Regulation of $[Ca^{2+}]_i$ Oscillations and Mitochondrial Activity by Various Calcium Transporters in Mouse Oocytes

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Research

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Abstract

Oocyte activation inefficiency is one of the reasons for female infertility and Ca 2+ functions play a critical role in the regulation of oocyte activation. We used various inhibitors of Ca 2+ channels and pumps located on the plasma membrane, the endoplasmic reticulum or both, including sarcoplasmic/endoplasmic reticulum Ca 2+ ATPases (SERCAs, the main Ca 2+ pumps which decrease the intracellular Ca 2+ level by reaccumulating Ca 2+ into the sarcoplasmic reticulum), transient receptor potential (TRP) ion channel subfamily member 7 (TRPM7, a Ca 2+ /Mg 2+ -permeable non-selective cation channel), T-type Ca 2+ channels and calcium channel Orai1, to investigate their roles in[Ca 2+] i oscillation patterns and mitochondrial membrane potential during oocyte activation by real-time recording. Our results show that SERCAs, TRPM7 and T-type Ca 2+ channels are important for initiation and maintenance of [Ca 2+] i oscillations, which is required for mitochondrial membrane potential changes during oocyte activation, as well as for subsequent pronuclear formation and transition to embryo development, while the function of calcium channel Orai1 is not confirmed. Increasing the knowledge of these transporters may provide a theoretical basis for improving oocyte activation in human assisted reproduction clinics.

Introduction

According to reports by the World Health Organization in 2016, at least one of ten couples in developed countries cannot have children within five years of marriage, half of which are due to female infertility [1]. Oocyte activation inefficiency is a major problem causing female infertility [2]. Oocytes are arrested at metaphase of the second meiosis until fertilization takes place. Directly following sperm penetration, oocyte activation begins with a series of crucial steps triggered by periodical repetitive increases and decreases of intracellular Ca^{2+} ($[Ca^{2+}]_i$) concentrations known as $[Ca^{2+}]_i$ oscillations [3, 4]. Although $[Ca^{2+}]_i$ oscillations are required for oocyte activation [5], very little is known about which Ca^{2+} transporter participates in producing $[Ca^{2+}]_i$ oscillations during oocyte activation.

The importance of Ca^{2+} functions in the regulation of oocyte activation is increasingly being recognized [6]. There are many important channel proteins involved in Ca^{2+} transportation in oocytes. Ca^{2+} transporters on the plasma membrane can be controlled by voltage, ligand, second messengers, Ca^{2+} concentration, or other interactions [7]. Ca^{2+} influx into the cytoplasm is mediated by a diverse population of Ca^{2+} transporters exhibiting significant diversities in their gating and activation mechanisms. Once the oocyte is activated, the $[Ca^{2+}]_i$ oscillations are produced by simultaneous intracellular Ca^{2+} storage release and the extracellular Ca^{2+} influx [8]. Hence, it is important to elucidate the molecular mechanisms of $[Ca^{2+}]_i$ oscillations at activation. According to our previous research, it can be seen that $[Ca^{2+}]_i$ oscillations are regulated by mitochondria during activation [9]. Mitochondria are the main organelles for energy supplement in the cell. We believe that $[Ca^{2+}]_i$ oscillations in the cytoplasm will have similar

effects as Ca^{2+} regulates mitochondria in cardiomyocytes [10]. At present, the transporters involved in the regulation of $[Ca^{2+}]_i$ oscillations and mitochondria activity have not been completely determined.

In this study, specific inhibitors for different Ca^{2+} transporters were introduced to detect the $[Ca^{2+}]_i$ oscillation patterns and mitochondria membrane potential dynamic changes, in order to evaluate the role of Ca^{2+} transporters in oocyte activation. Studying the function and regulatory mechanisms of these transporters have great importance for both understanding oocyte activation mechanisms and for improving clinical assisted reproductive technologies (ART).

Materials And Methods

Ethics statement

Female 8 weeks ICR mice were purchased from the Beijing Vital River Laboratory Animal Technology Co., Ltd. All mice were handled in accordance with the institutional animal care policies of the Institute of Zoology, Chinese Academy of Sciences. Mice were maintained under a 12-h light and 12-h darkness cycle in a specific pathogen-free stage at the Central Animal Laboratory of the Institute of Zoology. The Laboratory Animal Care and Use Committee of the Institute of Zoology approved this study.

Oocytes Collection And Parthenogenetic Activation

Female mice were injected with 10 IU pregnant mare serum gonadotropin (PMSG, Ningbo Hormone Product Co. Ltd., Cixi, China) followed 44 to 48 hours later by injection of 10 IU human chorionic gonadotropin (HCG, Ningbo Hormone Product Co. Ltd., Cixi, China). After 15 h of HCG, ovulated cumulus-oocyte complexes (COCs) were collected and denuded in 1 mg/ml hyaluronidase. $[Ca^{2+}]$i oscillations induced by 10 mM strontium chloride ($SrCl_2$, 10025-70-4, Sangon Biotech, Shanghai, China) in Ca^{2+}-free CZB incubating medium containing various Ca^{2+} channel modulators four hours. After parthenogenetic activation, embryos were moved into KSOM (MR-106, Merck Millipore, USA) for further culture.

Real-time Recording Of $[Ca^{2+}]_i$ Changes

Oocyte $[Ca^{2+}]_i$ oscillations were detected using 2 μg/mL Fluo-4 AM (488 nm excitation, 525 nm emission) in the partheno-activation solution. Real-time images after parthenogenetic activation of oocytes which were co-incubated with various Ca2 + channel modulators were obtained using a time-lapse confocal laser microscope (UltraVIEW-VoX; PerkinElmer, MA, USA) and recorded at 2 frames per minute. $[Ca^{2+}]_i$ intensity was detected using an argon laser. A software Volocity was used to analyze fluorescence intensity.

Real-time Recording Of Oocyte Mitochondrial Potential

In our study, we set out to confirm the $[Ca^{2+}]_i$ oscillations regulatory function in mitochondrial activity during oocyte activation. Oocytes were denuded, and pre-incubated in M2 culture medium (M7167, Sigma-Aldrich, USA) with 2 μg/mL JC-1 (C2005, Beyotime, Beijing, China), a cell permeable voltage-sensitive fluorescent mitochondrial dye, for 15 minutes. In order to reduce the toxicity of JC-1 for long-term observation under laser confocal microscopy, we reduced the concentration of JC-1 to 0.5 μg/ml in the activating solution. Briefly, the lower potential mitochondria were imaged as JC-1 monomer emitting in green (488 nm excitation, 561 nm emission), and energized highly potential mitochondria were imaged in aggregated JC-1 that emits in red (488 nm excitation, 561 nm emission) as fluorescence transporters. Oocyte mitochondrial potential dynamic changes after treatment with various Ca2 + channel modulators were recorded after partenogenetic activation using a time-lapse confocal laser microscope (UltraVIEW-VoX; PerkinElmer, MA, USA) at identical magnification and gain settings throughout the experiments. A software Volocity was used to analyze fluorescence intensity.

