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# 17β-Estradiol inhibits the voltage-dependent L-type $Ca^{2+}$ currents in aortic smooth muscle cells

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**Abstract**

To elucidate the mechanisms of estrogens-induced relaxation effects on vascular smooth muscle cells, the effects of estrogens and the related hormones were examined in cultured rat thoracic aortic smooth muscle cell lines (A7r5), using the whole-cell voltage clamp technique. The patch pipette was filled with 140 mM CsCl- or KCl-containing internal solution. With CsCl-internal solution, 17β-estradiol and synthetic estrogens, ethynylestradiol and diethylstilbestrol (0.1–30 μM) inhibited the $Ba^{2+}$ inward current ($I_{Ba}$) through the voltage-dependent L-type $Ca^{2+}$ channel in a concentration-dependent and reversible manner. The potency of the inhibitory effects on $I_{Ba}$ was 17β-estradiol < ethynylestradiol < diethylstilbestrol. 17β-Estradiol (10 μM) appeared to reduce the maximal conductance of $I_{Ba}$ with only a slight shift of voltage-dependency of inactivation and to affect $I_{Ba}$ in a use-independent fashion. On the other hand, testosterone and progesterone (30 μM) failed to affect $I_{Ba}$. At a holding potential of −40 mV, both vasopressin and endothelin-1 (100 nM) activated a long-lasting inward current. After endothelin-1 (100 nM) activated the current, the additional application of vasopressin (100 nM) could not induce it furthermore, suggesting that each agonist activates the same population of the channels. The reversal potential of the current was about 0 mV and was not significantly altered by replacement of $[Cl^-]_i$ or $[Cl^-]_0$ and the inward current was also observed even when extracellular cations are $Ca^{2+}$, proposing that it was a $Ca^{2+}$-permeable non-selective cation channel ($I_{N.S.}$). $La^{3+}$ or $Cd^{2+}$ (1 mM) completely abolished $I_{N.S.}$, however, nifedipine (10 μM) failed to inhibit it at all. Diethylstilbestrol (1–30 μM) suppressed the $I_{N.S.}$ evoked by both endothelin-1 and vasopressin in a concentration-dependent manner, while 17β-estradiol, ethynylestradiol, progesterone and testosterone (30 μM) failed to inhibit it significantly. In addition, at a holding potential of +0 mV, 17β-estradiol by itself did not affect the holding currents, and did not inhibit $K^+$ currents evoked by endothelin-1 or vasopressin, possibly due to the $Ca^{2+}$ release from the storage sites. These results suggest that 17β-estradiol may play a role in regulating vascular tone, selectively by inhibiting the voltage-dependent L-type $Ca^{2+}$ current in vascular smooth muscle cells.

*Keywords:* 17β-Estradiol; Estrogen; Vascular smooth muscle; $Ca^{2+}$ current, L-type, voltage-dependent; Endothelin-1; Vasopressin; $Ca^{2+}$-permeable non-selective cation current

## 1. Introduction

Estrogen regulates vascular tone in endothelial-dependent (Gisclard et al., 1988; Van Buren et al., 1992; Williams et al., 1992; Shay et al., 1994; Collins et al., 1994; Reis et al., 1994) and independent fashions (Harder and Coulson, 1979; Chang et al., 1980; Stice et al., 1987a,b; Jiang et al., 1991; Jiang et al., 1992a; Collins et al., 1993; Shay et al., 1993). The former may be linked to the modulation of the production of nitric oxide (Van Buren et al., 1992; Collins et al., 1994) and an endothelium-derived vasoactive intermediary that induces vascular smooth muscle relaxation and inhibits vascular smooth muscle cell proliferation and mitogenesis (Garg et al., 1989), while the latter may be due to the stimulation of prostacyclin production (Chang et

