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# Effect of β-estradiol on voltage-gated $Ca^{2+}$ channels in rat hippocampal neurons: a comparison with dehydroepiandrosterone

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**Abstract**

We investigated the effects of β-estradiol, dehydroepiandrosterone and dehydroepiandrosterone sulfate on intracellular calcium concentration ($[Ca^{2+}]_i$) increases induced by γ-aminobutyric acid (GABA), high $K^+$ and *N*-methyl-D-aspartate acid (NMDA) in cultured hippocampal neurons. Acute treatment with β-estradiol, dehydroepiandrosterone and dehydroepiandrosterone sulfate inhibited the GABA-induced $[Ca^{2+}]_i$ increases to the similar extent. Tamoxifen, an estrogen receptor antagonist, did not block the inhibitory effects of β-estradiol. On the other hand, GABA type A ($GABA_A$) receptor antagonists, picrotoxin and bicuculline, blocked the GABA-induced $[Ca^{2+}]_i$ increases. Previously, we demonstrated that GABA- and high $K^+$-induced $[Ca^{2+}]_i$ increases were commonly mediated by voltage-gated calcium channels (VGCCs). Therefore, we examined the effects of these steroids on the high $K^+$-induced $[Ca^{2+}]_i$ increases. The inhibitory effect of β-estradiol on the high $K^+$-induced $[Ca^{2+}]_i$ increases was much greater than that of dehydroepiandrosterone and dehydroepiandrosterone sulfate. β-Estradiol inhibited the NMDA-induced $[Ca^{2+}]_i$ increases with an $IC_{50}$ of 51.8 μM and NMDA responses were reduced to half in the presence of 10 μM nifedipine, indicating that the NMDA-induced $[Ca^{2+}]_i$ increases also involved VGCCs. Further, we examined the inhibitory effect of β-estradiol on the high $K^+$-induced $[Ca^{2+}]_i$ increases in the presence of a N-type VGCCs antagonist, 1 μM ω-conotoxin, or a L-type VGCCs antagonist, 10 μM nifedipine. The $IC_{50}$ value of β-estradiol alone (45.5 μM) was similar to that of ω-conotoxin (33.1 μM), while the value combined with nifedipine was reduced to 2.2 μM. β-Estradiol also abolished the positive modulatory effect of L-type VGCCs agonist, 1,4-dihydro-2,6-dimethyl-5-nitro-4-[2-(trifluoromethyl)phenyl]-pyridine-3-carboxylic acid methyl ester (Bay K 8644). Our results showed that the inhibitory mechanism of β-estradiol is different from that of dehydroepiandrosterone and dehydroepiandrosterone sulfate and β-estradiol may act primarily at L-type VGCCs. 

*Keywords:* β-Estradiol; Dehydroepiandrosterone; Dehydroepiandrosterone sulfate; Neurosteroid; GABA (γ-aminobutyric acid); $Ca^{2+}$ intracellular; Hippocampal neuron

## 1. Introduction

Estrogen is one of the sex steroids that are mainly produced in the ovaries and is well known to have several important physiological functions in the central nervous system. It has been observed that estrogen increases the dendric spine density in hippocampal neurons (Gould et al., 1990; Murphy and Segal, 1996) and these neurotrophic effects are blocked by tamoxifen, an estrogen receptor antagonist, suggesting that estrogen exerts effects by mediating classical genomic pathways (Murphy and Segal, 1996). On the other hand, a number of studies suggest that estrogen also changes the electrical activity within milliseconds to minutes in neurons by mediating non-transcriptional mechanisms (Moss et al., 1997). During neuronal development, estrogen modulates neuronal and sexual differentiation by influencing the organization of neural circuits and neuroendocrine systems (Arnold and Gorski, 1984; Arnold and Breedlove, 1985). The estro-

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gen-synthesizing enzyme, aromatase, is expressed in many regions of the brain during neuronal development, including the hippocampus, hypothalamus and cortex. Aromatase activity is significantly greater during development than at any subsequent age (Harada and Yamada, 1992; Lephart et al., 1992; MacLusky et al., 1994), suggesting that estrogen plays an important role, especially in the developing brain.

The major precursors of estrogen, dehydroepiandrosterone and its sulfate ester, dehydroepiandrosterone sulfate, are members of neurosteroid families that are expressed in the central nervous system. Levels of dehydroepiandrosterone and dehydroepiandrosterone sulfate decline with age, and the enzymes for their synthesis are more abundant in the developing brain than in adult brain (Compagnone et al., 1995). Recent studies have shown that many neurosteroids modulate the functions of γ-aminobutyric acid type A ($GABA_A$) receptors allosterically (Majewska et al., 1986; Puia et al., 1990; Schmid et al., 1998). Several neurosteroids, such as progesterone, pregnenolone and its metabolizes, play an important role in promoting cell growth (Schumacher et al., 1996). In addition, dehydroepiandrosterone and dehydroepiandrosterone sulfate have been reported to provide neuroprotective effects in hippocampal neurons against excitatory amino acid-induced neurotoxicity as well as estrogen (Singer et al., 1996; Kimonides et al., 1998; Mao and Barger, 1998). Neurosteroids, including dehydroepiandrosterone and dehydroepiandrosterone sulfate, may be involved in neuronal plasticity through the modulating functions of $GABA_A$ receptors in the developing brain.

It is well known that γ-aminobutyric acid (GABA) is a major inhibitory neurotransmitter in the adult mammalian central nervous system. In contrast, GABA is reported to act as an excitatory neurotransmitter in the developing brain, including embryonic and postnatal stages (Cherubini et al., 1991; Ben-Ari et al., 1994). The excitatory effect of GABA in rat neurons results from its depolarization of the neuron through $GABA_A$ receptors (Segal., 1993; Ben-Ari et al., 1994; Takebayashi et al., 1996). GABA has not only an inhibitory effect on neuronal transmission, but also neurotrophic effects on developing neurons, such as promoting neurite outgrowth, differentiation and synaptogenesis (Spoerri, 1988; Barbin et al., 1993; Ben-Ari et al., 1994). These neurotrophic effects are thought to be mediated by $GABA_A$ receptors, because similar effects are obtained using $GABA_A$ receptor agonists and are blocked by $GABA_A$ receptor antagonists (Barbin et al., 1993). The mechanisms of these neurotrophic effects were due to GABA-induced $[Ca^{2+}]_i$ increases induced by GABA, because they are diminished by reducing $[Ca^{2+}]_i$ increases with either L-type $Ca^{2+}$ channel blockers or $Ca^{2+}$-chelating (Berninger et al., 1995; Behar et al., 1996). Furthermore, we reported that GABA depolarizes cultured neurons through $GABA_A$ receptors and induce $Ca^{2+}$ influx through voltage-gated calcium channels (VGCCs), resulting in an increase in $[Ca^{2+}]_i$ (Takebayashi et al., 1996).