Statistical Analysis

All experiments were conducted at least three times. No less than 40 oocytes were collected and examined in each group each time. We presented information of samples with Means and Standard Deviations (SD). Results were analyzed by SPSS 19.0. The significance of differences among groups was analyzed by the Chi-square test, and p values less than 0.05 were considered statistically significant.

Results

Inhibitors of Ca^{2+} channels affect the efficiency of oocyte activation

We firstly examined the dose-dependent effects of inhibitors of SERCAs, TRPM7, T-type Ca^{2+} channels and Orai1 on the efficiency of $SrCl_2$-induced oocyte parthenogenetic activation and found a suitable concentration. The percentages of survival oocytes showing clear cell membrane and high cytoplasmic refraction after activation, pronuclear formation and 2-cell cleaved embryos were shown in Table 1. We applied Thapsigargin (10522, Cayman, Michigan, USA), a SERCAs inhibitor, with gradient concentrations of 0.5, 1, and 10 μM. When its concentration rose to 10 μM, a suggested working concentration by manufacturer, [Ca2+]i would rise slowly, and oocytes died during the activation process. NS-8593 (N-195, Alomone, Jerusalem BioPark, Jerusalem, Israel), a TRPM7 inhibitor, was applied with gradient concentrations of 0.1, 1, and 5 μM. The oocyte survival rate was 37.5% when treating with 1 μM NS-8593. When treating with 5 μM NS-8593, half of suggested concentration by manufacturer, all oocytes died in a short time. Mibefradil (Mib, HY-15553, MCE, NJ 08852, USA), a T-type Ca2 + channels blcoker, was added with gradient concentrations of 0.5, 5, and 10 μM. Under suggested concentration of 10 μM, activation of most oocytes was blocked. When applying GSK-7975A (HY12507, MCE, NJ 08852, USA), an Orai1blocker,

with gradient concentrations of 10, 100 μM, and 1 mM. Oocytes were still activated at concentrations of 10 μM and 100 μM GSK-7975A. Pronuclear formation was suppressed significantly when GSK-7975A reached 1 mM, but this concentration had sevre cytotoxcity. We found that SERCAs, TRPM7 and T-type Ca^{2+} channels played an important role in activation, while inhibition of Orai1 does not seem to affect oocyte activation. In our subsequent experiments, we selected a moderate concentration of inhibitors to observe the effect of $[Ca^{2+}]_i$ oscillations on mitochondrial dynamic potential.

Table 1

Effect of calcium channel inhibitors on oocyte parthenogenetic activation

Inhibitor	Concentration	No. oocytes	Repeats	PA	PN	2-Cell
Ctrl		240	5	98.33 ± 1.24%	93.25 ± 5.32%	82.90 ± 7.53%
Tha	0.5 μM	240	5	84.2 ± 5.72%	65.7 ± 9.56%*	67.6 ± 7.59%
	1 μM	240	5	66.2 ± 9.24%	42.1 ± 8.56%*	26.1 ± 12.45%*
	10 μM	240	5	0	X	X
NS-8593	0.1 μM	240	5	83.3 ± 6.28%	75.2 ± 9.57%	86.7 ± 7.50%
	1 μM	240	5	37.5 ± 12.53%*	52.9 ± 7.84%*	57.7 ± 11.56%*
	5 μM	240	5	0	X	X
Mib	0.5 μM	240	5	77.3 ± 5.74%	55.6 ± 10.60%	67.5 ± 8.82%
	5 μM	240	5	36.7 ± 5.68%*	42.5 ± 5.93%*	66.4 ± 10.34%
	10 μM	240	5	3.6 ± 2.40%*	0	X
GSK-7975	10 μM	240	5	95.4 ± 3.21%	84.5 ± 6.34%	82.7 ± 7.26%
	100 μM	240	5	83.8 ± 5.21%	82.6 ± 5.83%	58.4 ± 11.57%
	1 mM	240	5	63.5 ± 7.95%*	0	X

Note：Tha indicates SERCAs inhibitor Thapsigargin. NS-8593 is a TRPM7 specific inhibitor. Mib indicates T-type Ca2+ channels inhibitor Mibefradil. GSK-7975 is an Orai1 specific inhibitor. PA indicates survival embryos of all activated oocytes after activation. PN indicates pronuclear embryos from surviving oocytes. 2-Cell indicates cleaved embryos in pronuclear fertilized eggs. “X” indicates none available data. The significance of differences between Inhibitors and Ctrl group were analyzed by the Chi-square test and $p<0.05$ (*) was considered statistically significant.

$[Ca^{2+}]_i$ And Mitochondrial Potential Dynamic Changes During Activation

Cytoplasmic Ca^{2+} concentration ($[Ca^{2+}]_i$) oscillations and mitochondrial dynamic potential changes during the activation of oocytes were observed by time-lapse confocal laser microscopy (UltraVIEW-VoX, PerkinElmer, Massachusetts, USA). Normal $[Ca^{2+}]_i$ oscillations were induced by $SrCl_2$ stimulation (Fig. 1A). We found that mitochondria were activated from the cortex to the internal area of the oocyte and finally aggregated around the chromosomes (Fig. 1B). In order to analyze changes in oocyte mitochondrial membrane potential, a relative fluorescence intensity analysis and a high membrane potential/low membrane potential ratio were introduced. Ordinate is marked as Relative Fluorescence Intensity (relative to the fluorescence intensity of the start point). The overall membrane potential showed two elevated peaks and then returned to a basal level (Fig. 1C, D).

Effect Of SERCAs Inhibitor Thapsigargin On Oocyte Activation

Endoplasmic reticulum (ER) is a major Ca^{2+} storage component in the cytoplasm. SERCAs are the main transporters for cytoplasmic Ca^{2+} refilling to the ER. We wanted to observe changes in $[Ca^{2+}]_i$ oscillations and mitochondrial activity during oocyte activation after the Ca^{2+} refill was blocked by SERCAs inhibitor Thapsigargin (Tha) (Fig. 2). The survival rate was 66.2% when treating with 1 μM Tha (Table 1), by which concentration $[Ca^{2+}]_i$ oscillations (Fig. 2A) and pronuclear formation were sufficiently suppressed. Mitochondrial membrane potential continued to decrease under Ca^{2+} refilling inhibition by 1 μM Tha (Fig. 2B). Under suggested working concentration by manufacturer, 10 μM Tha will completely inhibit Ca^{2+} refill into ER, and oocyte will die during activation (Fig. 2A). Ordinate is marked as Relative Fluorescence Intensity (relative to the fluorescence intensity of the start point). Thus, Ca^{2+} refilling to ER through SERCAs plays an important role in $[Ca^{2+}]_i$ oscillations and mitochondrial activation during oocyte activation.

