, we concluded that PLC4/ATL-II is the predominant PLC4 isoform expressed in sperm.

**ZP- and progesterone-induced acrosome reaction is impaired in PLC4^{−/−} sperm**

We have reported that PLC4 is an essential protein for ZP-induced acrosome reaction. To understand whether PLC4 is involved in this process, we first examined whether PLC4 is required for the acrosome reaction induced by progesterone and thapsigargin. A soybean trypsin inhibitor (SBTI) was used to monitor the acrosome reaction. SBTI is known to tightly bind to acrosin (Tollner et al., 2000), which is located on the inner acrosomal membrane and only becomes exposed extracellularly after the acrosome reaction; therefore, we could easily recognize acrosome-reacted live sperm using fluorescence-labeled SBTI (Fig. S1, available at http://www.jcb.org/cgi/content/full/jcb.200210057/DC1). Moreover, this method enabled us to avoid spontaneously acrosome-reacted sperm before the addition of the agonists. In this study we used Al- exa Fluor®594–labeled SBTI to monitor the acrosome reaction induced by ZP, progesterone, and thapsigargin (Fig. 2A). The fluorescence signal began to be detected at ~2–5 min after the addition of these reagents, and 31.7% of wild-type sperm completed the acrosome reaction within 15 min of addition of 3 ZP/μl, whereas only 6.2% of PLC4^{−/−} sperm underwent the acrosome reaction (Fig. 2B). 100 μM progesterone induced higher rates of acrosome reaction with 49.7% of PLC4^{+/+} sperms undergoing the reaction, whereas only 26.1% of the PLC4^{−/−} sperm completed the process. These results suggest that PLC4 has an important role in the progesterone- and ZP-induced acrosome reaction. Nonetheless, because some of the sperm from PLC4^{−/−} males underwent the acrosome reaction, we cannot exclude the possibility that other PLC isozymes might be involved in these acrosome reactions in the absence of PLC4, especially when progester-
one is used to induce the reactions. What is more, we also cannot discount the possibility that some of these sperm might have undergone spontaneous acrosome reactions during the monitoring. There was no apparent difference in the number of wild-type and mutant sperm that underwent the acrosome reaction in response to thapsigargin (58.4 and 49.7%, respectively) (Fig. 2 B).

**Events of capacitation are normal in PLCδ4−/− sperm**

Before successful acrosome reaction can take place, the sperm have to undergo capacitation, a series of poorly defined molecular and biochemical changes including efflux of cholesterol, changes in the phospholipid content of the plasma membrane, hyperpolarization, and tyrosine phosphorylation (Arnoult et al., 1996; Nolan and Hammerstedt, 1997; Darszon et al., 2001). Capacitation occurs in vivo during sperm transit through the female tract and is mimicked in vitro by incubation of epididymal sperm in medium containing HCO₃−, BSA, and Ca²⁺ (Breitbart, 1996; Nolan and Hammerstedt, 1997; Darszon et al., 2001). Capacitation occurs in vivo during sperm transit through the female tract and is mimicked in vitro by incubation of epididymal sperm in medium containing HCO₃−, BSA, and Ca²⁺ (Breitbart, 2002). Several putative assays, such as assessment of cholesterol efflux, cAMP concentration, and protein tyrosine phosphorylation, can be used to know whether or not capacitation has occurred (Visconti et al., 1995, 1999; Iborra et al., 2000). To determine whether defects in capacitation may explain, at least in part, the abnormal acrosome reaction in PLCδ4−/− sperm, we measured the content of cholesterol during capacitation. The decrease in cholesterol levels began to be observed 15 min after capacitation in PLCδ4+/+ sperm and by 30 min in PLCδ4−/− sperm, although the overall decrease in cholesterol content by 90 min of capacitation was similar in both groups (Fig. 3 A). In addition, time-dependent specific protein tyrosine phosphorylation began to be detected in both PLCδ4+/+ and PLCδ4−/− after ~60 min of incubation (Fig. 3 B, arrowheads). From these data, we conclude that PLCδ4 is likely not involved in the capacitation process.