In this context, we examined whether an acute treatment with estrogen would affect $[Ca^{2+}]_i$ induced by GABA, high $K^+$ and *N*-methyl-D-aspartate acid (NMDA). We also compared effects of estrogen with dehydroepiandrosterone and dehydroepiandrosterone sulfate in order to elucidate the regulatory effect of these steroids on intracellular $Ca^{2+}$ signaling systems and to examine the possibility of neurotrophic effects by these steroids.

## 2. Materials and methods

### 2.1. Cell culture

Primary cultures were prepared according to a method we have previously described (Takebayashi et al., 1996). Wister rats were removed from the mother at embryonic day 19 under ether anesthesia. Hippocampal tissue was dissected out and incubated in 0.25% trypsin and 0.02% deoxyribonuclease I for 25 min at 37°C on a shaker, followed by inactivation of the enzymes with fetal bovine serum. The hippocampal cells were mechanically dispersed by pipetting, and rinsed twice with culture medium, comprised of Dulbecco's modified Eagle's Medium (DMEM) supplemented with 5% fetal bovine serum, 5% horse serum, glutamine (2 mM), sodium pyruvate (1 mM), penicillin G (50 U/ml) and streptomycin sulfate (50 μg/ml). The cells were plated at a density of $8 \times 10^5$/ml on poly-L-lysine-coated wells (1.77 $cm^2$), and were maintained for 4–7 days in culture medium under a humidified atmosphere of 10% $CO_2$ at 37°C.

### 2.2. Intracellular $Ca^{2+}$ measurements in single cells

The $[Ca^{2+}]_i$ was measured by fluoromicroscopic determination using 1-[2-(5-carboxyoxazol-2-yl)-6-aminobenzofuran-5-oxy]-2-(2′-amino-5′-methylphenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid (fura-2) as a $Ca^{2+}$ indicator. Hippocampal cells were rinsed twice with a balanced salt solution (BSS), containing NaCl (130 mM), KCl (5.4 mM), $CaCl_2$ (1.8 mM), glucose (5.5 mM) and HEPES (20 mM), adjusted to pH 7.4 with NaOH, and then incubated with 5 μM of fura-2 acetoxymethyl ester in BSS for 60 min at 37°C. Fura-2-loaded cells were perfused with BSS, warmed to 37°C, and examined at a flow rate of 1.5 ml/min on the stage of a fluorescence microscope-video camera system (C-2000; Hamamatsu Photonics, Hamamatsu, Japan). The fluorescence intensity of fura-2 was measured with excitation wavelengths of 340 and 380 nm and an emission wavelength of 510 nm. Fluorescence was received at 5-s intervals and the ratio (340/380 nm) of the emitted fluorescence intensities was digitized by a color image processor (Argus 50; Hamamatsu Photonics). Ratios of emitted fluorescence were calculated using a digital fluorescence analyzer (Argus 50; Hamamatsu Photonics) and converted to $[Ca^{2+}]_i$.

Table 1
Effects of tamoxifen on the β-estradiol inhibition of 50 μM GABA-induced increases in $[Ca^{2+}]_i$
Values are means ± S.E.M. of four independent experiments.

| Treatment | GABA response (% of first response) |
|---|---|
| Control | 100 ± 1.6 |
| Tamoxifen (1 μM) | 94.2 ± 4.9 |
| β-Estradiol (30 μM) | 56.8 ± 2.1[a] (N.S.) |
| Tamoxifen + β-Estradiol | 52.9 ± 4.8[a] (N.S.) |

[a] $P < 0.01$ compared with control.

### 2.3. Materials

GABA, NMDA, picrotoxin, and ω-conotoxin GVIA were obtained from Nakarai Tesque (Kyoto, Japan); 3-hydroxy-5-aminoethylisoxazole hydrobromide (muscimol hydrobromide), 3-amino-2-(4-chlorophenyl)propylphosphonic acid (phaclofen), Bay K 8644 and 5-methyl-10,11-dihydro-5*H*-dibenzo[*a*,*d*]cyclo-hepten-5,10-iminemaleate (MK801) from Research Biochemicals (MA, USA); 1, 4-dihydro-2,6-dimethyl-4-(2-nitrophenyl)-3,5-pyridinedicarboxylic acid dimethyl ester (nifedipine), [*R*-(*R**,*S**)]-5-(6,8-dihydro-8-oxofuro[3,4-*e*]-1,3-benzodioxol-6-yl)-5,6,7,8-tetrahydro-6,6-dimethyl-1,3-dioxolo[4,5-*g*]isoquindinium iodide[(−)bicuculline methiodide], androstenedione, corticosterone, dehydroepiandrosterone, dehydroepiandrosterone sulfate, α-estradiol, β-estradiol, tamoxifen, verapamil, poly-L-lysine and deoxyribonuclease from Sigma (St. Louis, MO, USA); HEPES and fura-2 acetoxymethyl ester from Dojindo (Kumamoto, Japan); DMEM, horse serum and trypsin from Gibco (Grand Island, USA); fetal bovine serum from Filtron (Brooklyn, Australia); dimethyl sulfoxide from Wako (Osaka, Japan); potassium chloride from Kanto Chemical (Tokyo, Japan); penicillin and streptomycin from Meiji Seika, (Tokyo, Japan). Solutions of androstenedione, corticosterone, dehydroepiandrosterone, dehydroepiandrosterone sulfate, α-estradiol and β-estradiol were prepared in dimethyl sulfoxide (DMSO), at a final concentration of 0.5%. This concentration of DMSO was able to dissolve β-estradiol at the highest concentration of 60 μM.

### 2.4. Statistics

All experiments were conducted at least four times to ensure a consistency of observations. In each experiment, averaged data from five to seven cells was used for analysis. The data was presented as means ± S.E.M. The Student's unpaired *t*-test was used to compare between the two groups in Table 1, Figs. 5 and 6. Two-way analysis of variance (ANOVA) was also used in Fig. 6. For multiple comparison, data were analyzed by ANOVA followed by Fisher's procedural least significant difference (PLSD) test in Figs. 2, 4–7 and 9. A $p$ value of $< 0.05$ was considered statistically significant. The $IC_{50}$ values were determined using regression lines of the log concentration–response curves generated by computer.

