Role of the extracellular Ca^{2+} on the intracellular Ca^{2+} changes in fertilized and activated mouse oocytes

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Changes in intracellular calcium concentration ($[\text{Ca}^{2+}]_i$) in fertilized mouse oocytes were measured using the calcium-sensitive dye, fura-2. After fertilization, an initial long-lasting $[\text{Ca}^{2+}]_i$ increase was followed by a periodic $[\text{Ca}^{2+}]_i$ increase. The periodic increase in $[\text{Ca}^{2+}]_i$ persisted for 1 to 3 h and all fertilized oocytes extruded the second polar body within 4 h. The mean interval of periodic $[\text{Ca}^{2+}]_i$ increase was 470 ± 180 s (mean $\pm$ SD). The frequency of the periodic $[\text{Ca}^{2+}]_i$ increase depended on the extracellular calcium concentration. Verapamil and nifedipine inhibited the periodic $[\text{Ca}^{2+}]_i$ increase. Sixty-five per cent of tested cells extruded the second polar body within 90 min of exposure to 7% ethanol. In these activated oocytes, the long-lasting $[\text{Ca}^{2+}]_i$ increase was observed. However, no cells showed a repetitive increase in $[\text{Ca}^{2+}]_i$. Both release of calcium from intracellular stores and influx of extracellular calcium contribute to the increase in $[\text{Ca}^{2+}]_i$ induced by ethanol. We conclude that the extrusion of the second polar body requires an increase in $[\text{Ca}^{2+}]_i$ above a certain threshold level and the mobilization of calcium of both the intracellular and extracellular space in mouse oocytes.

Introduction

Intracellular calcium concentration ($[\text{Ca}^{2+}]_i$) starts to increase in a periodic manner after fertilization in oocytes of mammalian species including mice (Cuthbertson and Cobbold, 1985), hamsters (Miyazaki and Igusa, 1981, 1982) and rabbits (McCulloh *et al.*, 1983). In electrophysiological studies, it was found that fertilized hamster (Igusa and Miyazaki, 1983) and mouse (Igusa *et al.*, 1983) oocytes showed periodic hyperpolarization which was superimposed on the sustained hyperpolarization. Detailed studies of hyperpolarization responses in hamster oocytes revealed that both the sustained and periodic hyperpolarization depended on the extracellular calcium (Igusa and Miyazaki, 1983). Studies using a calcium-sensitive electrode in hamster oocytes showed that the increase in $[\text{Ca}^{2+}]_i$ synchronized with periodic hyperpolarization, which reflects the increase in calcium-activated potassium conductance (Igusa and Miyazaki, 1986). Thus, the increase in $[\text{Ca}^{2+}]_i$ is strongly related to the initiation of fertilization in hamster oocytes (Miyazaki and Igusa, 1981, 1982, 1983, 1986). A periodic $[\text{Ca}^{2+}]_i$ increase was also observed in mouse oocytes after the initiation of fertilization although the characteristics were not investigated in detail (Cuthbertson *et al.*, 1981; Cuthbertson and Cobbold, 1985).

Studies on mouse oocytes showed that the second meiosis can be resumed by the activator of oocytes without fertilization

(Steinhardt *et al.*, 1974; Kaufman, 1978, 1982; Siracusa *et al.*, 1978; Cuthbertson, 1983). The oocytes can extrude the second polar body after exposure to ethanol, an activator, and develop to the stage of blastomere. There was an increase in $[\text{Ca}^{2+}]_i$ when oocytes were stimulated by exposure to ethanol, whereas a periodic increase in $[\text{Ca}^{2+}]_i$ was not observed (Cuthbertson *et al.*, 1981; Cuthbertson, 1983). Thus, in spite of the absence of a periodic $[\text{Ca}^{2+}]_i$ increase, the second meiosis could be resumed.

In the present experiments, we investigated the characteristics of the increase in $[\text{Ca}^{2+}]_i$ after the initiation of fertilization in mouse oocytes using the calcium-sensitive dye, fura-2. We also studied the relationship between the increase in $[\text{Ca}^{2+}]_i$ and extrusion of the second polar body induced by exposure to ethanol as a model of fertilization (Kaufman, 1978, 1982).