Effect Of TRPM7 Inhibitor NS-8593 On Oocyte Activation

TRPM7 is highly expressed in the GV and MII oocytes, mainly distributed on the plasma membrane. We inhibited TRPM7 activity by its specific inhibitor NS-8593 to study its role in oocyte activation (Fig. 3). The $[Ca^{2+}]_i$ oscillations (Fig. 3A) and pronuclear formation were sufficiently suppressed when 1 μM NS-8593 was used to inhibit the transport of Ca^{2+} through TRPM7. The dynamic mitochondrial membrane potential of oocyte was observed after treatment with 1 μM NS-8593 (Fig. 3B). At this concentration, mitochondrial membrane potential continually decreased. Depolarization of mitochondria was found in the case of long-term inhibition (Fig. 3B). When treating with 5 μM NS-8593, half of suggested concentration given by manufacturer, $[Ca^{2+}]_i$ slowly rose and finally induced oocyte death. Ordinate is marked as Relative Fluorescence Intensity (relative to the fluorescence intensity of the start point). Thus,

$[Ca^{2+}]_i$ oscillations regulated through TRPM7 are important for mitochondrial activity during oocyte activation.

Effect of T-type Ca $^{2+}$ channels inhibitor Mibefradil on oocyte activation

T-type Ca^{2+} channels include Cav3.1, Cav3.2, and Cav3.3 subtypes are distributed on the plasma membrane. Mibefradil can suppress all three T-type Ca^{2+} channels. Mibefradil was introduced to study the role of T-type Ca^{2+} channels in $[Ca^{2+}]_i$ oscillations (Fig. 4). The mitochondrial membrane potential was observed in the 5 μM group, half of suggested concentration by manufacturer. We found that the mitochondrial membrane potential continued to decrease after inhibiting the transport of Ca^{2+} through T-type Ca^{2+} channels. When the concentration was increased to 10 μM, Ca^{2+} slowly increased, which induced rapid death of most oocytes. Ordinate is marked as Relative Fluorescence Intensity (relative to the fluorescence intensity of the start point). Based on these results it can be concluded that the transport of Ca^{2+} through T-type Ca^{2+} channels is important for $[Ca^{2+}]_i$ oscillations and mitochondrial activity in oocyte activation and survival.

Effect Of Orai1 Inhibitor GSK-7975a On Oocyte Activation

Orai1 is distributed on the plasma membrane. We studied the role of Orai1 in oocyte activation by treating oocytes with the specific inhibitor GSK-7975A. The mitochondrial membrane potential was observed at 1 mM, which showed irregular changes compared to the Ctrl group (Fig. 5). Most oocytes survived until 4 hours of activation, but none of them formed a pronucleus due to cytotoxicity. Ordinate is marked as Relative Fluorescence Intensity (relative to the fluorescence intensity of the start point). It was not confirmed whether this $[Ca^{2+}]_i$ oscillation pattern and mitochondria membrane potential changes were caused by complete inhibition of Orai1 or by cytotoxicity induced by the high concentration of 1 mM GSK-7975A. The role of Orai1 in the activation of oocytes requires additional evidence to confirm.

Effects Of Inhibitors On Long-lasting $[Ca^{2+}]_i$ Oscillations

The mechanisms of initiation and maintenance of $[Ca^{2+}]_i$ oscillations are believed to be different. However, it is unclear which transporters participate in the initiation or maintenance. The effects of several transporters on the maintenance of $[Ca^{2+}]_i$ oscillations were investigated in our study (Fig. 6). By using time-lapse confocal laser microscopy we observed the effect of various inhibitors on the maintenance of $[Ca^{2+}]_i$ oscillations after some initial oscillations (Fig. 6A). The same concentration was applied as in the mitochondrial membrane potential observation study mentioned above. Ruthenium Red (MACKLIN, Beijing, China) completely inhibited oocyte activation as a non-specific cation channels

inhibitor. It was found that the addition of Ruthenium Red, Thapsigargin and GSK-7975A inhibited Ca^{2+} transport, and the $[Ca^{2+}]_i$ oscillations were blocked from the time of inhibitor addition. After addition of Mibefradil and NS-8593, the intracellular Ca^{2+} increased continuously, and the cell death rate was higher than in the ctrl and other groups. Based on these results it can be concluded that the three Ca^{2+} transporters, SERCAs, TRPM7 and T-type Ca^{2+} channels, are not only involved in $[Ca^{2+}]_i$ oscillation initiation but also in $[Ca^{2+}]_i$ oscillations maintenance. Interestingly, the effect of Ruthenium Red and other inhibitor additions into culture of oocytes which had already initiated $[Ca^{2+}]_i$ oscillations in oocytes did not significantly influence oocyte activation (Fig. 6B), suggesting that oocyte activation and pronuclear formation require only a small amount of $[Ca^{2+}]_i$ oscillations. The function of long-lasting $[Ca^{2+}]_i$ oscillations in embryo development needs further study.

Discussion

Obese, diabetic and aging women typically suffer from abnormal body metabolism such as hypertension, hyperglycemia and hyperlipidemia [11–16], causing long-term stress in oocytes [17], which severely damages the quality of oocytes, thereby leading to lower pregnancy rate [18]. Insufficient oocyte activation and mitochondrial damage were considered to be major causes for embryo developmental disorders[19, 20].

Ca^{2+} is one of the major signal molecules that regulate various cell functions including cell cycle progression, arrest and apoptosis. Oocyte activation induces a continuous series of oocyte intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) increases and decreases known as $[Ca^{2+}]_i$ oscillations, which encode oocyte activation events, including liberation from the MII arrest, pronucleus formation and the transition to embryo development [21]. Release of Ca^{2+} from internal stores and Ca^{2+} influx from the extracellular matrix induce moderate increases in $[Ca^{2+}]_i$ levels. The increase of $[Ca^{2+}]_i$ generally lasts about 2 minutes. As Ca^{2+} refills back to the ER or efflux from the cytoplasm to prepare for the next peak of oscillation, the elevated $[Ca^{2+}]_i$ will return to baseline levels, resulting in an average of a ten to twenty minutes resting interval. $[Ca^{2+}]_i$ oscillations will last 5–6 hours until pronuclear formation [9]. The repeated elevation and recovery of Ca^{2+} signaling is tightly regulated, and the strictly ordered Ca^{2+} signal will coordinate the interaction of various organelles in the oocyte for its activation. Ca^{2+} transporters and regulators could become potential therapy targets for infertile women especially for in vitro fertilization failure.

During activation, Ca^{2+} shuttles through the cell, and the transporter is likely to be located near IP3R and downstream organelles [22]; the cell membrane, lysosomes, the nucleus, vesicles and mitochondria may be targets of Ca^{2+} release. Endoplasmic reticulum (ER) is a main intracellular Ca^{2+} store, where the Ca^{2+} concentration increases to 300 or even 1,000 μM [23]. The pathway for Ca^{2+} efflux from the ER into the cytoplasm has not yet been well identified. SOCE or Ca^{2+} release-activated Ca^{2+} channels (CRAC), were first described in immune cells where they have been shown to be critical for their function. Accordingly,