## 3. Results

### 3.1. The effect of β-estradiol on the GABA-induced increases in $[Ca^{2+}]_i$ in primary cultured rat hippocampal neurons

The application of GABA for 30 s induced a concentration-dependent increase in $[Ca^{2+}]_i$ in primary cultures of rat hippocampal neurons. The mean increase in $[Ca^{2+}]_i$ induced by 50 μM GABA was 70.6 ± 5.5 nM, as shown in Fig. 1A. In all of our current experiments, we routinely

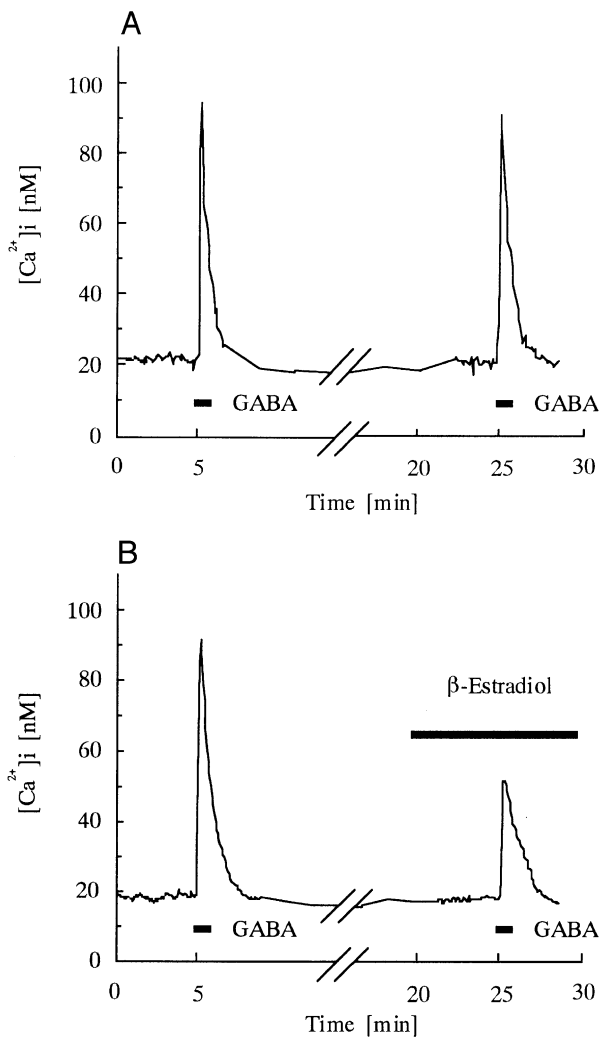


Fig. 1. The fluorometric tracing shows typical 50 μM GABA-induced $[Ca^{2+}]_i$ increases in primary cultured rat hippocampal neurons. Cells were exposed to two 30-s pulses of GABA in (A). In (B), cells were perfused with 30 μM β-estradiol 5 min prior to the second application of GABA. Experiments were repeated more than four times with similar results. Bars represent periods of agent exposure.

used a response to 50 μM GABA. More than 80% of the monitored cells were responsive. The response was fully recovered in 15–20 min after the previous application with GABA. Therefore, 25 min after measuring the first response to GABA, the cells were stimulated again for 30 s in the presence of various drugs to examine their effects on the GABA responses. Cells were perfused with the drug for 5 min prior to the second application of GABA. To exclude the influence of DMSO, cells were also exposed to BSS with 0.5% DMSO for 5 min before every stimulation with GABA and high $K^+$ in the steroids-related experiments.

We reported previously that a second stimulation of the cells with GABA produced a similar response to the first in rat primary cultured neurons (Takebayashi et al., 1996). We found that not only the area under the $[Ca^{2+}]_i$ curves, but also the peak heights of induction are similar between the first and second application with GABA. Therefore, we routinely evaluated the effect of drugs using the peak heights of the $Ca^{2+}$ responses. A typical example of the effect of 30 μM β-estradiol is shown in Fig. 1B.

### *3.2. An acute inhibitory effect of β-estradiol on the GABA-induced increases in $[Ca^{2+}]_i$*

As shown in Fig. 1B, β-estradiol inhibited the $[Ca^{2+}]_i$ increases induced by GABA. The inhibitory effect of β-estradiol on the GABA-induced $[Ca^{2+}]_i$ increases was concentration-dependent with an $IC_{50}$ of 75.2 μM (Fig. 2). Ten micromolars and higher concentrations of β-estradiol inhibited the GABA-induced $[Ca^{2+}]_i$ increases significantly (Fig. 2). To investigate the mechanism of inhibition by β-estradiol, the concentration–response curves for GABA were examined in the absence or presence of

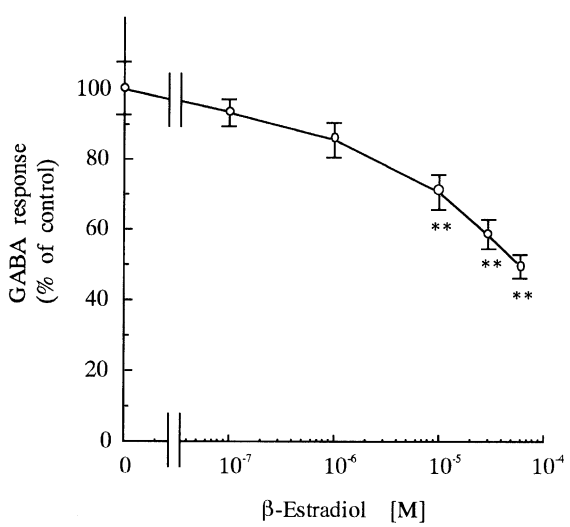


Fig. 2. Acute inhibitory effect of β-estradiol on 50 μM GABA-induced increases in $[Ca^{2+}]_i$ in primary cultures of rat hippocampal neurons. The effect of β-estradiol was assessed by comparison between the first and second GABA responses. Values represent the means ± S.E.M. of four independent experiments and $^{**}p < 0.01$ compared with control.