**PLCδ4 is required for [Ca²⁺]i mobilization and sustained Ca²⁺ influx in sperm**

As it has been reported that Ca²⁺ has a primary role in the execution of the acrosome reaction (Patrat et al., 2000; Barratt and Publicover, 2001; Darszon et al., 2001; Breitbart, 2002), we next examined the Ca²⁺ responses in single sperm treated with ZP, progesterone, thapsigargin, or ionomycin (Fig. 4 A). We selected a single excitation calcium indicator fluo-4, because we could obtain calcium images of high resolution with this dye, and the images were temporally and spatially comparable to those obtained with the ratiometric dye fura-2 (Fig. S2, available at http://www.jcb.org/cgi/content/full/jcb.200210057/DC1). PLCδ4+/+ sperm pre-loaded with fluo-4 and treated with ZP exhibited a continuous [Ca²⁺]i increase (Fig. 4 B). This [Ca²⁺]i rise peaked at ~30–60 s after the stimulation and lasted for more than 3–4 min before slowly returning to baseline. Approximately 50% of the sperm exhibited the described response (n = 120), and some sperm even showed repetitive [Ca²⁺]i increases after returning to baseline. In contrast, the addition of ZP induced a minor [Ca²⁺]i increase in PLCδ4−/− sperm, suggesting a role for PLCδ4 in the Ca²⁺ response during the ZP-induced acrosome reaction.

![Figure 4](http://www.jcb.org/cgi/content/full/jcb.200210057/DC1) **[Ca²⁺]i mobilization is impaired in PLCδ4−/− sperm during acrosome reaction.** Capacitated sperm were loaded with 4 μM fluo4-AM for 15 min, and Ca²⁺ responses were monitored every 2 s. (A) Ca²⁺ images of PLCδ4+/+ (+/+) and PLCδ4−/− (−/−) sperm before and after (10–30 s) treatment with 3 Zp/μl solubilized mouse ZP (ZP), 100 μM progesterone (PG), 5 μM thapsigargin (TG), or 5 μM ionomycin (Iono). The same microscopic fields are shown before and after the stimulus. The experiments were done at 32°C. Bar, 10 μm. (B) Ca²⁺ patterns of five sperm representative of the responses induced by the agonists used here. Levels of [Ca²⁺]i are expressed as F/F0 ratios. The data are representative from five (ZP), nine (PG), eight (TG), and three (Iono) independent experiments. Progesterone induced repetitive [Ca²⁺]i increases.
Figure 5. Spatio-temporal [Ca\(^{2+}\)]\(_i\) dynamics in a single sperm treated with acrosome reaction inducers. Capacitated sperm were loaded with 4 µM fluo4-AM for 15 min, and [Ca\(^{2+}\)]\(_i\) mobilization at the single-sperm level was monitored. Sperm from PLC64\(^{+/+}\) (A, C, and E) or PLC64\(^{-/-}\) (B, D, and F) were treated with 3 Zp/µl solubilized mouse ZP (A and B), 100 µM progesterone (C and D), or 5 µM thapsigargin (E and F). Images were collected every 2 s. The relative [Ca\(^{2+}\)]\(_i\) change is calculated as a ratio to F0 images and shown in pseudo-color. Bar, 10 µm. Figures show time (sec) after treatment of the reagents.
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The wave-like Ca\(^{2+}\) influx induced by progesterone has been demonstrated in human sperm (Meizel et al., 1997), although repetitive Ca\(^{2+}\) responses were not reported in that work. It is worth noting that the responses to progesterone seem to be dose dependent. For instance, Kobori et al. (2000) reported that addition of 40 \(\mu\)M progesterone induced rises of longer duration than those induced by 20 \(\mu\)M. In agreement with those findings, we observed longer and repetitive [Ca\(^{2+}\)] responses with higher doses of progesterone (unpublished data). Although progesterone induced a Ca\(^{2+}\) response in PLC\(^{−/−}\) sperm, the amplitude of the rise was smaller and the duration was very transient, returning to basal levels within 60–120 s.

Treatment of PLC\(^{+/+}\) sperm with thapsigargin or ionomycin induced continuous [Ca\(^{2+}\)] increases, and in most sperm, the [Ca\(^{2+}\)] rises were sustained for at least 240 s. Treatment of PLC\(^{−/−}\) sperm with these reagents produced a similar pattern, but with less amplitude (Fig. 4 B). These results raise the possibility that PLC\(^{+/+}\) may have an important role in the regulation of [Ca\(^{2+}\)], mobilization in sperm in response to numerous agonists.

