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Estrogen and bisphenol A disrupt spontaneous $[Ca^{2+}]_i$ oscillations in mouse oocytes☆

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Abstract

The present work aims to study the effects of estrogen or endocrine disrupters (EDs) on the dynamic changes in intracellular Ca^{2+} concentration of mouse immature oocytes (IOs) loaded with Ca^{2+}-sensitive dye Fura-2 using an image analyzer. The majority of IOs isolated from the ovary exhibited spontaneous Ca^{2+} oscillations at regular intervals. Entry of external Ca^{2+}, probably through gap junctions, contributes to Ca^{2+} oscillations since they were reversibly inhibited by removing Ca^{2+} from the bathing medium or by the application of a gap-junction inhibitor carbenoxolone (CBX, 30 μM). Both 17β-estradiol (E2) and E2–BSA, a membrane impermeable estrogen, shortened the duration of Ca^{2+} oscillations in a dose-dependent manner (1–1000 nM), and produced an irregular pattern of the oscillations, strongly suggesting that E2 acts on the plasma membrane of the oocyte. For bisphenol A (BPA), one of the estrogen-mimicking EDs, a 10,000-fold higher concentration (100 μM) was necessary to exert similar inhibitory action to that of E2.

Keywords: Mouse; Immature oocyte; Estrogen; 17β-estradiol (E2); Ca^{2+} oscillation; E2–BSA; Gap junction; Carbenoxolone; Bisphenol A

Immature mammalian oocytes grow, mature, and finally acquire the competence for fertilization in the ovary under the control of various hormones [1]. Immature oocytes (IOs) with intact germinal vesicle (GV) have been reported to exhibit spontaneous Ca^{2+} oscillations which are necessary for maturation [2–6]. Furthermore, it is suggested that the ability to generate Ca^{2+} transients in response to spermatozoa increases during oocyte maturation, especially at the final stage [7]. It should be remembered that Ca^{2+} oscillations at fertilization are important in order to induce various enzymatic responses [1]. Oocyte maturation or follicle development is controlled mainly by follicle stimulating hormone (FSH) and luteinizing hormone (LH). Estrogen is known to increase its plasma concentration during oocyte maturation and reaches a peak before ovulation. Thus, the increase of estrogen induces a surge of LH that continues oocyte maturation and leads to ovulation [1]. However, the precise mechanism of oocyte maturation and the significance of the spontaneous Ca^{2+} oscillations remain to be elucidated.

To date, many endocrine disrupters (EDs) are thought to interfere with various biological systems including the reproductive system by mimicking estrogen. Despite many studies of EDs on spermatozoa [8–10], few reports are available for oocytes. Exposure of bisphenol A (BPA) has recently been reported to cause meiotic aneuploidy in mouse oocytes [11]. Therefore, it is important to reproductive physiology as well as environmental sciences to investigate the effects of both

☆ *Abbreviations:* IO, immature oocyte; GV, germinal vesicle; FSH, follicle stimulating hormone; LH, luteinizing hormone; ED, endocrine disrupter; PMSG, pregnant mare serum gonadotropin; CBX, carbenoxolone; BSA, bovine serum albumin; $[Ca^{2+}]_i$, intracellular concentration of Ca^{2+}; BPA, bisphenol A.

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estrogen and estrogen-like EDs on oocyte maturation or follicle development. The present study is the first report of suppressive effects of 17-β-estradiol (E2) or BPA on spontaneous Ca^{2+} oscillations, which are a marker of competence for fertilization [3].

Materials and methods

Preparation of oocytes. Fully grown IOs with intact GV were obtained from ovaries of 8–12-week-old female mice (CD-1/ICR). Antral follicles in ovaries were ruptured with 31 gauge needles in M2 medium (94.7 mM NaCl, 4.8 mM KCl, 1.7 mM $CaCl_2$, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 4.2 mM $NaHCO_3$, 20.9 mM Hepes, 23.3 mM Na lactate, 0.3 mM Na pyruvate, and 5.6 mM glucose) [12] containing bovine serum albumin (BSA; 5 mg/ml) and IOs were collected using a fine-bore micropipette. IOs used for experiments were mechanically denuded of cumulus cells with needles or by pipetting. Mice were not primed with pregnant mare serum gonadotropin (PMSG), which is generally used to accelerate maturation of ovarian oocytes, in order to avoid any exogenous hormonal effects on oocytes in this experiment. This precaution may not be necessary, since the number of fully grown IOs with a GV from non-primed animals was not significantly different from that from animals primed with PMSG ($P > 0.4$). Average number of IOs from non-primed mice was 26 ± 2 ($n = 28$), whereas that was 26 ± 3 ($n = 18$) from those primed with 5-IU PMSG (Teikoku Hormone Mfg., Tokyo, Japan) 48–50 h before obtaining oocytes.