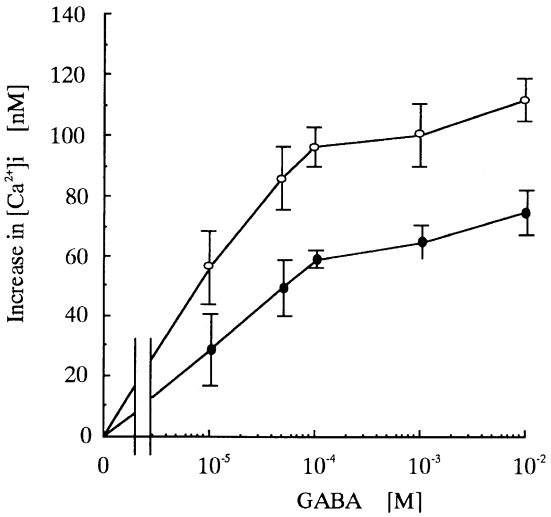


Fig. 3. Effect of β-estradiol on the concentration–response curves for the GABA-induced increases in $[Ca^{2+}]_i$. The GABA response was measured in the absence (○) or presence (●) of 30 μM β-estradiol. Values represent the means ± S.E.M. of four independent experiments.

β-estradiol. As shown in Fig. 3, GABA induced a concentration-dependent increase in $[Ca^{2+}]_i$, and the antagonism by 30 μM β-estradiol on the GABA-induced increase in $[Ca^{2+}]_i$ was conducted in a non-competitive manner. The inhibition of the GABA response by 30 μM β-estradiol was rapid, and the apparent inhibition occurred within 5 min of perfusion with β-estradiol (Fig. 4).

Tamoxifen is well known as an antagonist of the estrogen receptor. Tamoxifen is also known to modulate negatively the protein kinase C activity at high concentrations, with $IC_{50}$ values of approximately 15 μM in glioblastoma cell lines (Rocha et al., 1999). In the present results, 10 μM and higher concentrations of tamoxifen significantly inhibited the GABA-induced increase in $[Ca^{2+}]_i$ (data not

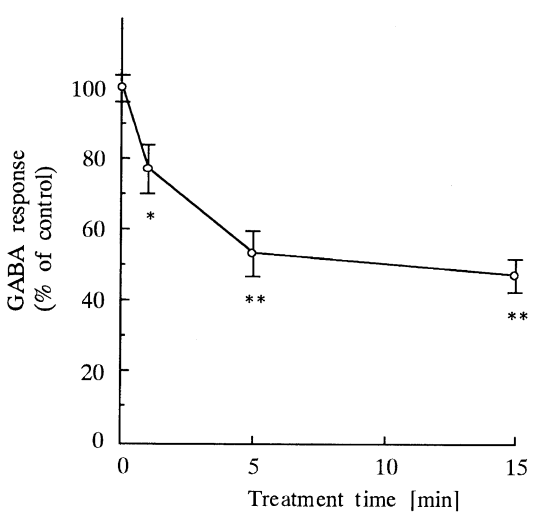


Fig. 4. Time course of the inhibition by 30 μM β-estradiol on 50 μM GABA-induced increases in $[Ca^{2+}]_i$. Values represent the means ± S.E.M. of four independent experiments and $^{*}p < 0.05$, $^{**}p < 0.01$ compared with control.

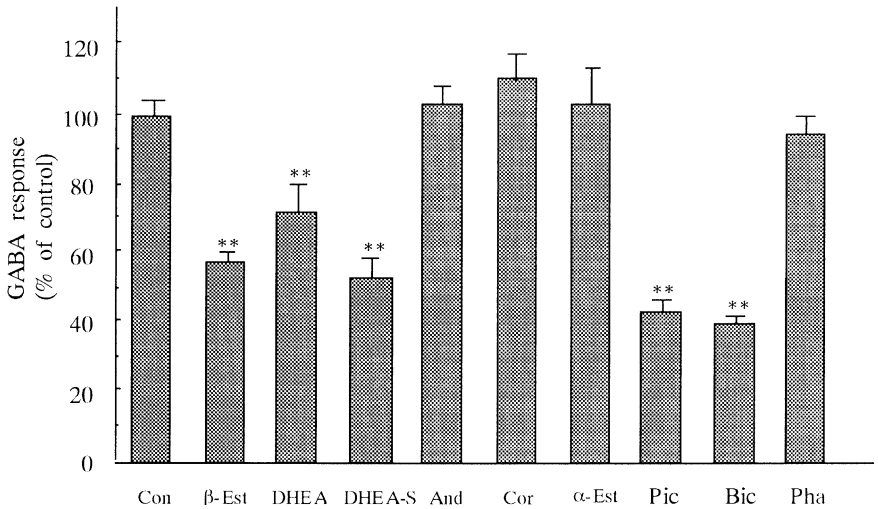


Fig. 5. Effects of β-estradiol, other steroids and GABA antagonists on the GABA-induced increases in $[Ca^{2+}]_i$. All steroids and GABA antagonists were used at a concentration of 30 μM. Values represent the mean ± S.E.M. of four independent experiments and $^{**}p < 0.01$ compared with control. Con: control; β-Est: β-estradiol; DHEA: dehydroepiandrosterone; DHEA-S: dehydroepiandrosterone sulfate; And: androstenedione; Cor: corticosterone; α-Est: α-estradiol; Pic: picrotoxin; Bic: bicuculline; Pha: phaclofen.

shown). However, 1 μM tamoxifen influenced neither the GABA-induced $[Ca^{2+}]_i$ increases nor the inhibitory effects of 30 μM β-estradiol on these increases (Table 1).

### 3.3. Inhibitory effects of dehydroepiandrosterone, dehydroepiandrosterone sulfate and other steroids on the GABA-induced increases in $[Ca^{2+}]_i$

The effects of 30 μM β-estradiol, other steroids (dehydroepiandrosterone, dehydroepiandrosterone sulfate, androstenedione, corticosterone, α-estradiol) and GABA receptor antagonists (picrotoxin, bicuculline, phaclofen) on the GABA-induced increases in $[Ca^{2+}]_i$ are shown in Fig. 5. β-Estradiol, dehydroepiandrosterone and dehydroepiandrosterone sulfate inhibited the GABA-induced $[Ca^{2+}]_i$ increases significantly. The inhibitory potency of dehydroepiandrosterone sulfate was significantly stronger than that of dehydroepiandrosterone at 30 μM ($p < 0.05$), while α-estradiol, corticosterone and androstenedione had no effect on the GABA-induced increases in $[Ca^{2+}]_i$.

