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Intracellular Ca^{2+} during fertilization and artificial activation in mouse oocytes[1]

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KEY WORDS mice; oocytes; ovum; calcium; fertilization; electric stimulation

AIM: To study the mechanism of oocyte activation in mammals. **METHODS**: Mouse oocytes arrested at metaphase of the second meiotic division were loaded with Fura 2-AM and then activated with ethanol, calcimycin, electric pulse or fertilization. Intracellular free Ca^{2+} during activation were measured by Spex AR-CM-MIC cation system. Cortical granule exocytosis after activation was detected under electron microscopy. **RESULTS**: Sperm penetration initiated a long lasting Ca^{2+} oscillation in Ca^{2+} containing IVF medium in mouse ova. The Ca^{2+} oscillation lasted for over 3 - 4 h until the ova developed to pronuclear stage. The Ca^{2+} oscillated faster as extracellular Ca^{2+} concentration was increased from normal 1.7 mmol·L^{-1} to 5.0 mmol·L^{-1} and ceased to oscillate when extracellular Ca^{2+} was removed. The ova resumed Ca^{2+} oscillation after transferred back to IVF (Ca^{2+} 1.7 mmol·L^{-1}). The ova which exhibited Ca^{2+} oscillation all extruded second polar body and formed pronuclei. Suppression the Ca^{2+} oscillation by intracellular injection of egtazic acid (2 - 10 pL, 200 μmol·L^{-1}) blocked the activation of oocytes. Heparin (100 μmol·L^{-1}) injection failed to prevent the Ca^{2+} oscillation. In artificial activation, ethanol, calcimycin, and a single electric pulse usually induced a monotonic Ca^{2+} rise and resulted in the activation only in older oocytes (> 18 h after CG injection) Activation of freshly ovulated oocytes required multiple intracellular Ca^{2+} increases induced by repetitive electric pulses. Artificial activation elicited the similar cortical granule exocytosis in oocytes as occuring at fertilization. Suppression of the intracellular Ca^{2+} elevation by introduction of egtazic acid into the oocytes blocked the activation process. **CONCLUSIONS**: The increase of intracellular free Ca^{2+} is the primary signal responsible for the initiation of oocyte activation but there are distinct differences between fertilization and artificial activation both in Ca^{2+} change patterns and Ca^{2+} sources. Young oocytes require oscillatory Ca^{2+} signals for activation.

Mammalian unfertilized ova can be artificially activated by a variety of chemical and physical stimuli such as ethanol, calcimycin, and electric stimulation. Mammalian oocyte exhibits a regular Ca^{2+} oscillation in intracellular Ca^{2+} ($[Ca^{2+}]_i$) during fertilization[1,2]. It was assumed that this Ca^{2+} oscillation played an important role in inducing the activation process[1-4]. If artificial stimulation can induce exactly the same intracelluar changes as occuring at fertilization is an open question. In this report we investigated the $[Ca^{2+}]_i$ change patterns and Ca^{2+} sources in fertilized and artificially activated mice oocytes and studied the role of Ca^{2+} in oocyte activation.

MATERIALS AND METHODS

Preparation of oocytes Oocytes were collected and manipulated in M_2[4], and were recovered by flushing the oviducts from mated and unmated Kunming albino mice, superovulated by injection of equine gonadotrophin (EG) 10 IU followed 48 h later by injection of chorionic gonadotrophin (CG) 10 IU. *In vivo* fertilized ova were retrieved from mated mice with visible vaginal plug 13 - 14 h after CG injection as fertilization usually occurs 13 h after the injection of CG or mating[7]. Oocytes used for artificial activation were recovered 13 h and 18 h after CG injection. The cumulus cells surrounding the oocytes were removed by culturing in M_2 containing 0.1 % hyaluronidase for 3 - 5 min. Following each treatment the oocytes were extensively washed with M_2.

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Media and reagents Fertilized ova and artificially activated oocytes were cultured in an improved medium by Charot, Ziomek, and Bavister (CZB)[5]. The intracellular free Ca^{2+} in fertilized ova was measured in *in vitro* fertilization medium (IVF)[4]. Electric pulsing medium was composed of mannitol 0.3 mol · L^{-1} & Ca^{2+} 50 μmol · L^{-1} [6]. All media were prepared with AR and ultra-pure water (>18.0 MΩ). Bovine serum albumin was added to the medium prior to use. Fura-2 acetoxymethyl ester (Fura 2-AM, Sigma) was used as Ca^{2+} fluorescent probe, which was disolved in anhydrous Me_2SO (2 mmol · L^{-1} stock solution). Heparin (Sigma, M_r = 3500) was used as the inhibitor of IP_3 activated intracellular calcium store.