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al., 1980; Makila et al., 1982), or the direct action of estrogens on vascular smooth muscle cells (Stice et al., 1987a,b; Jiang et al., 1991, Jiang et al., 1992a; Collins et al., 1993; Shay et al., 1993). In isolated rabbit coronary artery and rat aortic preparations without endothelium, 17β-estradiol reduces the contraction induced by membrane depolarization in a high $K^+$ medium (Jiang et al., 1991). 17β-Estradiol also has an inhibitory effect on $Ca^{2+}$-induced contraction of vein smooth muscle (MacCalden, 1975). Furthermore, it has been reported that 17β-estradiol decreases the contraction of perfused rabbit hearts and guinea-pig isolated ventricular cells (Raddino et al., 1986; Jiang et al., 1992b). These data suggest that a reduction of $Ca^{2+}$ entry through the $Ca^{2+}$ channels may be one of the direct mechanisms by which 17β-estradiol relaxes smooth muscle. Actually, it has been reported that 4-hydroxylated estradiol reduced $Ca^{2+}$ uptake of uterine arterial smooth muscle cells through potential-sensitive channels (Stice et al., 1987a,b) and 17β-estradiol inhibits the voltage-dependent L-type $Ca^{2+}$ current in guinea-pig ventricular myocytes (Jiang et al., 1992b). However, little is known about the ionic mechanisms of estrogen action on vascular smooth muscle cells.

17β-Estradiol also reduces contraction caused by endothelin-1 in rabbit coronary and basilar artery (Jiang et al., 1991; Shay et al., 1993), and antagonizes a vasopressin-induced coronary spasm in isolated rabbit heart (Raddino et al., 1986). In addition, diethylstilbestrol, a synthetic estrogen, inhibits noradrenaline-induced contraction in rat aortic strips (Roesch and Borowitz, 1981). In vascular smooth muscle, these vasoactive hormones induce $Ca^{2+}$ release from internal $Ca^{2+}$ storage sites and $Ca^{2+}$ influx through the membrane associated with contraction (Bolton, 1979; Somlyo and Himpens, 1989; Van Breemen and Saida, 1989). Since in coronary arterial cells, endothelin increases voltage-dependent $Ca^{2+}$ currents (Goto et al., 1989; Silberberg et al., 1989), it is likely that endothelin causes extracellular $Ca^{2+}$ influx by activation of voltage-dependent $Ca^{2+}$ channels. On the other hand, independently from $Ca^{2+}$ entry through the voltage-dependent $Ca^{2+}$ channels, receptor-activated $Ca^{2+}$ entry in vascular smooth muscle cells is mediated by $Ca^{2+}$-permeable non-selective cation channels which are insensitive to classical $Ca^{2+}$ channel blockers like nifedipine (Benham and Tsien, 1987; Ruegg et al., 1989; Wallnofer et al., 1989; Amedee et al., 1990; Shimpson et al., 1990; Loirand et al., 1991; Wang and Large, 1991). It has been reported that endothelin activates $Ca^{2+}$-permeable non-selective cation channels in coronary arterial cells and aortic smooth muscle cells (Van Renterghem et al., 1988a; Chen and Wagoner, 1991), and noradrenaline activates the channels in rabbit ear arterial cells and portal vein cells (Amedee et al., 1990; Wang and Large, 1991; Inoue and Kuriyama, 1993). In this paper, to elucidate the mechanism of the direct relaxation effects of estrogen on vascular smooth muscle cells, the actions of estrogens on ionic currents were investigated in aortic smooth muscle cells, using the patch clamp technique. In particular, the effects of estrogens on the voltage-dependent $Ca^{2+}$ currents were compared with those on receptor-operated $Ca^{2+}$-permeable non-selective cation channels. Here we reported that 17β-estradiol inhibited the voltage-dependent L-type $Ca^{2+}$ currents, but it failed to affect receptor-operated $Ca^{2+}$-permeable non-selective cation channels in aortic smooth muscle cells.