Picrotoxin and bicuculline, $GABA_A$ receptor antagonists, inhibited the GABA-induced $[Ca^{2+}]_i$ increases significantly, but phaclofen, a $GABA_B$ receptor antagonist, did not, as shown in Fig. 5. Ten-micromolar nifedipine, a L-type VGCCs antagonist, inhibited the GABA-induced $Ca^{2+}$ responses to 39.9 ± 4.4% of the initial responses. The removal of $CaCl_2$ from BSS inhibited the GABA-induced $Ca^{2+}$ responses to 12.6 ± 1.2% of the first responses (data not shown).

### 3.4. The effects of β-estradiol, dehydroepiandrosterone and dehydroepiandrosterone sulfate on the high-$K^+$-induced increases in $[Ca^{2+}]_i$

To clarify the mechanisms of actions of β-estradiol, dehydroepiandrosterone and dehydroepiandrosterone sulfate, we investigated the effects of these steroids on the increases in $[Ca^{2+}]_i$ induced by depolarizing concentrations of 25 mM $K^+$. The mean increase of the high $K^+$-induced increase in $[Ca^{2+}]_i$ was 82.3 ± 4.1 nM. Concentrations of β-estradiol greater than 1 μM inhibited the high $K^+$-induced $[Ca^{2+}]_i$ increases significantly in a concentration-dependent manner, with an $IC_{50}$ of 45.5 μM (Fig. 6). Although concentrations of dehydroepiandrosterone and dehydroepiandrosterone sulfate greater than 10 and 60 μM, respectively, had inhibitory effects on the high $K^+$-induced $[Ca^{2+}]_i$ increases (ANOVA followed by Fisher's PLSD), the inhibitory effects of dehydroepiandrosterone and dehydroepiandrosterone sulfate were significantly smaller than that of β-estradiol (two-way ANOVA

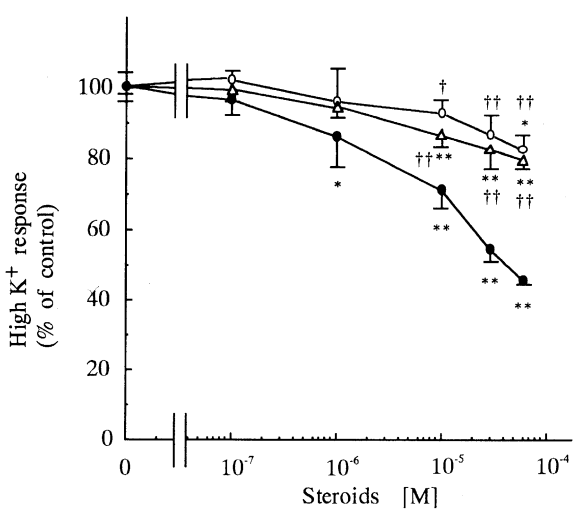


Fig. 6. Effects of β-estradiol (●), dehydroepiandrosterone (△) and dehdyroepiandrosterone sulfate (○) on 25 mM $K^+$-induced increases in $[Ca^{2+}]_i$. Values represent the mean ± S.E.M. of four independent experiments. $^{*}p < 0.05$, $^{**}p < 0.01$ compared with each control and $\dagger p < 0.05$, $\dagger\dagger p < 0.01$ compared with β-estradiol at the same concentrations.

analysis, dehydroepiandrosterone; $p < 0.01$, dehydroepiandrosterone sulfate; $p < 0.05$). At concentrations of 10, 30, 60 μM, the inhibitory effects of dehydroepiandrosterone and dehydroepiandrosterone sulfate were significantly less potent than that of β-estradiol on the high $K^+$-induced $[Ca^{2+}]_i$ increases (Student's *t*-test).

### *3.5. The effect of β-estradiol on the NMDA-induced increases in $[Ca^{2+}]_i$*

We examined the effect of β-estradiol on 10 μM NMDA-induced $[Ca^{2+}]_i$ increases (Fig. 7A), because NMDA receptors were targets of glutamates, one of the major neurotransmitters in the central nervous system, and well known to induce $Ca^{2+}$ influx. The mean increase of the NMDA-induced $[Ca^{2+}]_i$ increases was $75.8 \pm 7.0$ nM and $Ca^{2+}$ response was reduced to $15.9 \pm 2.7\%$ in the presence of 10 μM MK801 (Fig. 7B). Although 1 nM–1 μM β-estradiol did not show any significant effects, 10 μM and higher concentrations of β-estradiol inhibited the NMDA-induced $[Ca^{2+}]_i$ increases significantly. The inhibitory effect of β-estradiol on the NMDA-induced $[Ca^{2+}]_i$ increases was concentration-dependent with an $IC_{50}$ of 51.8 μM. These $[Ca^{2+}]_i$ increases induced by NMDA were also reduced to $47.4 \pm 5.6\%$ in the presence of 10 μM nifedipine (Fig. 7B), suggesting that the NMDA-induced $[Ca^{2+}]_i$ increases were mediated by not only NMDA receptors but also L-type VGCCs.