Artificial activation methods and apparatus Oocytes were stimulated by exposing to 7 % - 8 % ethanol[8] or calcimycin 0.95 μmol · L^{-1} [4] for 5 - 6 min. After several rinses in M_2 the oocytes were cultured in CZB at 5 % CO_2 + 95 % air at 37 ℃ for 3 - 4 d to assess their developmental capacities. For electric activation, oocytes were transferred to a pulsing chamber containing the pulsing medium[6] and stimulated by DC pulse (at field strength of 170 kV/m and duration of 80 - 100 μs). The pulsing chamber was arranged in parallel by platinum electrodes glued to a thin glass coverlip (0.2 mm depth). The gap between the 2 electrodes was 0.75 mm. The diameter of platinum wire was 0.3 mm. This modified chamber made oocytes pulsed and their $[Ca^{2+}]_i$ changes monitored simultaneously under the same set of conditions. Oocyte activation was evaluated according to the formation of normal pronucleus 4 - 6 h after stimulation.

Measurement of intracellular Ca^{2+} The collected *in vivo* fertilized ova and unfertilized ova waiting for artificial activation were preloaded with Fura 2-AM 5 μmol · L^{-1} in M_2 medium + 10 % fetal calf serum at 37 ℃ for 30 min. After loading the oocytes were extensively washed with M_2. The fluorescence intensities of fertilized ova were monitored for >1 h. The Fura 2-AM loaded unfertilized oocytes were artificially activated and $[Ca^{2+}]_i$ was measured by an inverted Nikon diaphot epifluorescence microscope equipped with a Spex (USA) made AR-CM-MIC cation monitoring system. The fluorescence of single oocyte was recorded every 2 s.

Microinjection of drugs into oocytes To suppress the intracellular increase of $[Ca^{2+}]_i$, egtazic acid 200 μmol · L^{-1} was injected to the cytoplasm of the oocytes. IP_3 activated intracellular calcium store was inhibited by injection of heparin 100 μmol · L^{-1} into the oocytes. The injection volume was 2 - 10 pL.

To assess the cortical granule exocytosis following artificial activation and fertilization, the oocytes were fixed in 3 % glutaraldehyde[9] after fertilization or artificial stimulation.

RESULTS

Intracellular Ca^{2+} in fertilized ova We detected 20 *in vivo* fertilized ova and 16 of them exhibited Ca^{2+} oscillations (from basal level of 40 - 90 nmol · L^{-1} to the peak level of 500 - 800 nmol · L^{-1}) in IVF medium containing Ca^{2+} 1.7 mmol · L^{-1} (Fig 1).

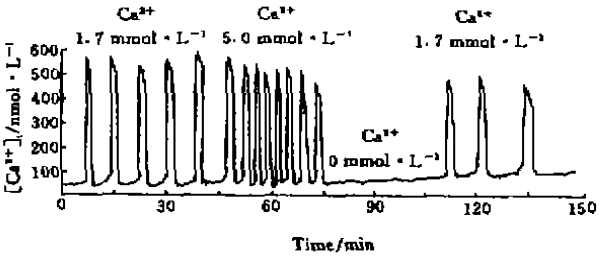

Fig 1. Effect of extracellular Ca^{2+} on fertilization-induced Ca^{2+} oscillation.

The Ca^{2+} oscillation continued until pronucleus was formed in the ova. The increase of $[Ca^{2+}]_i$ was not observed in other 4 ova during the experiment. After the measurement of Ca^{2+}, the oocytes were evaluated for sperm penetration and pronuclear formation under phase contrast microscope. The 4 ova showing no $[Ca^{2+}]_i$ changes contained visible pronuclei. The $[Ca^{2+}]_i$ oscillated faster when the fertilized ova were transferred to the IVF containing Ca^{2+} 5.0 mmol · L^{-1} and vanished after transferred to IVF without Ca^{2+} and later resumed Ca^{2+} oscillation after extracellular Ca^{2+} was restored to control level (Fig 1). Introduction of heparin (100 μmol · L^{-1}) to cytoplasm of oocytes failed to prevent the Ca^{2+} oscillation.