Materials and Methods

Preparation of media

The composition of the solutions used in the present experiments was as follows (in mmol l⁻¹). Modified human tubal fluid medium (HTF medium): NaCl 101.6, KCl 4.69, MgSO₄ 0.20, KH₂PO₄ 0.37, sodium lactate 21.4, glucose 2.78, NaHCO₃ 25.0, sodium pyruvate 0.33, CaCl₂ 2.04, penicillin (GIBCO Laboratories, NY) 100 u ml⁻¹, and saturation with 5% CO₂ in air. HTF-Hepes medium: NaCl 101.6, KCl 4.69, MgSO₄ 0.20, KH₂PO₄ 0.37, sodium lactate 21.4, glucose 2.78, NaHCO₃ 4.0, sodium pyruvate 0.33, CaCl₂ 2.04, sodium-Hepes 21.0, penicillin 100 u ml⁻¹, and the pH was adjusted to 7.5 with HCl. The

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calcium concentration in HTF–Hepes medium was changed with CaCl_2 if it was necessary, without changing the composition of other ions. NaOH–Hepes medium: NaCl 137.0, KCl 5.0, MgCl_2 1.0, CaCl_2 5.0, glucose 10.0, Hepes 5.0, and the pH was adjusted to 7.35 with NaOH. The final osmolarity of solutions was adjusted to 280 mosmol with distilled water. The final volume of distilled water added to adjust the osmolarity corresponded to 7.2% of an HTF medium, 11.3% of an HTF–Hepes medium and 3.6% of a NaOH–Hepes medium. All the solutions contained 4 mg BSA ml^{-1} (A4503, Sigma Chemical Co., St Louis, MO). Solutions used for the treatment of the oocyte with enzyme or Cell-Tak (Collaborative Research Inc., Bedford, UK) did not contain BSA. For calibration of calcium concentration K–Hepes medium was used (in mmol l^{-1}): KCl 130.0, NaCl 20.0, Hepes 10.0, and the pH was adjusted to 7.05 with KOH. Calcium concentrations of the K–Hepes medium varied from 10 nmol l^{-1} to 1 mmol l^{-1} . Unless otherwise mentioned, salts used in the present experiments were purchased from Wako Pure Chemical Industries Co. Ltd, Osaka.

Preparations

Oocytes. Unfertilized oocytes were obtained from 6–8-week-old B6C3F1 female mice by the hyperstimulation method (Yanagimachi, 1969). Pregnant mares' serum (PMS; 10 iu, Teikokuzohki, Tokyo) was injected into the peritoneal space. Two days later 10 iu human chorionic gonadotrophin (hCG; Mochida, Tokyo, Japan) was injected into the peritoneal space. Fifteen to sixteen hours after the second injection, the super-ovulated mice were killed by dislocation of the neck, and mature oocytes were collected from the oviducts. Oocytes were freed from cumulus cells by repeated aspiration with a glass capillary (bore size 100–130 μm) in an HTF medium containing 0.1% hyaluronidase (H3506, Sigma Chemical Co., St Louis, MO) at room temperature for dye loading.

Spermatozoa. ICR male mice, 8–12 weeks old, were killed by dislocation of the neck, and the cauda epididymis and spermiduct were resected. These were cut into small pieces in an HTF medium and kept at 37°C for 60–150 min in an incubator (gas phase, 5% CO_2 in air) to allow capacitation (Yanagimachi, 1969). After the capacitation the supernatant containing the spermatozoa was collected and used for the insemination.

Loading of fura-2

The oocytes were incubated in an HTF medium containing 5 $\mu\text{mol fura-2 AM l}^{-1}$ (Dojindo Laboratories, Kumamoto) for 30 min at 37°C in an incubator (gas phase, 5% CO_2 in air). The dye loaded oocytes were then treated with 25 iu α -chymotrypsin ml^{-1} (C4129, Sigma Chemical Co., St Louis, MO) in an HTF medium for 10–15 s at room temperature. The swollen zona pellucida was mechanically removed by aspiration with a glass capillary (bore size 100–130 μm).

Cuvette and perfusion of the solution

In the present experiments, aluminium (width 50 mm $\times$ 70 mm, depth 13 mm) with a centre hole (width 10 mm $\times$

6 mm, depth 13 mm) was used for the cuvette. The bottom of the centre hole was covered with quartz glass for the optical recording. During the experiments the upper side of the cuvette was covered with slide glass to prevent vaporization. The surrounding aluminium body was warmed by the water circulation set at 37°C by the heater (Digital Uni Ace type UA-1, Tokyo Rikakikai Co., Tokyo). The flow of the solution in the cuvette was controlled by two stainless steel tubes (inlet and outlet; inner diameter 1 mm) connected to the peristaltic pump (Micro tube pump type 201, Tokyo Rikakikai Co., Tokyo). The volume of the solution in the cuvette was controlled at about 600 μl . The temperature of solutions was controlled at 37°C. Exchange of the solutions in the cuvette was completed within 3 min in our system when solution was perfused at 3 ml min^{-1} . However, since the movement of the oocyte sometimes occurred when the solution was continuously perfused, experiments were carried out in a static condition if the perfusion was not necessary. If the solution exchange was necessary, the peristaltic pump was moved for 3–4 min. Solutions containing verapamil (V4629, Sigma Chemical Co.), nifedipine (N7634, Sigma Chemical Co.) or Bay K 8644 (Bayer, Wuppertal, Germany) were continuously perfused to keep the drug concentration at a steady level since they were light sensitive.