Solutions. 17β-estradiol (E2; E 2257, cell culture tested, Sigma Chemical) was dissolved in dimethyl sulfoxide (DMSO) as a stock solution, and diluted 10,000 times in M2 medium to the desired concentrations (1 nM–1 μM). 17β-estradiol 6-(*o*-carboxymethyl)oxime:BSA (E2–BSA; Sigma Chemical), a membrane impermeable estrogen conjugated with BSA, was first dissolved in deionized distilled water as a 190 μM stock solution and diluted to 1 nM to 1 μM in M2 for application. Ca^{2+}-free solution was prepared by substituting 1.71 mM $CaCl_2$ with 2.5 mM NaCl considering the osmolality contribution to the medium. BPA was first dissolved in DMSO and diluted 10,000 times to the desired concentrations.

$[Ca^{2+}]_i$ measurement. Isolated IOs were loaded with Fura-2 AM (Molecular Probes) at 32 °C for 10 min. Oocytes were washed twice with M2 + 5 mg/ml BSA and twice with M2 only, and they were transferred to a glass-bottomed experimental chamber on the stage of an inverted microscope (TE300 Nikon, Tokyo, Japan) with a 10× objective lens (CFI S Fluor 10× Nikon). Fluorescence $[Ca^{2+}]_i$ images were obtained every 9 or 10 s using a high-speed Ca^{2+} imaging system (ARGUS/HiSCA; Hamamatsu Photonics, Hamamatsu, Japan). Bright-field images of IOs were simultaneously monitored to check morphological changes of oocytes and recorded on a video tape recorder [13] or on a hard disk video recorder (LVC-HD V01A Logitech, Japan).

The protocol of experiments was as follows: (1) observation of an IO condition for 5 min in M2 solution (experimental standard solution), (2) application of test solution for 60 min, and (3) washout with M2 for 30 min. Oocytes not used to collect experimental data were: (1) IOs which showed no Ca^{2+} oscillations, (2) IOs which stopped Ca^{2+} oscillations within 5 min, and (3) morphologically young and small IOs with eccentrically located GVs. In order to compare records obtained in standard M2 and test solutions, the duration of Ca^{2+} oscillations is defined as the time from the starting point minus 8.3 min (the initial 5 min is spent judging the condition of an IO as stated above, and it took 3.3 min for the test solution to reach a dish on the stage).

Frequency of Ca^{2+} oscillations was calculated as a reciprocal of interspike interval using the macro-software, which runs on the Microsoft Excel 2000, developed by Dr. K. Sutani.

Analysis of Ca^{2+} oscillations. The first frequency calculated was not appropriate to use as the initial frequency because the starting time of measurement was usually in the middle of the period of Ca^{2+} oscillations. It was normally higher than later ones. We therefore ignored the first frequency. Instead, the second frequency should be regarded as the "first effective frequency" by taking a reciprocal of interval between the first Ca^{2+} spike and the second one. Changes in frequencies were plotted with time.

To examine to what extent Ca^{2+} oscillations were affected after application of E2, E2–BSA, or BPA, we evaluated the frequency change during the Ca^{2+} oscillations. "Coefficient of variation" (CV) of frequency, i.e., SD/mean, was calculated to assess the grade of firing irregularity. Thus, the larger the value of CV is, the greater the extent of perturbation of Ca^{2+} oscillations is.

Statistical analysis. The data were expressed as means ± SE. The significance of difference between means is analyzed using Student's *t* test (two-sample assuming unequal variance, statistical analysis tool of Microsoft Excel 2000), where *P* values less than 0.05 were considered as significant and marked with *.