defects in SOCE in humans are associated with severe immune-deficiencies [24]. In oocytes, the predominant $[Ca^{2+}]_i$ increase pathway appears to be achieved through store-operated Ca^{2+} entry (SOCE) processes. Ca^{2+} enters the cytosol from the endoplasmic reticulum (ER), which in turn opens one of ER channel, sarcoplasmic reticulum/ER Ca-ATPase (SERCAs), to transport Ca^{2+} back to ER [25]. Total cellular Ca^{2+} was estimated by the addition of 10 μM Thapsigargin (Tha), a SERCAs inhibitor, which induced complete release of Ca^{2+} from ER [26]. When ER Ca^{2+} stores had been significantly depleted by Tha, sperm no longer triggered $[Ca^{2+}]_i$ oscillations [27]. In our study, we used three gradient concentrations of 0.5, 1 and 10 μM of Tha. Ten μM Tha kept oocytes at a higher $[Ca^{2+}]_i$ and all oocytes died before the end of the activation process (Fig. 2). However, when oocyte $[Ca^{2+}]_i$ oscillations were suppressed by 1 μM Tha, more than half of the oocytes survived more than 4 hours. Under such moderate concentration, the effects of SERCAs on mitochondrial activity can be observed for a long term. Oocyte mitochondria membrane potential continued to decrease under Ca^{2+} refilling inhibition with 1 μM Tha incubation. Not only $[Ca^{2+}]_i$ oscillations but also mitochondria activity were suppressed by Tha that induced blocking of ER Ca^{2+} refilling. Finally, oocytes cannot be activited under SERCAs inhibition.

Recently, a member of the TRP channels family, TRPM7, was found to be expressed in mouse GV, MII oocytes and 2-cell embryos [28]. TRPM7 belongs to the subfamily of melastatin and exhibits a ubiquitous tissue distribution. Trpm7 knock-out caused E14.5 embryonic lethality [29]. Using inhibitor NS-8593 suppression of the transporter hours after activation reduced progression to the blastocyst stage [28]. Oocytes treated with 10 μM NS-8593 and fertilized in vitro display impaired Ca^{2+} oscillations [30]. We applied NS-8593 with gradient concentrations of 0.1, 1, and 5 μM (Table 1 and Fig. 3A). After treating with 5 μM NS-8593, $[Ca^{2+}]_i$ slowly rose, which finally induced oocyte death. Treatment with 1 μM NS-8593 kept the survival rate at 37.5% (Fig. 3), while the $[Ca^{2+}]_i$ oscillations and pronuclear formation were sufficiently suppressed. Under such moderate concentration of 1 μM NS-8593, the effects of TRPM7 on mitochondrial activity can be observed for a long term. Mitochondrial activity did not exhibit the same activation state as the Ctrl in the case of TRPM7 inhibition with NS-8593. Mitochondrial membrane potential continually decreased when inhibiting the transport of Ca^{2+} through TRPM7 with 1 μM NS-8593 (Fig. 3). The TRPM7 on the cell membrane of oocytes has a significant effect on the $[Ca^{2+}]_i$ oscillation patterns in oocytes, and $[Ca^{2+}]_i$ oscillations achieved through TRPM7 is important for mitochondrial activity and oocyte activation.

Mibefradil is a T-type channel inhibitor. Mibefradil was developed as a cardiovascular hypertension and angina remedy [31]. Mibefradil has been repurposed as an anti-cancer drug [32]. However, its underlying mechanisms are still unclear. The mechanism of the anti-cancer therapy is thought to be via the blockage of Ca^{2+} influx through T-type channels. Mibefradil blocks all three subtypes of T-type channels, including Cav3.1, Cav3.2, and Cav3.3, with an IC50 (Semi-lethal concentration) of 5.8–7.2 μM. In our study, inhibition of Ca^{2+} release by high concentrations of Mibefradil impaired intracellular Ca^{2+} dynamics and thus affected cell viability. We applied Mibefradil at three gradient concentrations of 0.5, 5, and 10 μM

(Table 1 and Fig. 4). In order to observe for a long term, a moderate concentration of 5 μM NS-8593 was selected to study the effects of T-type channels on mitochondrial activity. The mitochondrial membrane potential continued to decrease after inhibiting the transport of Ca^{2+} through T-type Ca^{2+} channels with 5 μM Mibefradil. When the concentration reached 10 μM, Ca^{2+} rapidly increased, which induced rapid death in most oocytes. Thus, transport of Ca^{2+} through T-type Ca^{2+} channels is important for mitochondrial activity and oocyte activation.

Orais are 4 transmembrane proteins that form highly Ca^{2+}-selective channels [33]. Orais has three family members, ORAI1, 2 and 3 [34]. Loss-of-function mutation of ORAI1 caused immune deficiency [35] and dysfunction of thrombus formation [36]. SOCE is also mediated through the ORAI channels at the outer membrane. 10 μM GSK-7975A has been reported to induce maximal inhibition of Ca^{2+} influx in Jurkat T-cells [37]. We applied GSK-7975A at gradient concentrations of 10, 100 μM, and 1 mM. We found that oocytes could not be activated when GSK-7975A below 1 mM was used to effectively inhibit Ca^{2+} influx. Mitochondrial dynamic membrane potential showed irregular changes compared to the Ctrl group (Fig. 5). Most of the oocytes survived up to 4 hours post activation even at a concentration as high as 1 mM, but none of them formed a pronucleus. Female mice were fertile after knocking out ORAI1 [30]. It is not yet clear whether this $[Ca^{2+}]_i$ oscillation pattern and membrane potential changes were caused by complete inhibition of Orai1 or by cytotoxicity induced by the high concentration of 1 mM. The role of Orai1 in the activation of oocytes requires additional evidence to confirm.

The mechanisms of initiation and maintenance of $[Ca^{2+}]_i$ oscillations are different [38]. However, it is unclear which transporters participate in the initiation or maintenance of $[Ca^{2+}]_i$ oscillations. The effects of several transporters on the maintenance of $[Ca^{2+}]_i$ oscillations were investigated in our study (Fig. 6). We found that the addition of Ruthenium Red, Thapsigargin and GSK-7975A all inhibited the maintenance of $[Ca^{2+}]_i$ oscillations. After addition of Mibefradil and NS-8593, the intracellular Ca^{2+} increased continuously, and the cell death rate was higher than in the ctrl and other groups. All these results suggest that three Ca^{2+} transporters, SERCAs, TRPM7 and T-type Ca^{2+} channels are invilved in both the initiation and maintenanceof $[Ca^{2+}]_i$ oscillations. Interestingly, the addition of Ruthenium Red and other inhibitors into culture of oocytes which had initiated $[Ca^{2+}]_i$ oscillations did not significantly influence oocyte activation (Fig. 6B), suggesting that oocyte activation required only a small amount of $[Ca^{2+}]_i$ oscillations.

In summary, we applied ER-associated Ca^{2+} transporter SERCAs inhibitor Thapsigargin, TRPM7 inhibitor NS-8593, T-type Ca^{2+} channels inhibitor Mibefradil, and Orai1 inhibitor GSK-7975A to understand the regulation of $[Ca^{2+}]_i$ oscillations and mitochondrial activity during oocyte activation, and showed inbibition of SERCAs, TRPM7 and T-type Ca^{2+} channels caused Ca^{2+} signaling disturbances, mitochondrial activity and subsequent oocyte activation and ebryinic development.