Fixation of oocytes on glass

Since movement of the oocytes by the flagellar motion of spermatozoa hampers stable optical measurement, nylon mesh (no 311, Swiss Nylon Monofilament, Switzerland) was used to fix the oocytes. Nylon monofilament net (5 mm $\times$ 7 mm) with a mesh opening size of 71 μm was immersed in 50 μl HTF medium containing 5 μl Cell-Tak for 30 min at 37°C. The nylon mesh was then anchored on the quartz glass in the cuvette by small pieces of stainless steel. The cuvette was set on an inverted microscope (TMD-EF, Nikon, Tokyo) and filled with an HTF–Hepes medium. The dye-loaded oocytes were placed on nylon mesh with a glass capillary (bore size 100–130 μm) and left for 10–15 min to allow adhesion to the nylon mesh to occur in a static condition. By this procedure oocytes could be settled stably in the nylon mesh and their fluorescent images were easily distinguished from one another. The fluorescent image of the nylon mesh was too weak to interfere with the optical measurement. Under these conditions, the change in fluorescence intensity of more than 10 oocytes could be measured simultaneously.

Insemination

Before insemination the volume of HTF–Hepes medium in the cuvette was corrected to 400 μl . Spermatozoa suspension, 60 μl , was added to the cuvette for insemination. The final concentration of the spermatozoa in the chamber was about 500 000 ml^{-1} . After the insemination oocytes were kept in a static state to allow fertilization.

Recording of the data

Fura-2 loaded to oocytes was excited by a Xe lamp with a wavelength of 340 nm or 380 nm. The duration of excitation

light was 130 ms for each wavelength. The emitted light was filtered by a dichroic mirror (passing wavelength > 420 nm). The intensity of the emitted light was digitized by a random access camera (C3329, Hamamatsu Photonics, Hamamatsu, Japan), and digitized data were sampled by computer (PC-9801, NEC, Tokyo). Sampling size of one digitized recording was $20\ \mu\text{m}$ in diameter at magnification scale of $\times 20$ (CF Fluor, Nikon, Tokyo). Data were sampled every 3 s for a 90 min recording and every 10 s for a 240 min recording. The sampled data were analysed by an off-line computer (PC-9801, NEC, Tokyo). Optical measurement was started within 4 min of insemination. All the experiments were carried out at 37°C .

Calibration of $[\text{Ca}^{2+}]_i$

In the present experiments, an *in vivo* calibration method reported in cardiomyocytes (Li *et al.*, 1987; Shibuya *et al.*, 1991) was applied. In brief, oocytes were incubated in K-Hepes medium containing $2\ \mu\text{mol}$ carbonyl cyanide-*m*-chlorophenyl-hydrazone l^{-1} (Aldrich Chemical Co., Milwaukee, WI), $0.5\ \text{mmol}$ monoiodoacetic acid l^{-1} (Wako Pure Chemical Industries, Osaka) and $2\ \text{mmol}$ NaCN l^{-1} (Wako Pure Chemical Industries) for 30 min at room temperature to eliminate energy dependent $[\text{Ca}^{2+}]_i$ changes (Li *et al.*, 1987). After the incubation, ionomycin ($2\ \mu\text{mol}\ \text{l}^{-1}$ at a final concentration in the chamber, Calbiochem Corporation, La Jolla, CA) was added to equilibrate $[\text{Ca}^{2+}]_i$ with the extracellular calcium concentration ($[\text{Ca}^{2+}]_o$). Fura-2 signal intensity recorded at excitation wavelength of 340 nm was normalized by that of 380 nm (F340:F380). $[\text{Ca}^{2+}]_i$ was expressed by the value of F340:F380.