Results

Spontaneous Ca^{2+} oscillations in immature oocytes

To examine whether Ca^{2+} oscillations are due to their development or their growth, the diameters of 74 oocytes were measured in separate experiments. The average diameters of oocytes with or without Ca^{2+} oscillations were 75.4 ± 1.7 μm ($n = 36$) and 78.4 ± 1.1 μm ($n = 38$), respectively. There was no significant difference between these groups ($P < 0.06$), indicating that occurrence of Ca^{2+} oscillations does not simply depend on the oocyte growth. Seventy-three percent (716/978) of fully grown IOs (75–78 μm in diameter) exhibited Ca^{2+} oscillations. The duration of Ca^{2+} oscillations varied from 5 to 87 min (29.5 ± 4.6 min, $n = 37$). Oocytes selected using our criteria given in Materials and methods, were subjected to analysis. Oocytes produced spikes with various periods from 0.7 to 11 min, i.e., 0.2–1.4 min^{-1} or 0.003–0.023 Hz ($n = 37$), and the mean frequency value was 0.37 ± 0.02 min^{-1}. IOs showed relatively regular Ca^{2+} oscillations followed by an abrupt cessation (Fig. 1A) or a gradual prolongation of interval before cessation (Fig. 2A). During the experimental period of 95 min, only one oocyte (1/37) showed a germinal-vesicle breakdown (GVBD) while it was oscillating, and 25 oocytes out of 37 exhibited GVBD after termination of Ca^{2+} oscillations. Most isolated IOs which were left in an incubator for longer than 2 h exhibited GVBD but they did not exhibit Ca^{2+} oscillations. In addition, the Ca^{2+} oscillations were never detected at a later stage of meiosis (data not shown). These results suggest that Ca^{2+} oscillations are related to cell cycle as well as GVBD.

Effect of extracellular Ca^{2+} on spontaneous Ca^{2+} oscillations

To examine the mechanism of spontaneous Ca^{2+} oscillations, Ca^{2+} was removed from the M2 medium.