Abbreviations

GV	Germinal Vesicle
GVBD	Germinal Vesicle Breakdown
MII	Metaphase II
$[Ca^{2+}]_i$	Intracellular Ca^{2+} Concentration
$[Ca^{2+}]_m$	Mitochondrial Matrix Ca^{2+}Concentration
SERCAs	sarcoplasmic/endoplasmic reticulum Ca^{2+}ATPases
TRPM7	transient receptor potential (TRP) ion channel subfamily member 7
ER	Endoplasmic reticulum
ART	assisted reproductive technologies

Declarations

Consent for publication

The manuscript is approved by all authors for publication, and it is original research that has not been published previously, and is not being submitted to any other journal in whole or in part.

Availability of Supporting Data

Some or all data used during the study are available from the corresponding author by request.

Competing Interests

I would like to state that there is no conflict of interest or financial disclosure to declare for this manuscript.

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Author contributions

Feng Wang, Qing-Yuan Sun and Xiang-Hong Ou conceived and designed the experiments. Feng Wang and others conducted experiments. Feng Wang and Qing-Yuan Sun analyzed the data. Feng Wang, Heide Schatten, Qing-Yuan Sun and Xiang-Hong Ou wrote the manuscript.

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Figures

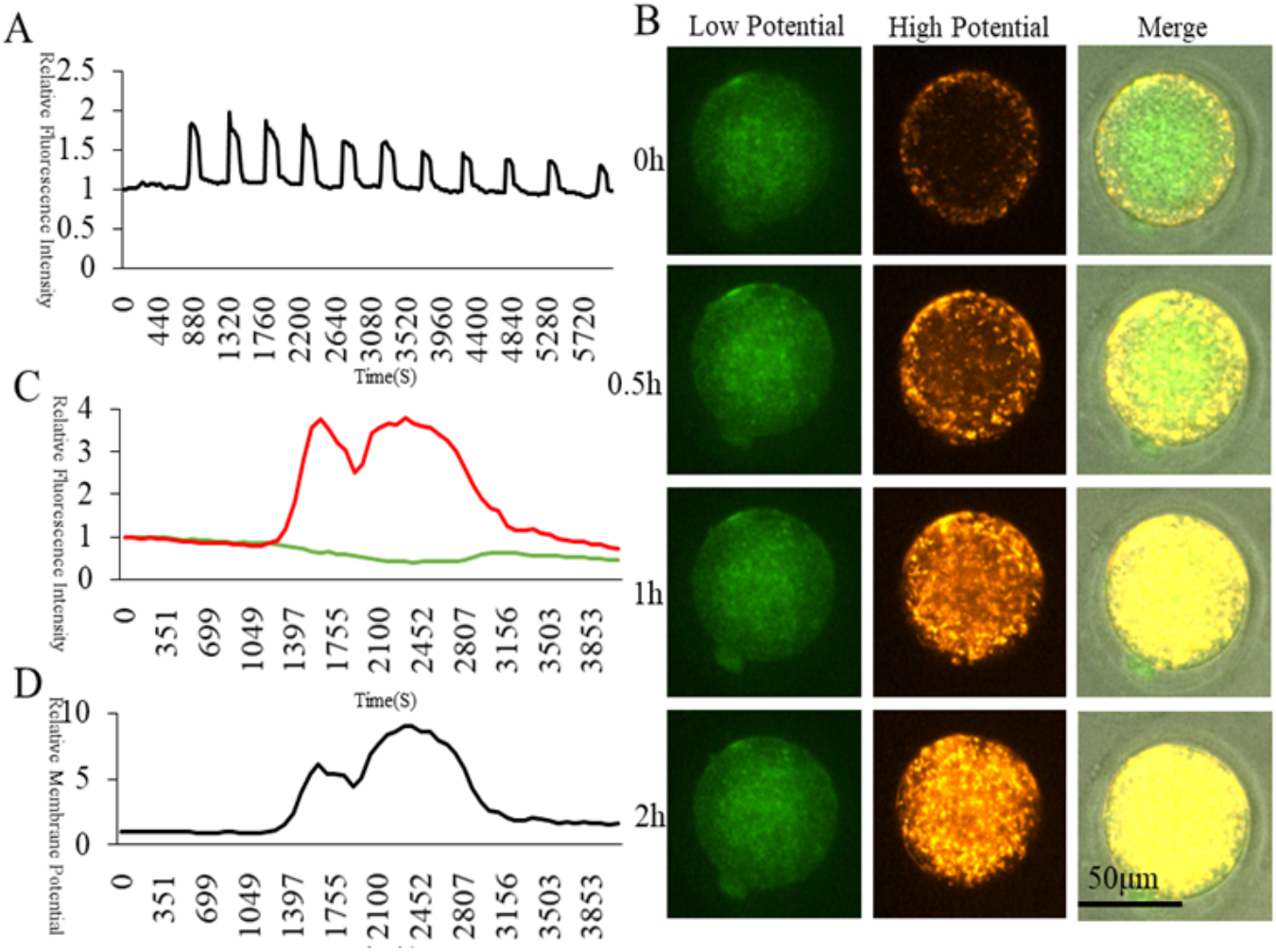


Figure 1

[Ca2+]i and mitochondrial membrane potential of wildtype activated oocytes. (A) Cytoplasmic calcium concentrations ([Ca2+]i) showing dynamic changes in wildtype oocytes during activation. (B) Oocyte mitochondrial membrane potential of wildtype oocytes during activation. (C) Membrane potential fluorescence intensity during oocyte activation. The green and red curves represent labeling with JC-1, indicating relative fluorescence intensities of low membrane potential (488nm excitation, 525nm emission) and high membrane potential (561 excitation, 590 emission), respectively. (D) The black curve shows the ratio of high membrane potential to low membrane potential indicating relative mitochondrial membrane potential.