$[\text{Ca}^{2+}]_i$ calibration curve in the mouse oocyte

When $2\ \mu\text{mol}$ ionomycin l^{-1} was added to the chamber with oocytes at $[\text{Ca}^{2+}]_o$ of $11.3\ \text{nmol}\ \text{l}^{-1}$, $[\text{Ca}^{2+}]_i$ decreased gradually and reached a steady state in 45 min (Fig. 1a). However, when the oocytes were bathed in $1\ \text{mmol}\ [\text{Ca}^{2+}]_o\ \text{l}^{-1}$, $[\text{Ca}^{2+}]_i$ increased quickly and reached a steady state within 30 min. Since the steady $[\text{Ca}^{2+}]_i$ state was assumed to be equilibrated with $[\text{Ca}^{2+}]_o$, $[\text{Ca}^{2+}]_i$ at the steady value may reflect the $[\text{Ca}^{2+}]_i$ equivalent to the $[\text{Ca}^{2+}]_o$ (Shibuya *et al.*, 1991). The threshold for the detection of $[\text{Ca}^{2+}]_i$ was at about $50\ \text{nmol}\ \text{l}^{-1}$. The value of F340:F380 increased in a sigmoidal fashion with increase in $[\text{Ca}^{2+}]_o$ and reached the maximum value at about $100\ \mu\text{mol}\ \text{l}^{-1}$ (Fig. 1b). The fitting of the sigmoid curve was carried out using the following theoretical equation (Grynkiewicz *et al.*, 1985):

$$p\text{Ca}_i = pK_F - \log\{(R_F - R_{F\min})/(R_{F\max} - R_F)\}, K_F = K_d \times \beta$$

where pK_F : negative logarithm of the apparent dissociation constant (K_F) for fura-2; $R_{F\min}$ and $R_{F\max}$: R_F values for unbound and bound forms of the fura-2- Ca^{2+} complex, respectively; K_d : dissociation constant for the fura-2- Ca^{2+} complex; β : ratio between maximum and minimum fluorescence intensities of fura-2 at 380 nm excitation. By numerical calculation for curve fitting, pK_F , $R_{F\min}$ and $R_{F\max}$ were determined as 5.63, 0.95 and 3.87, respectively. These values were used to calibrate $[\text{Ca}^{2+}]_i$ from the value of F340:F380 in the following

Fig. 1. (a) Time course of $[\text{Ca}^{2+}]_i$ change after the application of $2\ \mu\text{mol}$ ionomycin l^{-1} with various $[\text{Ca}^{2+}]_o$. At steady state $[\text{Ca}^{2+}]_i$ was assumed to be equilibrated with $[\text{Ca}^{2+}]_o$. The arrow indicates the application of ionomycin. Oocytes were kept at 37°C and extracellular pH was controlled at 7.05. Data were sampled every 3 s. (b) $[\text{Ca}^{2+}]_i$ calibration curve in the mouse oocyte. F340:F380 at steady state was plotted against $[\text{Ca}^{2+}]_o$. Symbols shown in the figure are average values and bars indicate SEM. Numbers of tested cells are shown in parentheses. Theoretical curve was fitted by eye ($n = 155$).

experiment. The basal value of $[\text{Ca}^{2+}]_i$ was measured before the experiment. The average value of basal $[\text{Ca}^{2+}]_i$ was $220 \pm 140\ \text{nmol}\ \text{l}^{-1}$ (mean $\pm$ SD, $n = 223$).

Results

Changes in $[\text{Ca}^{2+}]_i$ after fertilization

As the extrusion of the second polar body is a characteristic feature of fertilization, it was used to differentiate fertilized from unfertilized oocytes (Hogan *et al.*, 1986). In 65% of tested oocytes fertilization occurred during the 4 h observation ($n = 99$). In all of the fertilized oocytes an initial long-lasting $[\text{Ca}^{2+}]_i$ increase was followed by periodic $[\text{Ca}^{2+}]_i$ increases (calcium oscillation). This calcium oscillation lasted for 1–3 h (Fig. 2a, upper panel). However, no changes in $[\text{Ca}^{2+}]_i$ were observed in unfertilized oocytes until 4 h after the insemination. Thus calcium oscillation was strongly related to fertilization. Oocytes

Fig. 2. (a) $[Ca^{2+}]_i$ change (calcium oscillation) in the fertilized mouse oocyte. Oocytes used were collected 15–16 h after injection of human chorionic gonadotrophin. Upper panel, observation for 240 min. Lower panel, first 90 min observation. Solution: HTF–Hepes medium. (b) Distribution of latency. Recordings were made for 240 min ($n = 64$).

that showed calcium oscillation were therefore classified as fertilized in the following experiment. In the present experiments oocytes were inseminated 70–90 min after they were collected.

The latency of calcium oscillation after the insemination varied among oocytes (Fig. 2a) and ranged from 4 to 208 min (Fig. 2b, $n = 64$). In 53% of the tested oocytes calcium oscillation occurred within 30 min and in 75% calcium oscillation started within 60 min. However, the pattern of calcium oscillation was not modified by the duration of latent period.