Agonist-specific spatial distribution of [Ca\(^{2+}\)]

Typical [Ca\(^{2+}\)] increase patterns of sperm in response to ZP, progesterone, or thapsigargin over time are shown in Figs. 5 and 6 and Video 1 (available at http://www.jcb.org/cgi/content/full/jcb.200210057/DC1). When PLC\(^{+/+}\) sperm preloaded with fluo-4 were treated with ZP, the [Ca\(^{2+}\)] increase was first detected in the acrosome area (2 s), then in the equatorial segment (4 s), and finally extended to the whole sperm head (around 14 s) (Fig. 6 A). Although there are a few reports that describe the global [Ca\(^{2+}\)], elevations in bovine or hamster sperm head during the ZP-induced acrosome reaction (Florman, 1994; Shirakawa and Miyazaki, 1999), this is the first observation that clearly notes the sequential [Ca\(^{2+}\)], mobilization induced by ZP. These data support the notion that the acrosome vesicle serves as the intracellular Ca\(^{2+}\) store and that ZP mobilizes [Ca\(^{2+}\)], before promoting Ca\(^{2+}\) influx during acrosome reaction. Interestingly, the acrosome reaction always occurred after peak [Ca\(^{2+}\)], values were attained, and the occurrence of the reaction was confirmed by simultaneous monitoring of fluo-4 and Alexa Fluor\(^{®}\) 594–labeled SBTI (unpublished data). As expected, only a minor [Ca\(^{2+}\)], increase was detected in PLC\(^{−/−}\) sperm after the addition of ZP (Fig. 5 B; Fig. 6 B).

The Ca\(^{2+}\) responses induced by progesterone and thapsigargin in PLC\(^{+/+}\) sperm appeared to have a different spatial distribution than those triggered by ZP. Addition of progesterone or thapsigargin intensively induced a Ca\(^{2+}\) response at the base of the sperm head (2–6 s) (Fig. 6, C and E; Videos 2 and 3, available at http://www.jcb.org/cgi/content/full/jcb.200210057/DC1). It is known that thapsigargin induces Ca\(^{2+}\) influx through SOC by inhibiting the Ca\(^{2+}\)-ATPase on Ca\(^{2+}\) stores; therefore, we suspect that this post-acrosomal region near the neck region may be the site of the primary Ca\(^{2+}\) influx. Because progesterone promotes a [Ca\(^{2+}\)], rise with similar spatial distribution to that of thapsigargin, our results suggest that the progesterone-induced [Ca\(^{2+}\)], increase is primarily due to Ca\(^{2+}\) influx. This notion is further supported by the finding that [Ca\(^{2+}\)], increases in response to progesterone were not detected in PLC\(^{−/−}\) sperm when progesterone was applied in Ca\(^{2+}\)-free medium (unpublished data). Remarkably, the second Ca\(^{2+}\) wave also originated from the same post-acrosomal area (132 s) (Fig. 5 C). The spatial distribution of the [Ca\(^{2+}\)], increase induced by progesterone and thapsigargin in PLC\(^{−/−}\) sperm was also initiated in the post-acrosomal region (Fig. 5, D and F; Fig. 6, D and F), although the magnitude of the Ca\(^{2+}\) influx was very limited. It was also evident that the progesterone-induced [Ca\(^{2+}\)], increase was less sustained in PLC\(^{−/−}\) sperm. In spite of the lower Ca\(^{2+}\) responses induced by thapsigargin in PLC\(^{−/−}\)-

Figure 6.

Magnification of the initial images of sperm treated with acrosome reaction inducers.

Sperm from PLC\(^{+/+}\) (A, C, and E) or PLC\(^{−/−}\) (B, D, and F) were treated with ZP (A and B), progesterone (C and D), or thapsigargin (E and F). Bar, 10 \(\mu\)m. Figures show time (sec) after treatment of the reagents.
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weak and slow increases in fluorescence in PLC corresponding to a 96-well plate. We detected comparatively by the same agonists using a fluorescence microplate reader.

Monitoring of \( [Ca^{2+}] \), mobilization on sperm at the population level suggests a role of PLC\(_{64} \) on \( Ca^{2+} \) influx

To further understand the role of PLC\(_{64} \) in sperm [\( Ca^{2+} \)], mobilization and to confirm the validity of the \( Ca^{2+} \) responses obtained using single-sperm measurements, we examined at the population level the [\( Ca^{2+} \)], changes induced by the same agonists using a fluorescence microplate reader corresponding to a 96-well plate. We detected comparatively weak and slow increases in fluorescence in PLC\(_{64}^{-/-} \) sperm after addition of ZP (Fig. 7 A), reflecting, perhaps, the fact that the response to ZP may start over a course of several minutes within a population of sperm, as shown by others.