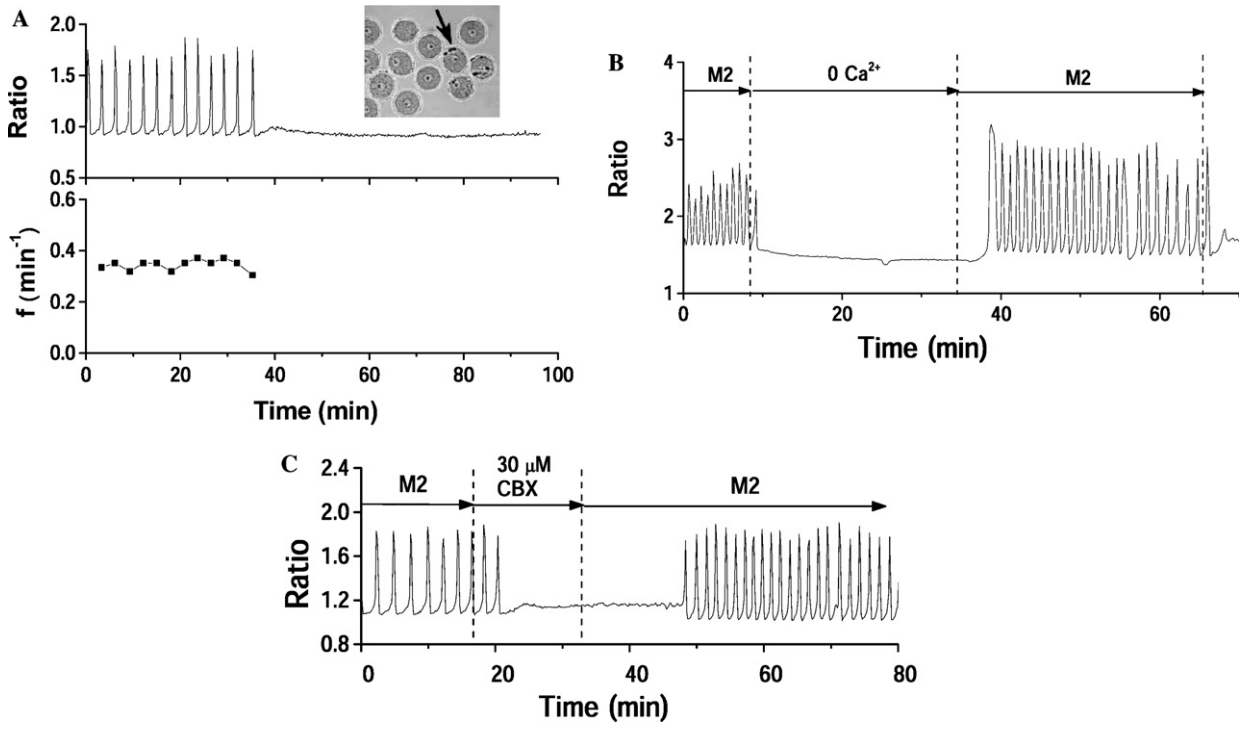

Fig. 1. (A) Spontaneous Ca^{2+} oscillations observed in immature oocytes (IOs) of the mouse. The upper panel displays the intracellular Ca^{2+} concentration by the ratio of Fura-2 fluorescence intensity, and the frequency of Ca^{2+} oscillations per minute is shown in the lower panel. Inset: photomicrograph of isolated IOs. The data shown here were obtained from the oocyte indicated by the arrow. (B) The effect of removing Ca^{2+} from the bathing medium. Each arrow bar shows the duration when the indicated solution was perfused. (C) The effect of carbenoxolone (CBX), an inhibitor of gap junctions, on spontaneous Ca^{2+} oscillations.

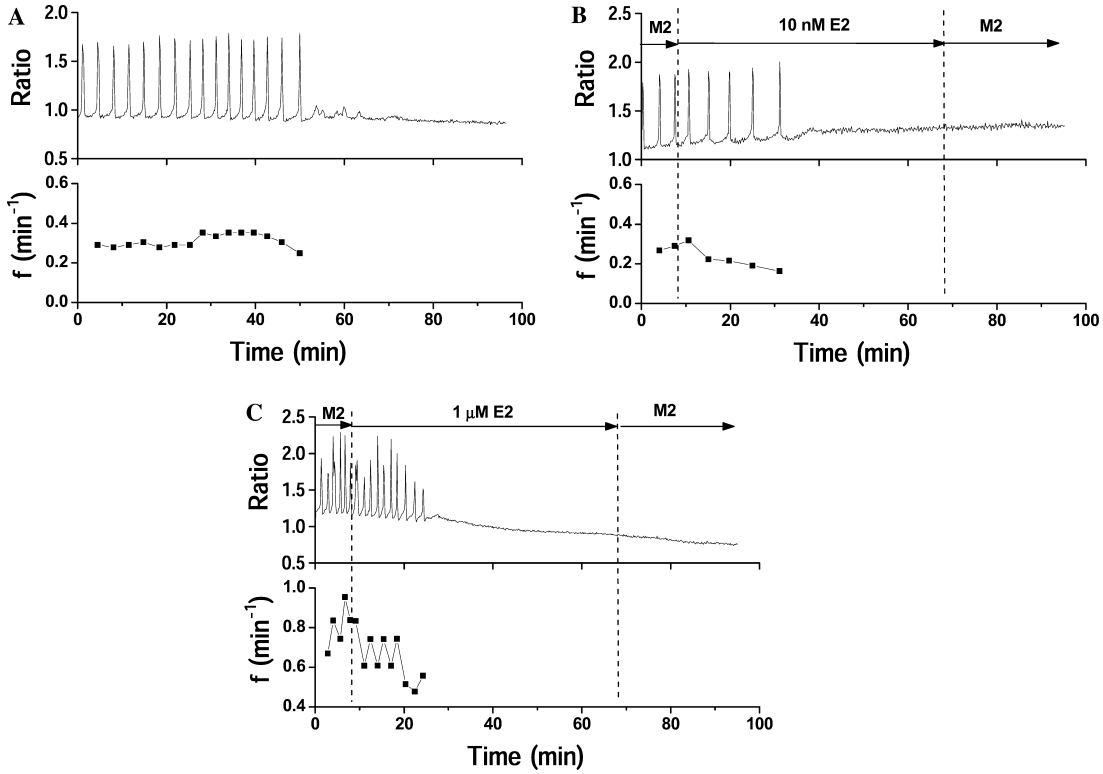

Fig. 2. (A) Control. (B,C) Dose-dependent effects of estradiol (E2) on $[Ca^{2+}]_i$ (upper) and the frequency (lower) of spontaneous Ca^{2+} oscillations. Note E2 not only shortened the duration of Ca^{2+} oscillations but also changed their firing pattern.

According to our experimental protocol, M2 solution was perfused for 5 min to collect control data and it was then switched to test solution (Fig. 1B). The first vertical dotted line, marked at 8.3 min, indicates the time when Ca^{2+}-free M2 solution reached the oocytes. The time lag between 8.3 and 5 min indicates the dead space of the perfusion system. Fig. 1B illustrates that Ca^{2+} oscillations were reversibly inhibited under Ca^{2+}-free condition, concluding that extracellular Ca^{2+} is required to maintain spontaneous Ca^{2+} oscillations in immature oocytes.