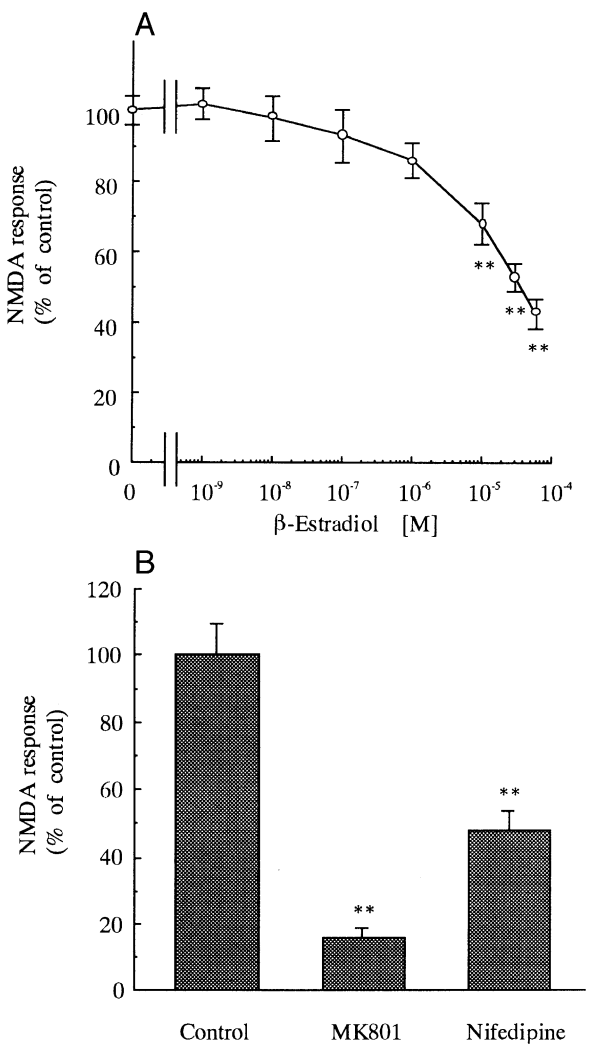


Fig. 7. Acute inhibitory effect of β-estradiol on 10 μM NMDA-induced increases in $[Ca^{2+}]_i$. Cells were perfused with β-estradiol for 5 min prior to the second application of NMDA in (A). In the same way, cells were perfused with 10 μM MK801 and 10 μM nifedipine in (B). Values represent the mean ± S.E.M. of four independent experiments and $^{*}p < 0.05$, $^{**}p < 0.01$ compared with control.

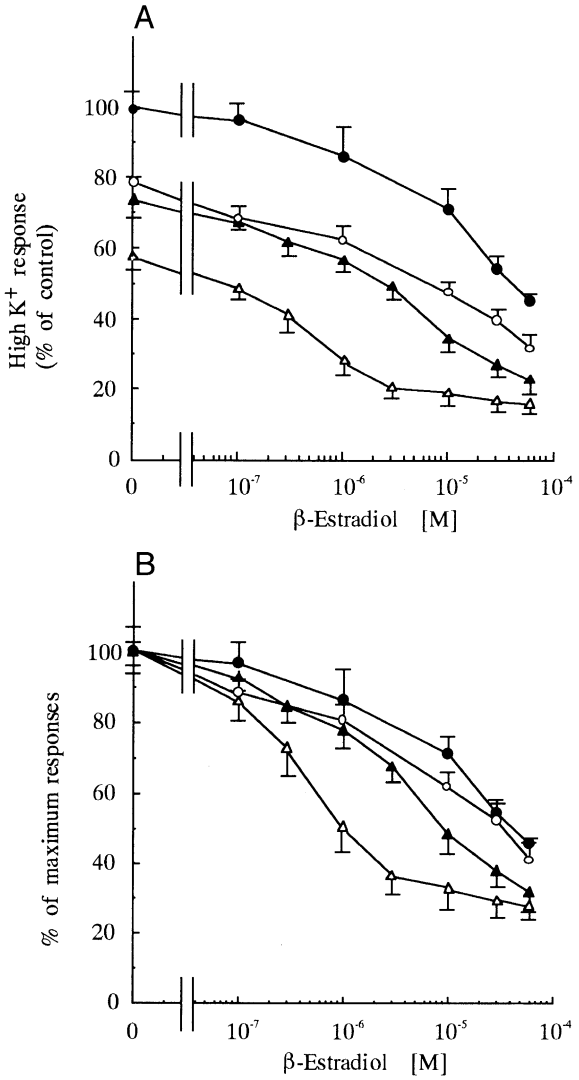


Fig. 8. Effects of β-estradiol alone (●) and β-estradiol plus 1 μM ω-conotoxin (○), 3 μM nifedipine (▲), 10 μM nifedipine (△) on 25 mM $K^+$-induced increases in $[Ca^{2+}]_i$. Values represent the mean ± S.E.M. of four independent experiments in (A). In (B), each curve was redrawn as a percentage of the respective $[Ca^{2+}]_i$ increases in the absence of β-estradiol.

### *3.6. The effects of β-estradiol on VGCCs*

Since both the GABA- and NMDA-induced $[Ca^{2+}]_i$ increases were mediated by VGCCs, it is suggested that

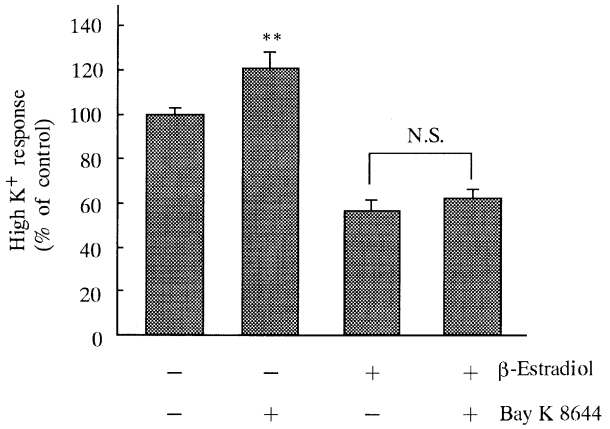


Fig. 9. Effect of 50 nM Bay K 8644 on the 25 mM $K^+$-induced increases in $[Ca^{2+}]_i$ in the absence or presence of 30 μM β-estradiol. Cells were perfused with Bay K 8644 with or without β-estradiol for 5 min prior to the second application of high $K^+$. Values represent the mean ± S.E.M. of four independent experiments. $^{**}p < 0.01$ compared with control.

β-estradiol inhibits $Ca^{2+}$ currents via VGCCs. In order to characterize VGCCs subtypes that are affected by β-estradiol, we examined the inhibitory effect of β-estradiol on the high $K^+$-induced $[Ca^{2+}]_i$ increases in the presence of channel-specific VGCCs antagonists. In the presence of 1 μM ω-conotoxin, a N-type VGCCs antagonist, high $K^+$-induced $[Ca^{2+}]_i$ increases were inhibited with an $IC_{50}$ of 33.1 μM. On the other hand, the $IC_{50}$ values of β-estradiol combined with 3 and 10 μM nifedipine were 10.5 and 2.2 μM, respectively (Fig. 8A,B). The $IC_{50}$ value of 10 μM verapamil, a L-type VGCCs antagonist, was 3.6 μM (data not shown).

To further test the action of β-estradiol on L-type VGCCs, we used a L-type VGCCs agonist, Bay K 8644. Cells were treated with 50 nM Bay K 8644 with or without 30 μM β-estradiol for 5 min prior to the second application of high $K^+$. While Bay K 8644 potentiated the high $K^+$-induced $[Ca^{2+}]_i$ increases by 20.3 ± 7.4%, those potentiation induced by Bay K 8644 were abolished in the presence of β-estradiol (Fig. 9).