Figure 7. Measurement of [\( Ca^{2+} \)] increase in sperm responded to various acrosome inducers by mass level. Capacitated cauda epididymal sperm were loaded with 4 \( \mu M \) fluo4-AM for 15 min at 37°C. Approximately 1 \( \times \) 10^6 sperm in a 96-well plate were treated with ZP, progesterone (PG), thapsigargin (TG), or ionomycin (Iono). Increasing levels of intracellular [\( Ca^{2+} \)] are expressed as an F/F0 ratio. The data were collected from three independent experiments.

Figure 8. SOC activity is impaired in PLC\(_{64}^{-/-} \) sperm. (A) PLC\(_{64}^{+/+} \) and PLC\(_{64}^{-/-} \) sperm were incubated in \( Ca^{2+} \)-free HS buffer containing 1 mM EGTA; the \( Ca^{2+} \) stores were depleted by treatment with 5 \( \mu M \) thapsigargin (TG). SOC activity was measured by addition of 1 mM extracellular \( Ca^{2+} \). Ca\(^{2+} \) patterns of two representative sperm responses are shown. The arrowhead denotes the time of acrosome reaction. (B) Changes in [\( Ca^{2+} \)], increases in different areas within a sperm are shown by pseudo-color images and time course plots. Two spots of post-acrosome (1) and equatorial segment (2) were selected for images and time course plots. The images shown were collected 6 s after addition of thapsigargin (TG) and immediately after (2 s) addition of 1 mM extracellular \( Ca^{2+} \)(Ca\(^{2+} \)). The fluorescence intensity of images and plots is shown with different ratio scales.

that PLC\(_{64} \) may play in sperm a novel functional role in the regulation of [\( Ca^{2+} \)] influx.

SOC activity is impaired in PLC\(_{64}^{-/-} \) sperm

Having noticed a difference in the amplitude of the [\( Ca^{2+} \)], increase between PLC\(_{64}^{+/+} \) and PLC\(_{64}^{-/-} \) sperm in response to thapsigargin, we examined directly whether SOC activity was deficient in PLC\(_{64}^{-/-} \) sperm. To evaluate SOC activity, the sperm’s \( Ca^{2+} \) stores were depleted by exposure to thapsigargin in \( Ca^{2+} \)-free medium, followed by the addition of 1 mM extracellular \( Ca^{2+} \) (Fig. 8 A). The addition of thapsigargin induced a small [\( Ca^{2+} \)], increase in both wild-type and mutant sperm, suggesting that the content of the \( Ca^{2+} \) stores is comparable in both sperm populations. However, addition of extracellular \( Ca^{2+} \) induced a large \( Ca^{2+} \) influx and [\( Ca^{2+} \)], increase in PLC\(_{64}^{-/-} \) sperm that decreased after driving the acrosome reaction (Fig. 8 A, top, arrowhead), whereas it produced a weak and transient [\( Ca^{2+} \)], increase in PLC\(_{64}^{-/-} \) sperm, demonstrating...
that the absence of PLCδ4 significantly affected the function of SOC channels.

To attempt to pinpoint the site of Ca\(^{2+}\) influx, and possibly to determine the position of SOC channels in sperm, we next examined the spatial distribution of the Ca\(^{2+}\) entry promoted by the addition of extracellular Ca\(^{2+}\) in sperm previously treated with thapsigargin in the absence of extracellular Ca\(^{2+}\). [Ca\(^{2+}\)], changes were compared in two different areas of the sperm head, one in the post-acrosomal region near the neck of the sperm head (Fig. 8 B, 1), which was anticipated to report Ca\(^{2+}\) influx and hence SOC activity, and the second in the equatorial segment (Fig. 8 B, 2), which was expected to indicate intracellular Ca\(^{2+}\) release. When PLCδ4+/+ sperm were treated with thapsigargin, a small [Ca\(^{2+}\)], increase was observed at position 2, in the equatorial region, and after addition of extracellular Ca\(^{2+}\), a large [Ca\(^{2+}\)], increase was detected primarily at position 1, the post-acrosomal area, which then propagated to position 2 (Fig. 8 B). These results indicate that release of intracellular [Ca\(^{2+}\)], in sperm occurs primarily in the acrosome and in the equatorial area, and that the post-acrosomal region is the SOC-rich area, which mediates Ca\(^{2+}\) influx.