Effect of carbenoxolone on spontaneous Ca^{2+} oscillations

The contribution of gap junctions to spontaneous Ca^{2+} oscillations was examined using carbenoxolone (CBX), an inhibitor of gap junctions. Although 10 μM CBX showed little effect on spontaneous Ca^{2+} oscillations, reversible inhibition of Ca^{2+} oscillations was observed at 30 μM (Fig. 1C) in six out of eight IOs. It is suggested that gap junctions are involved in maintaining Ca^{2+} oscillations.

Effects of estrogen on Ca^{2+} oscillations

Oocytes were exposed to 17β-estradiol (E2) at 1 nM–1 μM for 60 min to study its hormonal effect on spontaneous Ca^{2+} oscillations. When the concentration of E2 was higher than 10 nM, the duration of Ca^{2+} oscillations was significantly shortened ($P < 0.05$) and the firing pattern was disturbed (Fig. 2B). Differences in the duration were assessed with one-sided tests because the duration was always decreased when E2/E2–BSA was perfused. Dose-dependent reduction in the duration of Ca^{2+} oscillations was evoked by E2 as shown in Fig. 4A. The interval of Ca^{2+} transients was progressively prolonged compared to control (compare Fig. 2A to Figs. 2B and C). The regular pattern of Ca^{2+} oscillations was also changed and became irregular (Figs. 2B and C).

To examine to what extent Ca^{2+} oscillations were affected after application of E2, we calculated the coefficient of variation (CV) of frequency changes. This parameter calculated for all concentrations of E2 and E2–BSA showed a significant difference compared to that of control (see Fig. 4B). In the presence of E2, the spike interval was gradually prolonged before its complete cessation and the duration of the Ca^{2+} oscillation was significantly shortened (Figs. 2 and 4).

During an experimental period of approximately 1.5 h, GVBD was observed in most IOs that exhibited similar Ca^{2+} oscillations to control, indicating that E2 did not affect GVBD. In order to examine the site of action of E2, membrane impermeable E2–BSA was applied to oocytes. And similar inhibitory actions of E2–BSA on the duration and frequency reduction of Ca^{2+} oscillations to those of E2 were observed at 1 nM–1 μM (see Figs. 3 and 4). The regular pattern of Ca^{2+} oscillations was also changed to be irregular (Figs. 3A and B). CV of frequency change for oocytes treated with E2–BSA at all concentrations examined were also significantly different from that of control as shown in Fig. 4B.

The results indicate that the inhibitory actions of E2 and E2–BSA are almost identical, and that suppression of Ca^{2+} oscillations occurs in a non-genomic fashion, i.e., E2 acts on the plasma membrane. GVBD was not disturbed by E2–BSA, because the phenomenon was seen in most oocytes (120/126) which were exposed to E2–BSA.

Effects of bisphenol A on spontaneous Ca^{2+} oscillations

The effect of bisphenol A (BPA), a representative endocrine disrupter (ED) mimicking estrogen-action, was studied as the next stage. Experiments for BPA were carried out separately. Therefore, control data were needed to be obtained from the same group of mice and the duration of Ca^{2+} oscillations was 18.8 ± 2.2 min ($n = 33$).

IOs were exposed to BPA (10 nM–100 μM) for 60 min as illustrated in Figs. 5A and B. In order to obtain a similar shortening of the Ca^{2+} oscillations as for E2 or E2–BSA, the concentration of BPA that was

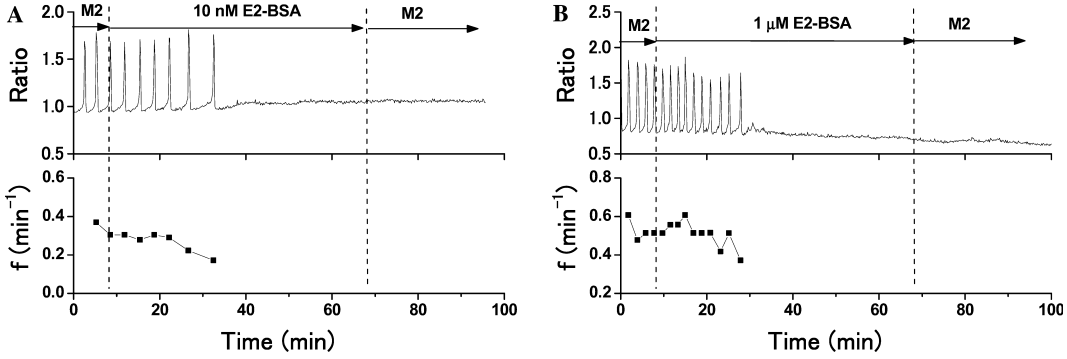

Fig. 3. (A,B) Dose-dependent effects of membrane impermeable E2–BSA on IOs. Note E2–BSA not only shortened the duration of Ca^{2+} oscillations but also changed their firing pattern.