## 4. Discussion

We have demonstrated that β-estradiol as well as its precursors, dehydroepiandrosterone and dehydroepiandrosterone sulfate, inhibited the GABA-induced $[Ca^{2+}]_i$ increases in primary hippocampal cultures. While β-estradiol inhibited the high $K^+$-induced $[Ca^{2+}]_i$ increases, both dehydroepiandrosterone and dehydroepiandrosterone sulfate inhibited to a lesser extent. β-Estradiol also inhibited the NMDA-induced $[Ca^{2+}]_i$ increases.

Acute treatment with β-estradiol inhibited the GABA-induced $[Ca^{2+}]_i$ increases in a concentration-dependent manner with an $IC_{50}$ of 75.2 μM. β-Estradiol showed rapid inhibitory effects within 5 min, suggesting that the effects of β-estradiol are non-genomic and occur at membrane levels. Tamoxifen did not interfere with the inhibitory effects of β-estradiol, suggesting that the inhibitory effects of β-estradiol on the GABA response were not mediated by estrogen receptors. Acute treatment with β-estradiol also inhibited the high $K^+$-induced $[Ca^{2+}]_i$ increases in a concentration-dependent manner with an $IC_{50}$ of 45.5 μM. Thus, acute treatment with β-estradiol inhibited both GABA- and high $K^+$-induced $[Ca^{2+}]_i$ increases with a relatively similar $IC_{50}$. Previously, we showed that the GABA-induced $[Ca^{2+}]_i$ increases share a common pathway of $Ca^{2+}$ current with the high $K^+$-induced $[Ca^{2+}]_i$ increases (Takebayashi et al., 1996). Because the inhibitory effects by β-estradiol on both GABA- and high $K^+$-induced $[Ca^{2+}]_i$ increases were similar, the inhibitory mechanism of β-estradiol on GABA responses may act primarily at VGCCs.

In order to clarify the mechanisms of actions of β-estradiol, we examined the inhibitory effect of β-estradiol on the high $K^+$-induced $[Ca^{2+}]_i$ increases in the presence of a N-type VGCCs antagonist, 1 μM ω-conotoxin, and a L-type VGCCs antagonist, 3 and 10 μM nifedipine and 10 μM verapamil. The $IC_{50}$ value of β-estradiol combined with ω-conotoxin (33.1 μM) was similar to that of β-estradiol alone (45.5 μM), indicating that β-estradiol did not modulate N-type VGCCs. In contrast, the $IC_{50}$ values of β-estradiol combined with 3 and 10 μM nifedipine that modulate dihydropyridine site of VGCCs were reduced to 10.5 μM and 2.2 μM in a dose-dependent manner. The $IC_{50}$ value of β-estradiol combined with verapamil was also reduced to 3.6 μM. β-Estradiol also abolished the positive modulatory effect of dihydropyridine agonist, Bay K 8644. Our data suggest that β-estradiol exerts the inhibitory effect on the high $K^+$-induced $[Ca^{2+}]_i$ increases by modulating both dihydropyridine site and verapamil binding site of VGCCs in primary cultured hippocampal neurons. Although there are few studies that refer to subtypes of VGCCs modulated by β-estradiol in neurons, Mermelstein et al. (1996) reported that β-estradiol acts in a steroid-specific manner to reduce $Ba^{2+}$ currents of L-type VGCCs in rat neostriatal neurons. In cells of peripheral organs, it is suggested that β-estradiol decreases the dihydropyridine and isoprenaline-sensitive $Ca^{2+}$ current in cardiac myocytes (Jiang et al., 1992), both nicardipine-sensitive and -resistant $Ca^{2+}$ current in a basilar artery (Ogata et al., 1996), L-type $Ba^{2+}$ current and T-type $Ca^{2+}$ current in vascular smooth muscle cells (Zhang et al., 1994). In most of these studies, β-estradiol at micromolar concentrations is required to maximally reduce $Ca^{2+}$ current via VGCCs and these results support our data in the present study.

Acute treatment with dehydroepiandrosterone and dehydroepiandrosterone sulfate inhibited the GABA-induced $[Ca^{2+}]_i$ increases in a similar manner as β-estradiol. However, the inhibitory potency of 30 μM dehydroepiandrosterone sulfate is significantly stronger than the same con-

centration of dehydroepiandrosterone. Recent studies have shown that many neurosteroids modulate GABA function allosterically through a unique binding site on the $GABA_A$ receptor complex (Majewska et al., 1986; Puia et al., 1990; Schmid et al., 1998). Acute treatment with dehydroepiandrosterone and dehydroepiandrosterone sulfate also inhibited the high $K^+$-induced $[Ca^{2+}]_i$ increases in a concentration-dependent manner. However, the inhibitory effect of β-estradiol on the high $K^+$-induced $[Ca^{2+}]_i$ increases was much stronger than that of dehydroepiandrosterone and dehydroepiandrosterone sulfate. As for the relationship between dehydroepiandrosterone, dehydroepiandrosterone sulfate and VGCCs, it is reported that 10–100 μM dehydroepiandrosterone sulfate rapidly and reversibly inhibits 43–73% of VGCCs currents in freshly isolated adult guinea-pig hippocampal CA1 pyramidal neurons (Ffrench-Mullen and Spence, 1991). Results reported here showed that dehydroepiandrosterone and dehydroepiandrosterone sulfate greater than 10 and 60 μM, respectively, had inhibitory effects on the high $K^+$-induced $Ca^{2+}$ responses. Acute treatment with dehydroepiandrosterone and dehydroepiandrosterone sulfate inhibited the GABA-induced $[Ca^{2+}]_i$ increases to the same extent as β-estradiol, however, the inhibitory effect of dehydroepiandrosterone and dehydroepiandrosterone sulfate on the high $K^+$-induced $[Ca^{2+}]_i$ increases was much less than that of β-estradiol. These findings suggest that the inhibitory mechanisms of β-estradiol, dehydroepiandrosterone and dehydroepiandrosterone sulfate on the GABA-induced $[Ca^{2+}]_i$ increases are different and that dehydroepiandrosterone and dehydroepiandrosterone sulfate may act primarily at $GABA_A$ receptors rather than at VGCCs.