**Discussion**

It is well known that Ca\(^{2+}\) release and Ca\(^{2+}\) influx play a significant role in the induction of the acrosome reaction (Patrat et al., 2000; Barratt and Publicover, 2001; Darszon et al., 2001; Breitbart, 2002). In this manuscript, we have characterized in sperm of wild-type and in PLCδ4-deficient mice the temporal and spatial distribution of the [Ca\(^{2+}\)], increases induced by treatment with ZP, progesterone, thapsigargin, or ionomycin (Figs. 4, 5, and 6). Our results show that absence of PLCδ4/ATII causes major impairment in the release of intracellular Ca\(^{2+}\) induced by the addition of ZP, which is the mechanism responsible for inducing the acrosome reaction during natural fertilization. Moreover, PLCδ4 appears to affect Ca\(^{2+}\) influx, which may be necessary to complete the acrosome reaction, as the Ca\(^{2+}\) responses induced by the above-mentioned agonists were altered in PLCδ4 mutant sperm. We can therefore conclude that PLCδ4/ATII may be involved in the regulation of both intracellular Ca\(^{2+}\) release and Ca\(^{2+}\) influx through SOC channels.

Using high resolution single-sperm Ca\(^{2+}\) imaging, we demonstrated that the agonists used in this study to induce the acrosome reaction trigger distinct Ca\(^{2+}\) responses, and that these responses are altered in intensity and duration in PLCδ4−/− sperm. First, we confirmed that in wild-type sperm, the addition of solubilized ZP induces the acrosome reaction by triggering Ca\(^{2+}\) release. We extended these observations by showing that the ZP-induced [Ca\(^{2+}\)], increase started in the acrosome region and spread to the whole sperm head (Fig. 6 A). As it has been reported that both the IP3 receptors (IP3Rs) and PLCδ4 localize to the acrosome (Walensky and Snyder, 1995; Fukami et al., 2001), our results suggest that the ZP induces [Ca\(^{2+}\)], mobilization initially from the acrosome and that PLCδ4 is responsible for the presumed generation of IP3. Evidence in support of this notion is provided by the findings that ZP almost failed to induce a [Ca\(^{2+}\)], increase in PLCδ4−/− sperm. Therefore, we conclude that the abnormal acrosome reaction induced by ZP in PLCδ4−/− sperm is most likely due to impaired intracellular [Ca\(^{2+}\)], mobilization in these sperm, and that this protein plays a crucial role in the acrosome reaction during natural fertilization.

Although the progesterone-induced [Ca\(^{2+}\)], increases in sperm exhibited different spatial and temporal patterns than those evoked by ZP, the intensity of these responses was still altered in PLCδ4−/− sperm. Our data show that most of the progesterone-induced Ca\(^{2+}\) response initiated in the post-acrosomal region, and that it was primarily due to Ca\(^{2+}\) influx, as it was not observed in Ca\(^{2+}\)-free medium. Remarkably, in PLCδ4−/− sperm, the amplitude and duration of the progesterone-induced Ca\(^{2+}\) response were greatly decreased, paralleled by a reduction in the number of sperm that underwent the acrosome reaction (Fig. 2 B), supporting the notion that a sustained [Ca\(^{2+}\)], increase is required for the completion of the acrosome reaction and that PLCδ4 may regulate this aspect of the progesterone-induced Ca\(^{2+}\) signal.

The thapsigargin-induced Ca\(^{2+}\) responses were similar to those evoked by progesterone in that they depended on Ca\(^{2+}\) influx to elevate [Ca\(^{2+}\)], in sperm. In addition, direct assessment of SOC function using thapsigargin showed that capacitative Ca\(^{2+}\) entry through SOC channels is severely impaired in PLCδ4−/− sperm (Fig. 8 A), further supporting a role of PLCδ4 in the regulation of Ca\(^{2+}\) influx through these channels. Moreover, in this manuscript, we determined for the first time that the site of Ca\(^{2+}\) influx after the addition of thapsigargin (and progesterone) appears to be the post-acrosomal region, near the connective piece. The question then arises as to why this site might mediate Ca\(^{2+}\) influx. It is possible that SOC channels may be localized to this region. Toward this end, it is worth noting that recent reports localize IP3Rs to an additional region on the base of the head and neck region in human and bovine sperm (Ho and Suarez, 2001; Naaby-Hansen et al., 2001). Whether there is a direct link between IP3R and SOC channels in sperm and whether the site of Ca\(^{2+}\) influx is conserved across species remain to be investigated.