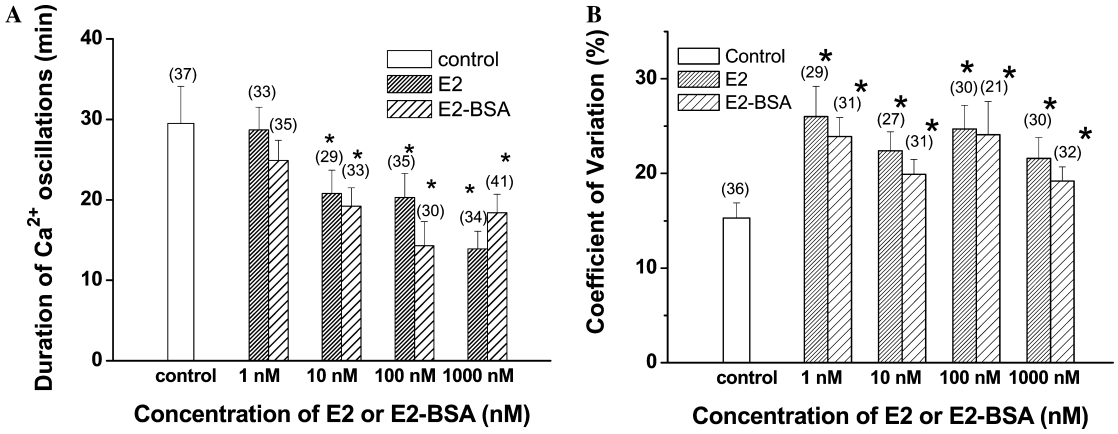

Fig. 4. Summary of E2 and E2–BSA experiments. (A) Average durations of Ca^{2+} oscillations obtained at different concentrations of E2 or E2–BSA. (B) Perturbation of frequency change was evaluated as coefficient of variation (CV) of total changes in frequency in each IO. CV for control IOs and IOs perfused with E2 or E2–BSA are calculated and expressed as a bar graph. There was significant difference in CV between control and IOs perfused with E2 or E2–BSA. Significant difference ($P < 0.05$) indicated with * was assessed with one-sided for duration and with two-sided for CV.

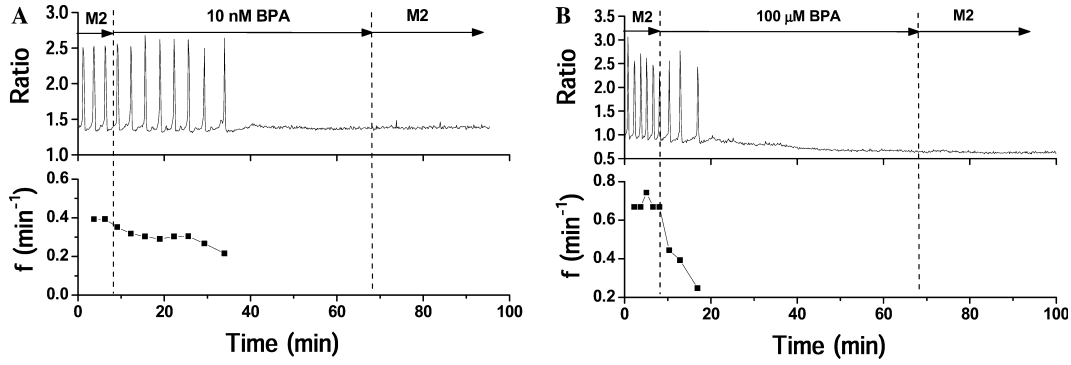

Fig. 5. Effects of bisphenol A, an estrogen-mimicking endocrine disrupter, on spontaneous Ca^{2+} oscillations at 10 nM (A) and 100 μM (B).

necessary was 100 μM (10,000-fold higher than E2 or E2–BSA) (compare Fig. 4A to Fig. 6A). As for the BPA effect on the pattern of the Ca^{2+} oscillations, again 100 μM was needed to produce an irregular pattern of oscillations (Figs. 5 and 6B). Although a cytotoxic effect was observed when the concentration of BPA exceeded 1 mM, i.e., the basal level of $[Ca^{2+}]_i$ was elevated and resulting in cell death (data not illustrated), oocytes exposed to BPA at lower than 100 μM survived and showed a polar-body emission.