After the long lasting Ca^{2+} oscillation the fertilized ova extruded the second polar body, formed the pronuclei and then cleavaged to blastocyst *in vitro* while injection of egtazic acid (200 μmol · L^{-1}) into the oocytes blocked the above mentioned process.

Intracellular Ca^{2+} during artificial activation When oocytes were submerged in M_2 containing 7 % - 8 % ethanol $[Ca^{2+}]_i$ rised to the peak and remained at that level (Fig 2) and declined to basal level after the oocytes were transferred to M_2 without ethanol.

Calcimycin induced a large monotonic Ca^{2+} increase in M_2 (Fig 2). In Ca^{2+}-free or Ca^{2+}-

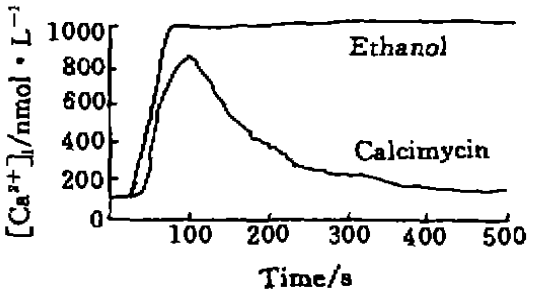

Fig 2. Oocytes in ethanol and after exposure to calcimycin.

chelated M_2, ethanol and calcimycin still induced a small $[Ca^{2+}]_i$ elevation. While injection of heparin to the oocytes suppressed the $[Ca^{2+}]_i$ increase.

An electric pulse induced an abrupt $[Ca^{2+}]_i$ increase and the amplitude of $[Ca^{2+}]_i$ transient was determined by both the electric parameters and the extracellular Ca^{2+} level. The intenser and the higher the electric stimulation was, the higher the $[Ca^{2+}]_i$ was induced. A single pulse failed to induce repetitive Ca^{2+} rises unless an additional stimulation was applied. There was an exact one-to-one correspondence between an electric pulse and a $[Ca^{2+}]_i$ transient (Fig 3).

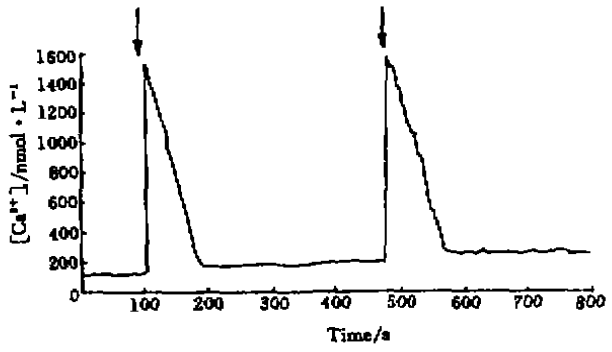

Fig 3. Multiple electric stimulations induced repetitive $[Ca^{2+}]_i$ transients in mouse oocyte. The arrows indicate electric discharge.

In pulsing medium devoid of Ca^{2+}, electric stimulation did not induce any $[Ca^{2+}]_i$ elevation even if stronger field strength and longer pulse duration were imposed.

A single increase of $[Ca^{2+}]_i$ was enough to induce activation in older oocytes (>18 h after CG) but was futile in activating the young oocytes (<13 h after CG). The young oocytes were activated only by multiple $[Ca^{2+}]_i$ rises induced by repetitive pulses.

Electron microscopy study showed that artificially activated oocytes exhibited a similar cortical granule exocytosis as *occurring* at fertilization. The parthenogenetically activated oocytes resumed second meiotic division and developed to the morphologically normal blastocysts *in vitro* just like fertilized ova.

DISCUSSION

In mammalian animals, fertilized ova showed a regular Ca^{2+} oscillation. Artificially increasing intracellular Ca^{2+} concentration in M Ⅱ oocytes induced the activation process such as cortical granule exocytosis, Pb Ⅱ extrusion, pronuclear formation, cleavage and embryonic development. Suppression the elevation of $[Ca^{2+}]_i$ blocked the activation of oocyte. This demonstrates that the increase of intracellular free Ca^{2+} is the primary signal responsible for the initiation of oocyte activation.