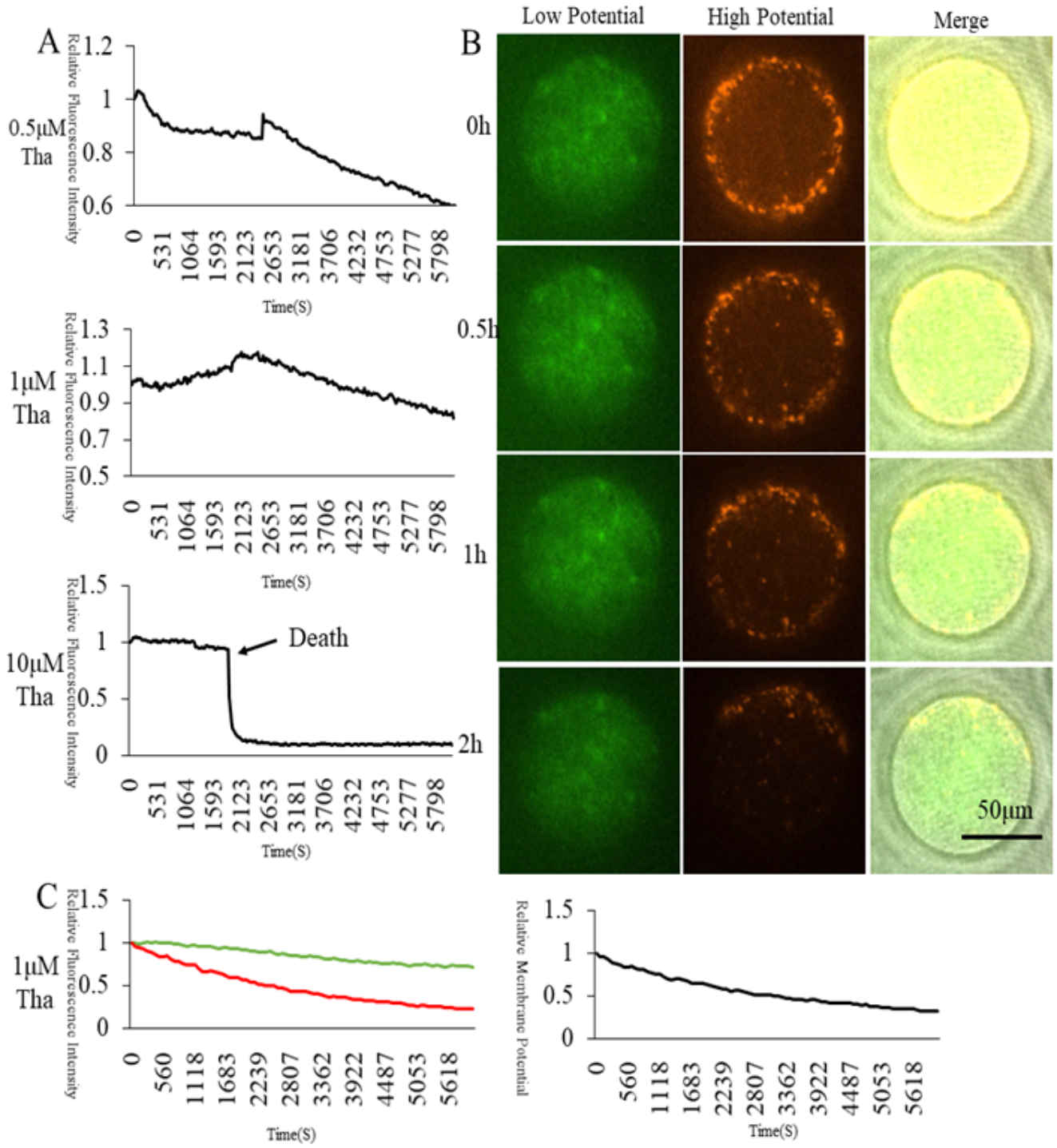


Figure 2

Effect of SERCAs inhibitor Thapsigargin on oocyte activation. (A) Cytoplasmic ([Ca2+]i) dynamic changes of Thapsigargin-inhibited oocyte during activation. (B) Mitochondrial membrane potential of 1μM Thapsigargin-inhibited oocytes during activation. (C) Mitochondrial membrane potential fluorescence intensity of 1μM Thapsigargin-inhibited oocytes. The green and red curves represent labeling with JC-1, indicating relative fluorescence intensities of low membrane potential and high

membrane potential, respectively. The black curve shows the ratio of high membrane potential to low membrane potential indicating relative mitochondrial membrane potential.

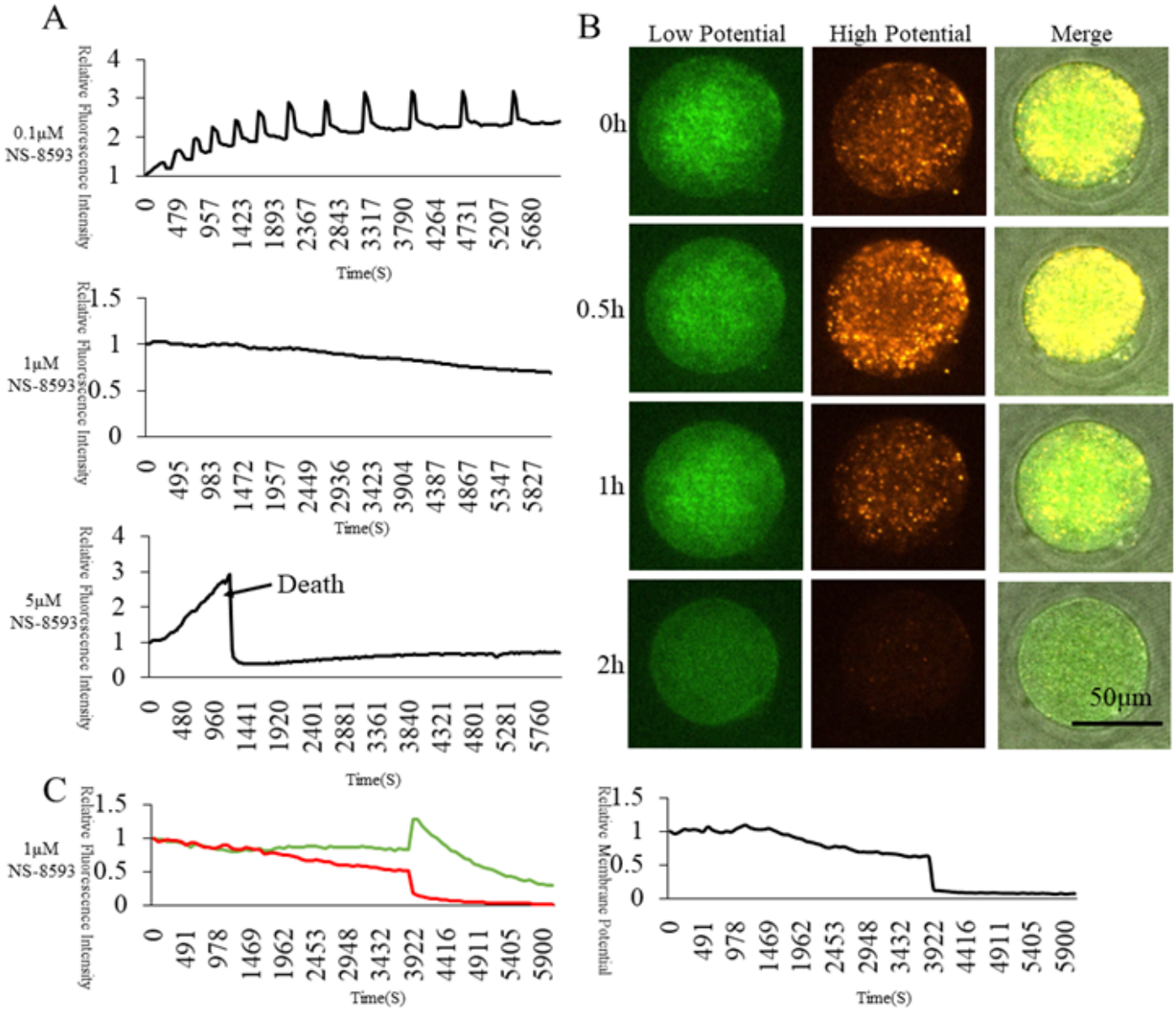


Figure 3

Effect of TRPM7 inhibitor NS-8593 on oocyte activation. (A) [Ca2+]i oscillations of NS-8593-inhibited oocytes. (B) Mitochondrial membrane potential dynamic changes of 1μM NS-8593-inhibited oocytes. (C) Mitochondrial membrane potential fluorescence intensity of 1μM NS-8593-inhibited oocytes. The green and red curves represent low and high membrane potential, respectively. The black curve shows the ratio of high membrane potential to low membrane potential indicating relative mitochondrial membrane potential.