The pattern of $[Ca^{2+}]_i$ increase was observed in detail by monitoring the change of $[Ca^{2+}]_i$ in the first 90 min in other fertilized oocytes ($n = 33$). The increase in $[Ca^{2+}]_i$ was initiated by a relatively long-lasting $[Ca^{2+}]_i$ increase (first response) (Fig. 2a). Soon after $[Ca^{2+}]_i$ increased, it reached a peak and showed small oscillations at the high value. The average value of $[Ca^{2+}]_i$ at the peak was $2.1 \pm 1.1 \mu\text{mol l}^{-1}$ and the duration of $[Ca^{2+}]_i$ increase was $320 \pm 100 \text{ s}$ ($n = 33$). The repetitive increase in $[Ca^{2+}]_i$ with a short duration followed the first response. In

most oocytes, within 30 min of the start of calcium oscillation the peak to peak time of the $[Ca^{2+}]_i$ increase (oscillation interval) was constant (upper panel, Fig. 2a). The oscillation interval was almost constant until 180 min after the insemination. In the steady state the oscillation interval was 230–840 s ($[Ca^{2+}]_o = 2.04 \text{ mmol l}^{-1}$, $470 \pm 180 \text{ s}$, mean $\pm$ SD, $n = 33$). The variation of the oscillation interval among oocytes was observed even when the calcium oscillation of more than ten oocytes was measured simultaneously, suggesting that the variation of the oscillation interval might reflect the difference of intrinsic factors among oocytes. However, the peak $[Ca^{2+}]_i$ concentration decreased gradually during the calcium oscillation.

The effect of $[Ca^{2+}]_o$ on the frequency of calcium oscillation

The frequency of calcium oscillation depended on $[Ca^{2+}]_o$ (Fig. 3). In this series of experiments oocytes were bathed in an HTF–Hepes medium containing 2.04 mmol l^{-1} $[Ca^{2+}]_o$ (control medium) for 60 min after insemination. The solution was then switched to an HTF–Hepes medium with different $[Ca^{2+}]_o$ (test medium). The frequency of calcium oscillation increased with 5 mmol l^{-1} $[Ca^{2+}]_o$ (Fig. 3a), but decreased with 0.5 mmol l^{-1} $[Ca^{2+}]_o$ (Fig. 3b). The oscillation interval in 5 mmol l^{-1} $[Ca^{2+}]_o$ was from 110 to 240 s ($n = 9$). When the solution was switched to a nominally calcium-free HTF–Hepes medium, calcium oscillation disappeared (Fig. 3c). Changes in the frequency of calcium oscillation were reversible (Fig. 3a–c).

The relationship between the oscillation interval and $[Ca^{2+}]_o$ is shown (Fig. 4). The mean oscillation interval in the control medium was calculated from the oscillation interval in the steady state. When a steady state was not achieved within the first 60 min, these preparations were not used for analysis. The mean oscillation interval in the test medium was normalized by the mean oscillation interval in the control medium. When the oscillation interval could not be measured because of a low frequency of calcium oscillation, the data were not used. The oscillation interval was longest at a concentration of 0.2 mmol l^{-1} $[Ca^{2+}]_o$ and became shorter with increasing $[Ca^{2+}]_o$.

Effects of calcium channel antagonists and agonist on the calcium oscillation

In this series of experiments oocytes were bathed in a control medium for 30 min after insemination. The medium was then switched to a NaOH–Hepes medium containing 5 mmol l^{-1} $[Ca^{2+}]_o$ to study the effect of drugs on the calcium oscillation.

Verapamil at $10 \mu\text{mol l}^{-1}$ inhibited the calcium oscillation (Fig. 5a, b). The calcium oscillation recovered after the washout of verapamil. The inhibitory effects of verapamil on the calcium oscillation were complete in seven oocytes (Fig. 5a) and partial in two oocytes (Fig. 5b). In partial inhibition, verapamil prolonged the interval of calcium oscillation. Nifedipine at $30 \mu\text{mol l}^{-1}$ also inhibited the calcium oscillation completely (Fig. 5c). After the washout of nifedipine, calcium oscillation recovered ($n = 6$). At $10 \mu\text{mol l}^{-1}$, nifedipine reduced the frequency of calcium oscillation ($n = 10$). Bay K 8644 at 100 nmol l^{-1} had no effects on calcium oscillation ($n = 7$).

Fig. 3. The effect of $[\text{Ca}^{2+}]_o$ on the frequency of calcium oscillation. (a) 5 mmol $[\text{Ca}^{2+}]_o \text{ l}^{-1}$, (b) 0.5 mmol $[\text{Ca}^{2+}]_o \text{ l}^{-1}$, (c) nominally calcium free. Oocytes were kept in a control medium (2 mmol $\text{Ca}^{2+} \text{ l}^{-1}$) for 60 min to allow fertilization and were then perfused with the test media. In test media oocytes were kept for (a) 20 min or (b and c) 60 min. Thereafter medium was switched to control medium again. Oocytes were collected 15–16 h after the human chorionic gonadotrophin (hCG) injection.