High level of extracellular Ca^{2+} accelerated the fertilization-induced Ca^{2+} oscillation while removal of the extracellular Ca^{2+} hampered the Ca^{2+} oscillation. It suggests that extracellular Ca^{2+} plays an important role in maintaining Ca^{2+} oscillation. The detailed pathway of Ca^{2+} influx is unknown. Some authors proposed that IP_3 activated intracellular calcium release (IICR) was the main mechanism in fertilization-induced Ca^{2+} oscillation[2]. In our experiment heparin failed to suppress the Ca^{2+} oscillation, implying that Ca^{2+} oscillation is not solely dependent on IICR in mouse ova. It is difficult to find a strong inhibitor to completely block the influx of extracellular Ca^{2+}, so the role of IICR remains to be investigated. Ethanol and calcimycin-induced monotonic $[Ca^{2+}]_i$ elevation was produced both by IP_3 activated intracellular calcium release and influx of extracellular Ca^{2+}. Electrically induced $[Ca^{2+}]_i$ increase came mainly from the influx of extracellular Ca^{2+} through electroporation on cell membrane[11]. This suggests that artificially induced $[Ca^{2+}]_i$ increases are different from fertilization-induced

Ca^{2+} oscillation both in Ca^{2+} change pattern and Ca^{2+} sources.

It seems paradoxical that if a single increase of $[Ca^{2+}]_i$ is enough to initiate the oocyte activation, what is the biological significance of Ca^{2+} oscillation in fertilized ova? It should be noted that artificially inducing the monotonic $[Ca^{2+}]_i$ elevation activated only older oocytes effectively but was futile in activating recently ovulated oocytes which were normally fertilized by spermatozoon. The freshly ovulated oocytes were activated by repetitive $[Ca^{2+}]_i$ rises induced by multiple electric pulses, suggesting that the activation of recently ovulated oocytes requires oscillatory Ca^{2+} signals.

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受精和人工激活诱导的小鼠卵内游离钙离子变化[1]

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关键词 小鼠; 卵母细胞; 卵; 钙; 受精; 电刺激

A **目的**: 研究哺乳类动物卵母细胞激活机制 **方法**: 将休止于第二次成熟分裂中期的小鼠卵母细胞负载 Fura 2-AM 后用乙醇, 钙离子载体, 电脉冲或受精等激活; 采用 Spex AR-CM-MIC 阳离子检测系统测定卵激活过程中细胞内游离 Ca^{2+} 浓度变化; 电镜下检测卵激活后皮质颗粒释放 **结果**: 在含 Ca^{2+} (1.7 mmol·L^{-1}) 的 IVF 液中, 精子受精诱发卵内 Ca^{2+} 浓度波动, 这种 Ca^{2+} 波动持续达 3 - 4 h 直至受精卵发育至原核期消失 当外 Ca^{2+} 升高至 5.0 mmol·L^{-1} 时 Ca^{2+} 波动加快; 去除外 Ca^{2+} 后 Ca^{2+} 波动很快消失; 将卵移入含 Ca^{2+} IVF 后又恢复 Ca^{2+} 波动 出现 Ca^{2+} 波动的卵随后均排出第二极体并形成原核 胞内注射 Ca^{2+} 整合剂依他酸 (200 μmol·L^{-1}) 抑制受精诱导的 Ca^{2+} 波动则阻止卵激活. 胞内注射肝素 (MW = 3500, 100 μmol·L^{-1}) 不能阻止受精 Ca^{2+} 波动 乙醇, 钙离子载体和 1 次电脉冲刺激诱导卵内 Ca^{2+} 浓度升高 1 次并且仅诱导大龄卵 (> 18 h CG) 激活 刚排出卵的激活则需要 $[Ca^{2+}]_i$ 多次升高诱导人工激活诱导卵出现与受精相似的皮质颗粒释放. 卵内注射 Ca^{2+} 整合剂抑制 $[Ca^{2+}]_i$ 升高则阻断卵激活的一系列反应. **结论**: 细胞内游离 Ca^{2+} 升高是诱导卵激活的重要信号; 受精与人工激活诱导的 $[Ca^{2+}]_i$ 升高及 Ca^{2+} 来源存在明显差异; 小龄卵激活需要 $[Ca^{2+}]_i$ 波动信号诱导

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