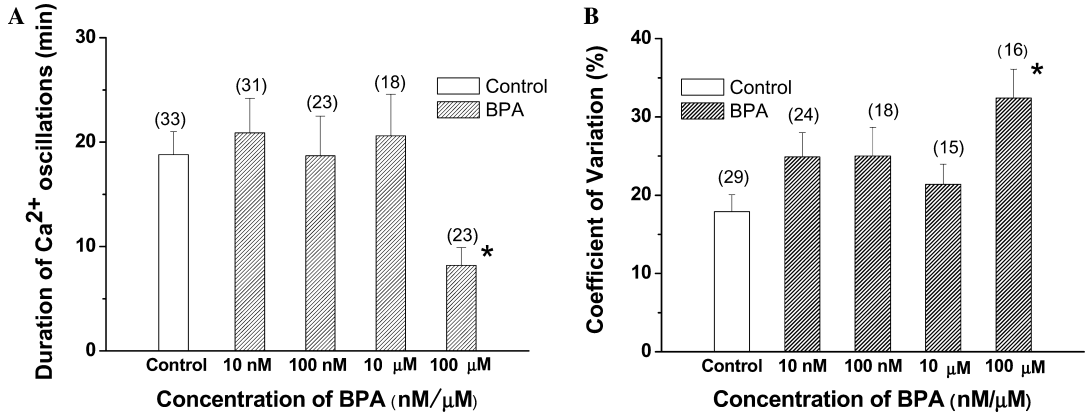

Fig. 6. (A) Summary of BPA effect on the duration of Ca^{2+} oscillations. (B) CV for control IOs and IOs perfused with BPA at different concentrations. Significant difference ($P < 0.05$) indicated with * was assessed with one-sided for duration and with two-sided for CV.

Discussion

Mechanism of spontaneous Ca^{2+} oscillations

Ca^{2+} oscillations are thought to be significant for further development or cytoplasmic maturation of oocytes [2,3,7]. In the present work, spontaneous Ca^{2+} oscillations in immature mouse oocytes were reversibly stopped by Ca^{2+}-free M2 medium (Fig. 1B), suggesting that extracellular Ca^{2+} is required for Ca^{2+} oscillations through $InsP_3$ (inositol trisphosphate)-induced Ca^{2+} release (IICR) as in the case of Ca^{2+} oscillations at fertilization [13–15]. In control oocytes, Ca^{2+} oscillations did not reappear once stopped. However, regained Ca^{2+} oscillations with larger amplitudes were observed in most IOs when returning to normal M2 after Ca^{2+}-free treatment (Fig. 1B). This may be ascribed to the activation of the capacitative Ca^{2+} channels as reported for mature eggs [13,16].

Immature oocytes in the ovary are connected to granulosa cells via gap junctions [17,18], and gap-junctional hemichannels present on the oocyte membrane in isolated oocytes may provide gates for Ca^{2+} entry as has been reported for isolated *Xenopus* oocytes [19–21]. Indeed, spontaneous Ca^{2+} oscillations were reversibly inhibited by carbenoxolone (CBX), an inhibitor of gap junction (Fig. 1C). Gap-junctional communication between granulosa cells and oocytes plays a significant role in the regulation of folliculogenesis, oocyte maturation, and atresia [17,22]. It has been reported that connexin 37 (Cx37) is present in oocyte–granulosa gap junctions and Cx37 knockout mice were infertile [23]. In addition, the follicle formation was suppressed by a gap-junction inhibitor lindane [24], and the amount of connexins was reduced in atretic follicles [25,26]. These reports plus the present results suggest that gap junctions are important for the oocyte maturation presumably via Ca^{2+} oscillations [17,22].

Other possible factors contributing to spontaneous Ca^{2+} oscillations are phosphatidyl inositol (PI) turnover and $InsP_3$ production which induce Ca^{2+} release from intracellular stores as suggested by experiments that show that microinjection of heparin into oocytes inhibited Ca^{2+} oscillations [3,6]. In the present study, however, any minor insertion of a micropipette, containing no heparin, into spontaneously oscillating IOs caused disruption or cessation of the Ca^{2+} oscillations. An excess amount of Ca^{2+} entry due to mechanical damage of the oocyte membrane by micropipette insertion is considered to close gap junctions responsible for the oscillation mechanism. Opening and closing of gap junctions are controlled by intracellular concentration of Ca^{2+} [20].