Activation of oocytes

Ethanol or A23187 induce the activation of unfertilized oocytes (Steinhardt *et al.*, 1974; Kaufman, 1978, 1982). Among four types of activation reported, the development of more than 80% of the oocytes was characterized by one pronucleus with the second polar body if the exposure time to ethanol was less than 3 min (Kaufman, 1982). We therefore used the extrusion of the second polar body as an indicator of the activation. Exposure to 7% ethanol induced the extrusion of the second polar body. With 15 s exposure to ethanol, 10% of the tested oocytes extruded the second polar body (Fig. 6). The ratio of extrusion of the second polar body was highest when oocytes were exposed to ethanol for 1 min. When the exposure time was longer than 5 min, the oocytes tended to degenerate. The ratio of degeneration was 85% with a 10 min exposure and

Fig. 4. The relationship between the interval of calcium oscillation and $[\text{Ca}^{2+}]_o$ in mouse oocytes. The mean oscillation interval in the test medium was normalized with the mean oscillation interval in the control medium (2.04 mmol $[\text{Ca}^{2+}]_o \text{ l}^{-1}$) and expressed as a percentage.

40% with a 5 min exposure. In the present experiments, oocytes exposed to ethanol for less than 3 min were analysed.

We also tested the effect of A23187. Exposure time of A23187 was 3 min. Fifty per cent of the tested oocytes extruded the second polar body within 90 min of exposure to 10 $\mu\text{mol A23187 l}^{-1}$ ($n = 18$). However, no oocytes extruded the second polar body even after 90 min when oocytes were exposed to 3 $\mu\text{mol A23187 l}^{-1}$ ($n = 12$).

Changes in $[\text{Ca}^{2+}]_i$ induced by ethanol

Exposure of the oocytes to 7% ethanol induced an increase in $[\text{Ca}^{2+}]_i$ (Fig. 7a). An increase in $[\text{Ca}^{2+}]_i$ started without detectable delay after exposure to ethanol and rapidly reached a peak. When the duration of exposure was shorter than 3 min, $[\text{Ca}^{2+}]_i$ returned to baseline values. However, the basal concentration of $[\text{Ca}^{2+}]_i$ after ethanol provocation was slightly higher than that of $[\text{Ca}^{2+}]_i$ before exposure. No calcium oscillation was observed. The peak $[\text{Ca}^{2+}]_i$ value and the duration of $[\text{Ca}^{2+}]_i$ increase were not different among tested oocytes when the exposure time was less than 3 min. An increase in $[\text{Ca}^{2+}]_i$ was observed in all exposed oocytes (Table 1).

In a calcium-EGTA-buffered HTF-Hepes medium (Ca-free HTF, $[\text{Ca}^{2+}]_o < 10^{-8} \text{ mol l}^{-1}$), the peak value of $[\text{Ca}^{2+}]_i$ and the duration of $[\text{Ca}^{2+}]_i$ increase were significantly reduced (Fig. 7b, Table 1). Extrusion of the second polar body was not observed at 90 min after exposure to ethanol in any of the tested oocytes. When 10 $\mu\text{mol verapamil l}^{-1}$ was simultaneously applied, the peak $[\text{Ca}^{2+}]_i$ and the duration of $[\text{Ca}^{2+}]_i$ increase were also significantly reduced (Fig. 7c, Table 1). None of the tested oocytes had extruded the second polar body at 90 min after exposure to ethanol.

Changes in $[\text{Ca}^{2+}]_i$ induced by A23187

A23187 at 10 $\mu\text{mol l}^{-1}$ induced an increase in $[\text{Ca}^{2+}]_i$. The pattern of $[\text{Ca}^{2+}]_i$ increase was quite similar to that observed

Fig. 5. The effect of calcium channel antagonist on the calcium oscillation. (a) and (b) 10 μmol verapamil l^{-1} . (c) 30 μmol nifedipine l^{-1} . Oocytes were collected 15–16 h after injection of human chorionic gonadotrophin (hCG). Oocytes were kept in a control medium for 30 min to allow fertilization to occur. At 30 min medium was switched to NaOH–Hepes medium ($[\text{Ca}^{2+}]_o = 5 \text{ mmol l}^{-1}$). Drugs were perfused for between 50–70 min.

during exposure to ethanol. In Ca-free HTF the peak value of $[\text{Ca}^{2+}]_i$ was reduced (Table 1). A23187 at 3 $\mu\text{mol l}^{-1}$ also induced an increase in $[\text{Ca}^{2+}]_i$. The peak value of $[\text{Ca}^{2+}]_i$ was 53% of the value observed during exposure to ethanol (Table 1). In the Ca-free HTF an increase in $[\text{Ca}^{2+}]_i$ was very small (Table 1).