Micromolar levels of β-estradiol inhibited GABA, high $K^+$ and NMDA-induced $[Ca^{2+}]_i$ increases with similar $IC_{50}$ values. These results indicate that β-estradiol has a general effect on changes in $[Ca^{2+}]_i$ regardless of their origins. However, we must consider several points in this issue. A recent electrophysiological study suggests that NMDA receptor-mediated excitatory postsynaptic potentials (EPSPs) or long-term potentiation (LTP) was enhanced by a treatment of 100 pM–1 nM β-estradiol (Foy et al., 1999) or estrous cycles of estrogen (Warren et al., 1995; Good et al., 1999). Other reports provide evidences that β-estradiol at 1 nM–1 μM concentrations regulates NMDA receptor-mediated functions and exerts a growth-promoting effect in cultured neurons (Brinton et al., 1997a,b; Pozzo-Miller et al., 1999). On the other hand, there is a report that β-estradiol at micromolar concentrations modulates NMDA receptor-mediated functions negatively in a dose-dependent manner similar to our results (Weaver et al., 1997). Previous reports suggest that the modulation of β-estradiol on the NMDA responses is different according to its concentrations. In contrast to previous reports, our results demonstrated that 1 nM–1 μM β-estradiol did not show any significant effects, whereas 10 μM and higher concentrations of β-estradiol inhibited the NMDA-induced $[Ca^{2+}]_i$ increases. These discrepancies may be due to several possibilities listed as follows: (1) Interaction between NMDA receptor and VGCCs: our results showed that the NMDA-induced $[Ca^{2+}]_i$ increases were also reduced to half in the presence of 10 μM nifedipine (Fig. 7B). This data suggests that NMDA responses are mediated by VGCCs as well as GABA responses. Some reports suggest that NMDA-induced $Ca^{2+}$ influx was partially blocked by nifedipine (Griffiths et al., 1998; Lachica et al., 1998). Therefore, one possibility is that β-estradiol may inhibit the NMDA-induced $[Ca^{2+}]_i$ increases via VGCCs. (2) Direct effect on NMDA receptor: in previous reports about the enhancement of NMDA function by β-estradiol (Brinton et al., 1997a,b; Foy et al., 1999; Pozzo-Miller et al., 1999), β-estradiol was treated for much longer time (30 min-8 days) than our report (5 min). Because it is reported that the longer treatment of estradiol is necessary to increase NMDA binding sites (Weiland., 1992) and both mRNA and protein levels of NMDAR1 in the rat hippocampus (Gazzaley et al., 1996), these mechanisms cannot be explained in our results. Besides, it is not yet known whether acute treatment (5 min) of estradiol directly affects NMDA receptors or indirectly affects via second messenger processes that in turn influence NMDA receptors. (3) Direct effect on the cell membrane: a number of studies suggest that estradiol acts not only by mediating estrogen receptors, but also directly affects cell membranes. β-Estradiol intercalates into bilayer of cell membranes and alters the membrane fluidity quickly, resulting in altering membrane functions through modification in the activity of membrane-bound enzymes (Schwartz et al., 1996; Whiting et al., 1995, 2000). Therefore, we assume that β-estradiol may have inhibitory effects in $[Ca^{2+}]_i$ increases induced by GABA, high $K^+$ and NMDA in nonspecific manners. However, further studies are required to clarify these mechanisms of β-estradiol.

In the present study, β-estradiol, dehydroepiandrosterone and dehydroepiandrosterone sulfate showed inhibitory effects on the GABA-induced $[Ca^{2+}]_i$ increases at a pharmacological concentration of about 30 μM. It is reported that these steroids are active at nanomolar concentrations in the brain (Foy et al., 1999; Kimonides et al., 1998). However, it is important to investigate the effects of β-estradiol at higher concentrations that we used in the present study because of the reasons mentioned as follows. (1) The regional levels of these neurosteroids in the brain are still uncertain. Since it is reported that the expressions of estrogen- and dehydroepiandrosterone-synthesizing enzymes are markedly increased in specific areas, including in hippocampus (Compagnone et al., 1995; Ivanova and Beyer, 2000; MacLusky et al., 1994), it is likely that the regional levels of β-estradiol, dehydroepiandrosterone and dehydroepiandrosterone sulfate may exceed those previously reported. (2) The serum level of β-estradiol in-

creases as much as 10 times in humans during pregnancy (Speroff et al., 1991). In addition, the fact that prenatal stresses impair regulation of hypothalamic–pituitary–adrenal axis (Weinstock, 1997) and hypothalamic–pituitary–gonad axis (Bonner., 1994) suggest that concentrations of β-estradiol, dehydroepiandrosterone and dehydroepiandrosterone sulfate may increase by prenatal stresses. (3) It is reported that more than 90% of plasma estrogen is bounded with plasma albumin or sex hormone binding globulin (Hsueh and Billig, 1995). Because protein-bound hormone can function in vivo as a free fraction, the actual concentration of free hormone may be many times greater than the plasma concentration of free hormones. Thus, higher concentrations of free hormones are required to more closely simulate actual physiological conditions (White et al., 1995).

In summary, β-estradiol, as well as dehydroepiandrosterone and dehydroepiandrosterone sulfate, has acute inhibitory effects on the GABA-induced $[Ca^{2+}]_i$ increases and the inhibitory effects of β-estradiol are not mediated by estrogen receptors. However, β-estradiol has much stronger inhibitory effects on the high-$K^+$-induced $[Ca^{2+}]_i$ increases than dehydroepiandrosterone and dehydroepiandrosterone sulfate. In contrast, β-estradiol inhibited the NMDA-induced $[Ca^{2+}]_i$ increases and NMDA responses were reduced to half in the presence nifedipine, indicating that the NMDA-induced $[Ca^{2+}]_i$ increases also involved VGCCs. The $IC_{50}$ value of β-estradiol combined with ω-conotoxin on the high $K^+$-induced $[Ca^{2+}]_i$ increase was similar to that of β-estradiol alone, but the $IC_{50}$ value of β-estradiol combined with nifedipine was reduced. β-Estradiol also abolished the positive modulatory effect of Bay K 8644, suggesting that the inhibitory effect of β-estradiol involves L-type VGCCs. Our results showed that the inhibitory mechanism of β-estradiol is different from that of dehydroepiandrosterone and dehydroepiandrosterone sulfate, suggesting β-estradiol may act primarily at L-type VGCCs.

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