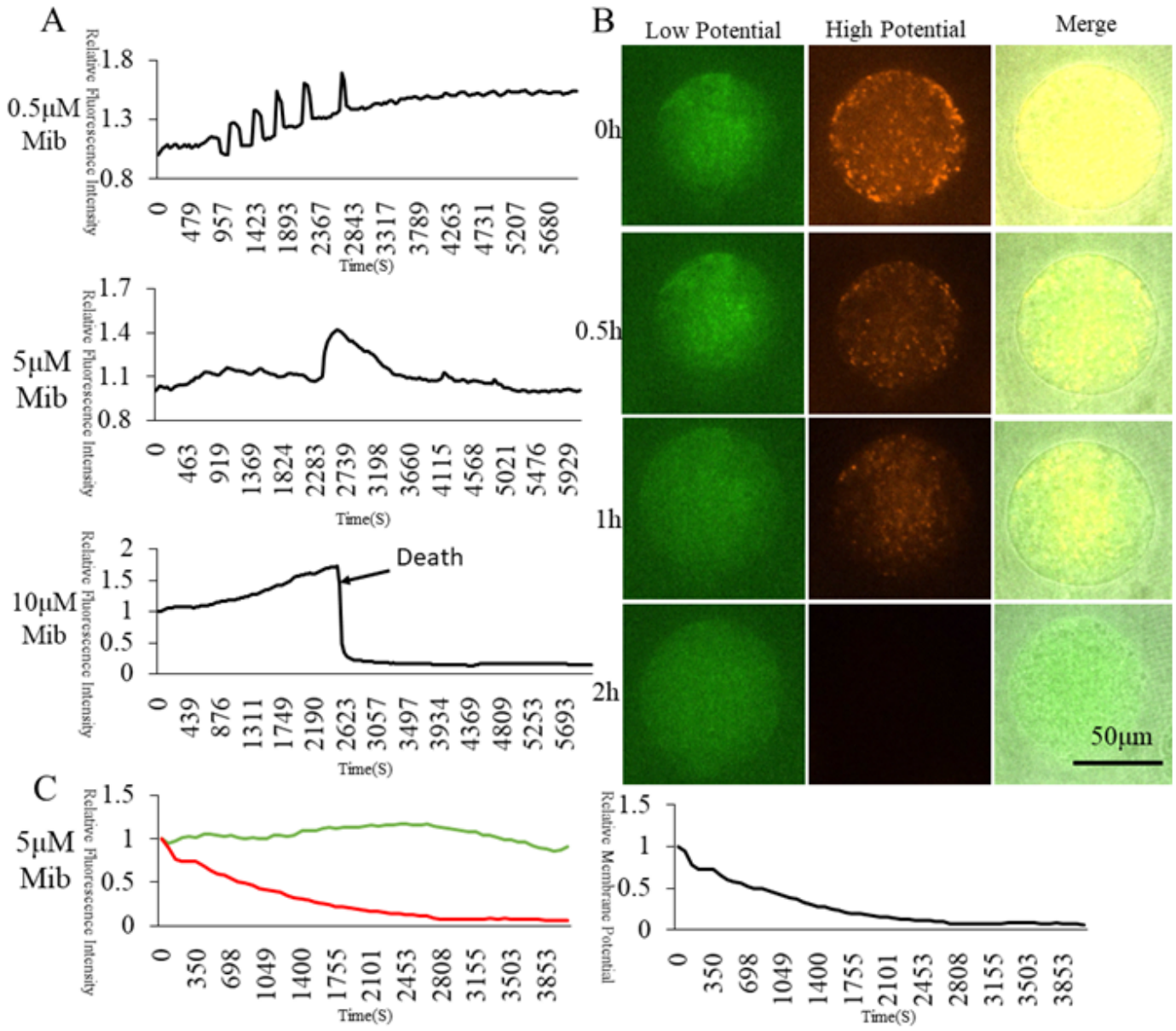


Figure 4

Effect of T-type Ca2+ channel inhibitor Mibefradil on oocyte activation. (A) [Ca2+]i oscillations of Mibefradil-inhibited oocytes. (B) Mitochondrial membrane potential dynamic changes of 5μM Mibefradil-inhibited oocytes. (C) Mitochondrial membrane potential fluorescence intensity of 5μM Mibefradil-inhibited oocytes. The green and red curves represent low and high membrane potential, respectively. The black curve shows the ratio of high membrane potential to low membrane potential indicating relative mitochondrial membrane potential.