Discussion

In the present experiments, $[\text{Ca}^{2+}]_i$ movement after fertilization of the mouse oocyte was analysed by optical measurement using fura-2. In fertilized oocytes an initial long lasting $[\text{Ca}^{2+}]_i$ increase and following calcium oscillation were observed. Average value of peak $[\text{Ca}^{2+}]_i$ was about 2 $\mu\text{mol l}^{-1}$. Oscillation interval in the steady state was constant and ranged from 4 to 14 min. However, there were no changes in $[\text{Ca}^{2+}]_i$ in unfertilized

Fig. 6. The relationship between the rate of activation and the duration of ethanol exposure. Ethanol (7%) was used for activation. Oocytes that extruded a second polar body were assumed to be activated oocytes. Numbers of tested oocytes are shown in parentheses.

oocytes. In fertilized mouse oocytes, similar results were reported by measurement of $[\text{Ca}^{2+}]_i$ using aequorin (Cuthbertson and Cobbold, 1985). In their experiments peak $[\text{Ca}^{2+}]_i$ was of the order of $\mu\text{mol l}^{-1}$ and calcium oscillation had a regular interval of 10–20 min. Thus, change of $[\text{Ca}^{2+}]_i$ is one of the characteristic features of fertilization in the mouse oocyte.

The latency of the onset of calcium oscillation after insemination was 4–208 min. In hamster oocytes, a correlation between the onset of fertilization and the onset of calcium oscillation has been suggested (Miyazaki and Igusa, 1981, 1982; Miyazaki *et al.*, 1986). If this is also true in the mouse oocyte, the observed variation in the latency suggests that the onset of fertilization occurs at random in the mouse oocyte.

Electrophysiological studies showed that periodic hyperpolarization synchronized with an increase in $[\text{Ca}^{2+}]_i$ (Igusa and Miyazaki, 1986) in hamster oocytes. In our experiments the oscillation interval in 5 mmol $[\text{Ca}^{2+}]_o \text{ l}^{-1}$ was 110–240 s. These data corresponded well to the interval of periodic hyperpolarization reported in mouse oocytes (120–360 s, $[\text{Ca}^{2+}]_o = 5 \text{ mmol l}^{-1}$; Igusa *et al.*, 1983). This correspondence therefore indicates that the $[\text{Ca}^{2+}]_i$ increase also synchronized with the periodic hyperpolarization, which may be ascribed to the calcium-activated potassium channel opening in the mouse oocyte.

Calcium oscillation was inhibited by a decrease in $[\text{Ca}^{2+}]_o$ and finally disappeared in the absence of $[\text{Ca}^{2+}]_o$. Calcium oscillation may therefore be controlled by calcium influx as reported in hamster oocytes (Igusa and Miyazaki, 1983). In addition, as calcium oscillation was inhibited by verapamil and nifedipine, calcium influx may occur through calcium channels. There is evidence for a calcium channel in the mouse oocyte (Okamoto *et al.*, 1977). Electrophysiological studies showed that the membrane potential of fertilized mouse oocytes is about -40 mV (Jaffe *et al.*, 1983; Peres, 1986). The steady state inactivation

Fig. 7. Change in $[\text{Ca}^{2+}]_i$ induced by exposure to 7% ethanol. Arrows indicate the start of exposure. (a) Control medium ($2.04 \text{ mmol } [\text{Ca}^{2+}]_o \text{ l}^{-1}$). (b) Calcium-EGTA-buffered HTF-Hepes medium. (c) Control medium containing $10 \mu\text{mol verapamil l}^{-1}$.

curve shows that 10% of calcium channels are still active at -40 mV (Peres, 1986). Thus, it is possible to consider that calcium influx occurs through calcium channels in mouse oocytes. However, in hamster oocytes the calcium channel is not considered to be a pathway of calcium influx (Igusa and Miyazaki, 1983).