It also remains unclear how PLCδ4 might regulate SOC channels. It has recently been shown that some Trp channels operate as SOC channels (Montell et al., 2002), and TrpC3 has been reported to associate directly with the IP3R (Boulay et al., 1999; Kiselyov et al., 1999). In addition, recent evidence has shown that TrpC2 is implicated in the acrosome reaction (Jungnickel et al., 2001) and the phosphoinositide pathway may regulate the activity of Trp channels (Montell et al., 2002; Runnels et al., 2002). Therefore it is possible that defective stimulation of the phosphoinositide pathway may be responsible for the abnormal Ca\(^{2+}\) responses detected in the mutant PLCδ4 sperm. Lastly, we cannot discount the possibility that in PLCδ4 mutant sperm, the number and/or function of SOC channels may be altered in a manner unrelated to the possibilities considered here. Nonetheless, our finding that PLCδ4 is one of the important enzymes in the regulation of [Ca\(^{2+}\)], mobilization in sperm significantly contributes to the elucidation of the molecular pathways of mammalian fertilization. In addition, its possible role in the regulation of Ca\(^{2+}\) influx suggests novel
areas of research. Regulation of Ca^{2+} influx is important for many vital cellular functions, such as cell growth, apoptosis, exocytosis, muscle contraction, and gene transcription (Berridge et al., 1999, 2000), and its abnormal function may lead to the development of disease (Peng and Hediger, 2002; Torbergsen, 2002). Further understanding of the relationship between phosphoinositide metabolism and Ca^{2+} channel regulation may contribute to elucidating the molecular basis of these diseases.

Materials and acrosome reactions

Detection of acrosomal reaction

Cauda epididymal sperm were obtained from 8–18 wk-old PLC\textsubscript{4}\& Schuell and capacitated for 1 h in HS medium (135 mM NaCl, 5 mM KCl, 1 mM MgCl\textsubscript{2}, 2 mM CaCl\textsubscript{2}, 30 mM Hepes, 10 mM glucose, 10 mM lactic acid, 1 mM purvic acid, 5 mg/ml BSA, 15 mM NaCO\textsubscript{3}, pH 7.4) at 37°C under CO\textsubscript{2}. Mouse ZP was isolated from the ovaries of 10–21 wk-old mice as previously described (Ward et al., 1992). Progesterone, thapsigargin, and ionomycin were purchased from Fuji Pharmaceutical and Sigma-Aldrich, respectively. The acrosome reaction was induced by the addition of the above-mentioned agonist, and it was performed at 32°C for 15 min in the presence of 2 μM SBTI (Nacalai Tesque), which was labeled using an Alexa Fluor\textsuperscript{594} protein labeling kit (Molecular Probes). After the acrosome reaction, the fluorescence-labeled sperm were counted under a fluorescence microscope with the same optical condition described below for the fluo-4 and Alexa Fluor\textsuperscript{594}–labeled SBTI double-labeling experiment. The ratio of the acrosome reaction was calculated by dividing the number of acrosome-reacted sperm by the number of total sperm.