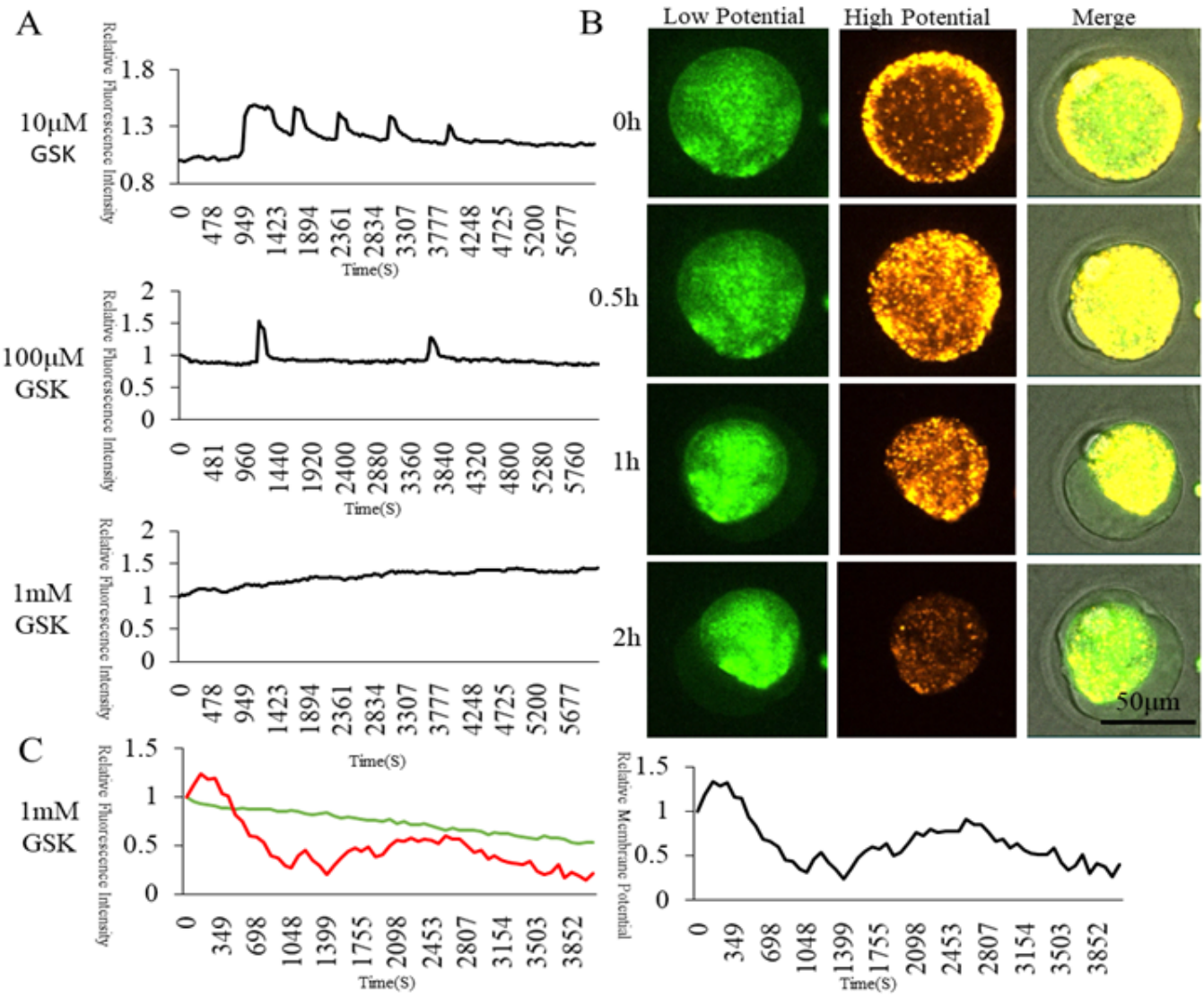
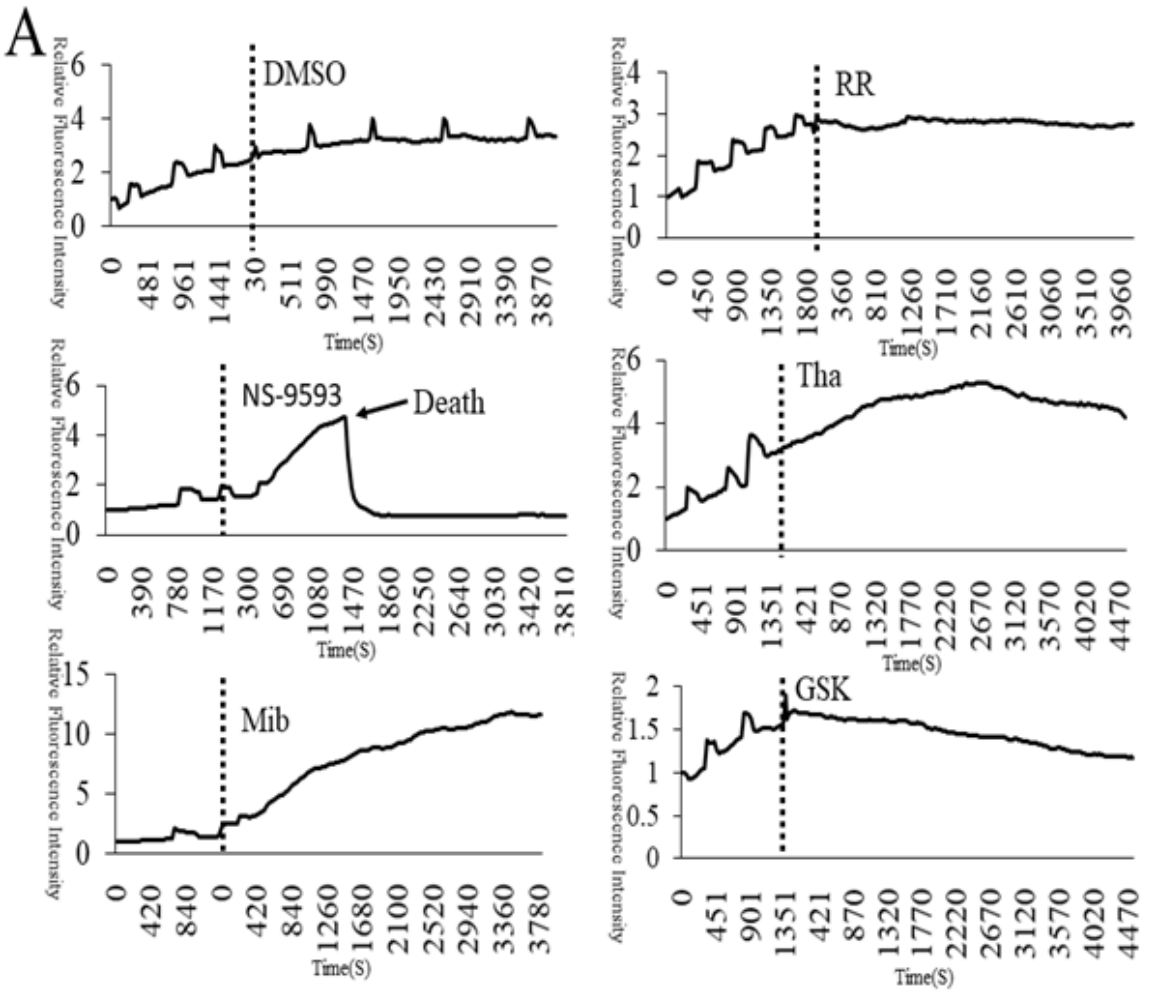


Figure 5

Effect of Orai1 inhibitor GSK-7975A on oocyte activation. (A) [Ca2+]i oscillations of GSK-7975A-inhibited oocytes. (B) Mitochondrial membrane potential dynamic changes of 100μM GSK-7975A-inhibited oocytes. (C) Mitochondrial membrane potential fluorescence intensity of 100μM GSK-7975A-inhibited oocytes. The green and red curves represent low and high membrane potential, respectively. The black curve shows the ratio of high membrane potential to low membrane potential indicating relative mitochondrial membrane potential.



B

	No oocytes	Repeats	PA	PN	2-Cell
Ctrl	60	3	96.1±5.72%	84.4±12.14%	85.7±8.26%
RR	60	3	72.4±11.50%	60.4±7.36%*	77.6±6.53%
Tha	60	3	71.6±4.34%	71.0±5.67%	50.0±11.50%*
GSK	60	3	85.1±11.36%	62.9±4.66%*	55.0±15.8%*
Mib	60	3	17.3±2.35%*	65.0±5.40%	62.6±11.40%
NS-8593	60	3	7.1±1.03%*	x	x

Figure 6

Inhibitor effects on on long-lasting [Ca2+]i oscillations. (A) Cytoplasmic [Ca2+]i dynamic changes after inhibitor addition. (B) Development of inhibitor addition following [Ca2+]i oscillations initiation. PA indicates survival of embryos of all MII oocytes. PN indicates pronuclear embryos of surviving oocytes. 2-Cell indicates cleaved embryos of pronuclear embryos. RR: 10μM Ruthenium Red; Tha: 1μM Thapsigargin; GSK: 100μM GSK-7975A; Mib: Mibefradil; N: 1μM NS-8593. The significance of differences

between groups was analyzed by the Chi-square test and $p<0.05$ (*) was considered statistically significant. (X) indicates data unavailable.