In hepatocytes, it is thought that phenylephrine or Arg^8 -vasopressin induced calcium influx triggers the increase in $[\text{Ca}^{2+}]_i$ and determines the speed of calcium oscillation (Woods *et al.*, 1990). In this case, calcium oscillation is also inhibited by reduction of $[\text{Ca}^{2+}]_o$ or application of verapamil. Since calcium influx stimulates phospholipase C and induces the production of inositol-1,4,5-triphosphate (InsP_3) (Meyer and Stryer, 1988), the speed of InsP_3 induced calcium release from the endoplasmic reticulum will be determined by the calcium influx (Berridge, 1987). In other words, the amount of influxed calcium controls the speed of InsP_3 production. If calcium oscillation is controlled in a similar manner in mouse oocytes, the effect of reduction of $[\text{Ca}^{2+}]_o$ or application of verapamil is explained by the

Table 1. Peak $[\text{Ca}^{2+}]_i$ concentration and duration of $[\text{Ca}^{2+}]_i$ increase in fertilized and activated mouse oocytes

	Peak $[\text{Ca}^{2+}]_i$ ($\mu\text{mol l}^{-1}$)	Duration (s)	Extrusion of second polar body
Insemination			
Control	2.1 ± 1.1	320 ± 100	100% (30)
Ethanol			
Control	3.6 ± 2.4	460 ± 70	46% (70)*
Ca-free HTF	0.5 ± 0.3	150 ± 20	0% (20)
Ca-free HTF + verapamil	0.6 ± 0.2	180 ± 20	0% (20)
A23187 ($3 \mu\text{mol l}^{-1}$)			
Control	1.9 ± 0.6	700 ± 120	0% (12)
Ca-free HTF	0.1 ± 0.1	550 ± 70	0% (5)
A23187 ($10 \mu\text{mol l}^{-1}$)			
Control	4.1 ± 2.4	540 ± 120	50% (18)*
Ca-free HTF	0.7 ± 0.2	360 ± 160	0% (5)

Data are expressed as means $\pm$ SD. Numbers in parentheses show the number of oocytes tested. Control was recorded in the HTF-Hepes medium. In fertilized oocytes first response was used for analysis. In activated oocytes data obtained by less than 3 min exposure were used for analysis. *Average values of activated oocytes. HTF: Modified human tubal fluid medium. Ca-free HTF: calcium-EGTA buffered HTF-Hepes medium.

inhibition of calcium influx which acts as a trigger to the calcium signalling system.

Resumption of the second meiosis by exposure to ethanol or A23187 is an established technique for activation of mouse oocytes (Steinhardt *et al.*, 1974; Kaufman, 1978, 1982; Cuthbertson, 1983). It is therefore possible that important factors for inducing the resumption of the second meiosis are reproduced in the activation, although the number of chromosomes is haploid. By exposure to ethanol or A23187 we observed the extrusion of the second polar body and a non-periodic $[\text{Ca}^{2+}]_i$ increase. In the mouse oocyte the non-periodic increase in $[\text{Ca}^{2+}]_i$ induced by exposure to ethanol was also reported by $[\text{Ca}^{2+}]_i$ measurement using aequorin (Cuthbertson *et al.*, 1981). This suggests that a single transient $[\text{Ca}^{2+}]_i$ increase may be enough to trigger the resumption of the second meiosis.

In the present experiments when the activation of oocytes was observed (ethanol and $10 \mu\text{mol A23187 l}^{-1}$), the duration of $[\text{Ca}^{2+}]_i$ increase and the peak $[\text{Ca}^{2+}]_i$ were comparable to those in fertilized oocytes in the control (Table 1). However, when calcium influx was inhibited (i.e. Ca-free HTF), no oocytes were activated although a small increase in $[\text{Ca}^{2+}]_i$ occurred. This result indicates that calcium influx is necessary to activate oocytes. However, no oocytes were activated by exposure to $3 \mu\text{mol A23187 l}^{-1}$, although the duration of $[\text{Ca}^{2+}]_i$ increase and the peak $[\text{Ca}^{2+}]_i$ were comparable to those in fertilized oocytes (Table 1). In this case, however, in Ca-free HTF no increase in $[\text{Ca}^{2+}]_i$ was observed. This result indicates that calcium mobilization from the intracellular calcium store is also necessary to activate oocytes. It is therefore possible that the role of intra- and extracellular calcium used for fertilization might be different in the mouse oocyte. However, in the rabbit it has been reported that rate of activation increases with increasing calcium influx (Ozil, 1990). The relationship between

peak $[Ca^{2+}]_i$ and rate of activation should also be considered in mouse oocytes.

In the present experiments, oocytes extruded second polar bodies in the presence and absence of calcium oscillation. There was no difference in the second meiosis of the fertilized and activated oocytes. Ozil (1990) reported that the pattern of calcium oscillation affects the development after the third cleavage division *in vitro* in rabbits. Calcium oscillation observed in fertilized mouse oocytes may therefore also affect the course of late developmental stages. However, further study will be needed to clarify the relationship between the extrusion of the second polar body and the pattern of increase in $[Ca^{2+}]_i$. These basic analyses of fertilization will be useful for developing a reliable technique for human *in vitro* fertilization.

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