Measurements of [Ca\textsuperscript{2+}] mobilization in single sperm

Capacitated cauda epididymal sperm were loaded with 4 μM fluo-AM (Molecular Probes) for 15 min at 37°C and immobilized on a laminin-coated glass-bottom dish just before calcium measurement. We monitored simultaneously in single sperm the fluorescence emission of two dyes, fluo-4 and Alexa Fluor\textsuperscript{594}–labeled SBTI. Fluorescence Ca\textsuperscript{2+} images of fluo-4 and Alexa Fluor\textsuperscript{594}–labeled SBTI images were collected alternately for 200 μsec every 2 s with altering excitation filters (470–490 nm for fluo-4 and 535–550 nm for SBTI), a dual-band dichroic mirror (51016bs; Chroma Technology Corp.), and a dual-band emission filter (51016em; Chroma Technology Corp.) on an inverted microscope (IX70; Olympus) through a 60× objective (n.a. 1.4; Olympus) with a digital CCD camera (ORCA-ERG; Hamamatsu Photonics). On-line control of the system, acquisition, and off-line analysis of the collected data were done with TI Workbench software (written by T. Inoue) running on a Macintosh computer. Levels of [Ca\textsuperscript{2+}], are calculated as F/F\textsubscript{0} ratios after background subtraction in both images and time course plots. Five image frames just before drug application were averaged and used as an F0 image. The experiments were performed at 32°C with a heating chamber covering the stage and objective lens of the microscope. For measurements of SOC activity, immobilized sperm were washed three times in Ca\textsuperscript{2+}-free HS buffer (containing 1 mM EGTA) and treated with thapsigargin, followed by the addition of 1 mM extracellular Ca\textsuperscript{2+}.

Measurements of [Ca\textsuperscript{2+}] mobilization at the population level

Capacitated cauda epididymal sperm were loaded with 4 μM fluo-AM for 15 min at 37°C. Approximately 1 × 10\textsuperscript{5} sperm were placed on a 96-well plate and treated with ZP, progesterone, thapsigargin, or ionomycin at 32°C. The fluorescence output changes were monitored at 5-s intervals using a Fusion-n fluorescence microplate analyzer (Packard BioScience) with a 495-nm excitation filter and a 515-nm emission filter. Increasing levels of [Ca\textsuperscript{2+}], were expressed as F/F\textsubscript{0} ratios.

Expression of PLC\textsubscript{4} isoforms, Western blot analysis, and RT-PCR analysis

COX-7 cells were maintained in DME supplemented with 10% FBS. PLC\textsubscript{4}, PLC\textsubscript{4}/ALT-I, and PLC\textsubscript{4}/ALT-II were subcloned into the expression vector pcDNA3 and transiently expressed in COS-7 cells by electroporation. At 48 h after transfection, cells were lysed with SDS sample buffer, and the proteins were separated by SDS-polyacrylamide gel electrophoresis, followed by transfer onto nitrocellulose membranes (Schleicher & Klampfer, 2002). Western blot analysis was performed using a specific polyclonal antibody against PLC\textsubscript{4}.

Sperm capacitated for the indicated period were collected by centrifugation, solubilized with SDS sample buffer, and subjected to electrophoresis. Tyrosine phosphorylation was assessed by immunoblot analysis using a 4G10 antibody (Upstate Biotechnology) that recognizes tyrosine-phosphorylated proteins.

Total RNA from testis and sperm was prepared using the Qiagen RNeasy kit. 10 μg RNA was used for reverse transcription. PCR was performed for 40 cycles of 30 s at 95°C, 30 s at 54°C, and 60 s at 72°C with 5 μCi [\textsuperscript{32}P]dCTP and PLC\textsubscript{4}–specific primers (forward primer, TGGCACAC- CATCTGATGCG; reverse primer, TACACCGCATACTGTCG) to produce 165-bp (PLC\textsubscript{4}), 387-bp (PLC\textsubscript{4}/ALT-I), 353-bp (PLC\textsubscript{4}/ALT-II), and 398-bp (PLC\textsubscript{4}/ALT-III) fragments. PCR products were separated on a 5.0% acrylamide gel, followed by autoradiography.

Cholesterol assay

Cauda epididymal sperm were capacitated for the indicated times in HS medium at 37°C under CO\textsubscript{2}. Sperm were collected by centrifugation, and lipids were extracted by chloriform/methanol/HCl as previously described (Schacht, 1978). An aliquot of the extracted lipids was used for measuring cholesterol content using an Amplex Red Cholesterol Assay kit (Molecular Probes). The cholesterol content was normalized by protein content and expressed relative to the concentration before capacitation.

Online supplemental material

The supplemental material (Figs. S1 and S2 and Videos 1–3, available at http://www.pjcb.org/cgi/content/full/jcb.200210057/D1C) shows [Ca\textsuperscript{2+}], mobilization in wild sperm treated with acrosome reaction inducers. Capacitated wild sperm were loaded with 4 μM fluo4-AM for 15 min, and Ca\textsuperscript{2+} images were monitored every 2 s after treatment with 3 μg/ml solubilized mouse ZP (JP/WT), 100 μM progesterone (PG/WT), or 5 μM thapsigargin (TG/WT).

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