As for the relation between Ca^{2+} oscillations and germinal vesicle breakdown (GVBD), Lefèvre et al. [5] proposed that GVBD was dependent on the spontaneous Ca^{2+} oscillations since inhibition of GVBD by dibutyryl cyclic AMP stopped Ca^{2+} oscillations. However, our data as well as the report by Deng et al. [4] indicate that spontaneous Ca^{2+} oscillations in immature oocytes often continued after GVBD or under the inhibitive condition of GVBD.

Novel role of estrogen for oocyte maturation

Appropriate changes in the plasma estrogen concentration play an important role in the folliculogenesis and the maintenance of ovarian somatic cells [27]. The recent developments of knockout mice for estrogen receptor α (ERα), estrogen receptor β (ERβ), and aromatase (ArKO) reveal that the actions of estrogen are essential for normal fertility in both males and females [28] as well as regulation of ovulation [29]. It is estimated that the concentration of E2 in human follicular fluid is higher than 4 μM without establishment of pregnancy or 3 μM with pregnancy [30], and the estrogen level in follicular fluid of the mouse ovary is approximately 1.1 μM [31]. Such a high level of estrogen must stop Ca^{2+} oscillations according to our results (Figs. 2 and 3), resulting in maintaining meiotic arrest in vivo [31].

Non-genomic action of estrogen, evoking intracellular Ca^{2+} rise, has been reported in many types of cells such as pancreatic β-cells [32], T cells [33], colonic epithelium [34], and reproductive cells [35]. Non-genomic effect of estrogen is also reported in maturing human oocytes, and Ca^{2+} oscillations were generated by the application of E2 [36]. In contrast, genomic action of estrogen is described in osteoblasts to regulate the expression of $InsP_3R$ by transcriptional repression [37]. Although the inhibitory action of a gap-junction blocker CBX was reversible (Fig. 1C), no recovery was observed when oocytes were exposed to E2 or E2–BSA (Fig. 2). This does not imply a cytotoxic effect of E2 or its BSA form, because no sign of degeneration was detected in the oocyte morphology. One possible explanation is that both E2 and E2–BSA bind tightly to estrogen receptors on the plasma membrane [38,39] and are not easily washed out. Another possibility is that E2 and E2–BSA activate an intracellular signal transduction system which cannot be interrupted even when they are washed out from the plasma membrane.

Effects of bisphenol A on spontaneous Ca^{2+} oscillations

Bisphenol A (BPA) is a monomer used in the manufacture of many types of products including polycarbonate plastic food storage containers. Although the effects of BPA on maturing mouse oocytes have been recently reported to cause meiotic aneuploidy in mouse oocytes, few reports are available for oocytes [11].

In the present study, E2 or E2–BSA at concentrations higher than 10 nM significantly reduced duration of Ca^{2+} oscillations in a dose-dependent manner (Fig. 4A), whereas as high as 100 μM BPA was necessary for a similar inhibition (Fig. 6A), supporting the in vitro assay that the potency of estrogenic effect for BPA is approximately 10,000 times weaker than that of estradiol [40]. Although no significant differences were seen in BPA-treated oocytes at concentrations less than 10 μM, such oocytes showed a tendency to fire at irregular patterns. In fact, 50 % (12/24), 44 % (8/18), and 60 % (9/15) of oocytes exposed to 10 nM, 100 nM and 10 μM BPA, respectively, exhibited an irregular pattern. These results suggest that even lower concentration of BPA can affect Ca^{2+} oscillations. This kind of perturbation of Ca^{2+} oscillations by estrogen and EDs including BPA may lead to disruption of Ca^{2+} oscillations at fertilization because spontaneous Ca^{2+} oscillations in IOs were required for normal oocyte maturation [2,3]. Further studies are necessary to examine the actions of EDs including BPA in order to avoid serious problems for human health.

Working hypothesis concerning estrogen and estrogen-like endocrine disrupters

The results indicating the inhibitory action of E2 lead us to a working hypothesis that (1) estrogen plays an important role in oocyte maturation by regulating spontaneous Ca^{2+} oscillations, (2) immature oocytes, producing spontaneous Ca^{2+} oscillations small in amplitude, are preparing for large Ca^{2+} oscillations at fertilization, and (3) estrogen-like endocrine disrupters (EDs) would adversely affect oocyte maturation by interfering with the estrogen action.

Acknowledgments

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