## 2. Materials and methods

### 2.1. Cell preparation

A7r5 cells, established vascular smooth muscle cells line obtained from embryonic rat thoracic aorta (Kimes and Brandt, 1976) were obtained from American Type Culture Collection through Dainippon Seiyaku (Kyoto, Japan). Cells were grown in Dulbecco's modified Eagle's medium (DMEM, Nissui Seiyaku, Tokyo, Japan) supplemented with 10% fetal bovine serum (M.A. Bioproducts, Walkersville, MD), 50 units/ml of penicillin and 50 μg/ml of streptomycin at 37°C in a fully humidified atmosphere of 5% $CO_2$ in air. Confluent cell layers were serum-derived by culturing in DMEM containing 0.3% bovine serum albumin for 24 h. Cells were isolated by an enzymatic procedure, using trypsin, and used for the experiments. All experiments were performed at 35–37°C.

### 2.2. Solution and drugs

The composition of the standard extracellular solution was as follows (in mM): NaCl 136.5, KCl 5.4, $CaCl_2$ 1.8, $NaCl_2$ 0.53, glucose 5.5 and Hepes-NaOH 5.5 (pH 7.4). The pipette solution contained (in mM): CsCl 130, EGTA 0.15, $MgCl_2$ 2, $Na_2ATP$ 3, guanosine 5′-triphosphate (sodium salt, Sigma) 0.1 and Hepes-CsOH buffer 5 (pH 7.2). To record $K^+$ currents, the patch pipette contained (in mM): KCl 130, EGTA 0.15, $MgCl_2$ 2, $Na_2ATP$ 3, guanosine-5′-triphosphate 0.1 and Hepes-KOH buffer 5 (pH 7.2). The $Ba^{2+}$-containing bathing solution was the same as the control bathing solution, with the exception that $CaCl_2$ was replaced with $BaCl_2$ (5 mM). When external or internal $Cl^-$ concentration was changed, $Cl^-$ was replaced with equimolar aspartic acid. 17β-Estradiol, ethynylestradiol, diethylstilbestrol, testosterone, progesterone, vasopressin and endothelin-1 were obtained from Sigma (St. Louis, MO, USA). 17β-Estradiol, ethynylestradiol,

diethylstilbestrol, testosterone, or progesterone was dissolved in ethanol to give a stock solution of 10 mM.

### 2.3. Recording technique and data analysis

The membrane currents were recorded using the tight-seal whole cell clamp technique (Hamill et al., 1981; Kurachi et al., 1986; Nakajima et al., 1992). The heat-polished patch electrode, filled with the artificial internal solution (for composition, see above), had the tip resistance of 3–5 MΩ. The series resistance was compensated. The membrane currents were continuously monitored with a high-gain storage oscilloscope (COS 5020-ST, Kikusui Electronic, Tokyo, Japan). The data were stored on a videotape using the PCM converter system (RP-880, NF electronic circuit design, Tokyo, Japan). The data were reproduced, low-passed filtered at 1 kHz (−3dB) with a Bessel filter (FV-625, NF, 48 dB/octave slope attenuation), sampled at 5 kHz and analyzed off-line on a computer using p-Clamp software (Axon Instruments, CA). The quasi-steady state inactivation parameters ($f_\infty$) of the $Ba^{2+}$-inward current through voltage-dependent $Ca^{2+}$ channel ($I_{Ba}$) at various membrane potentials were estimated using a double-pulse protocol. The conditioning voltage pulses (3 s in duration) to various membrane potentials between −70 and +30 mV were applied from a holding potential of −80 mV. At 10 ms after the end of each conditioning pulse, a test pulse to +10 mV (200 ms in duration) was applied to evoke $I_{Ba}$. The ratio of the amplitudes of $I_{Ba}$ with and without the conditioning pulse was plotted at the membrane potential of each conditioning pulse (inactivation curve). The interval between the sets of double pulses was 60 s. Statical data were expressed as means ± S.D. Student's *t*-test was used for statistical analysis of the data and a value of $P < 0.05$ was considered significant.

## 3. Results

### 3.1. Effects of 17β-estradiol and the related hormones on the voltage-dependent L-type $Ca^{2+}$ current in vascular smooth muscle cells

In A7r5 cells, it has been shown that the voltage-dependent $Ca^{2+}$ current is mainly composed of a dihydropyridine-sensitive high-threshold $Ca^{2+}$ channel, which can be classified as the L-type (McCarthy and Cohen, 1989; Marks et al., 1990; Giannattasio et al.,

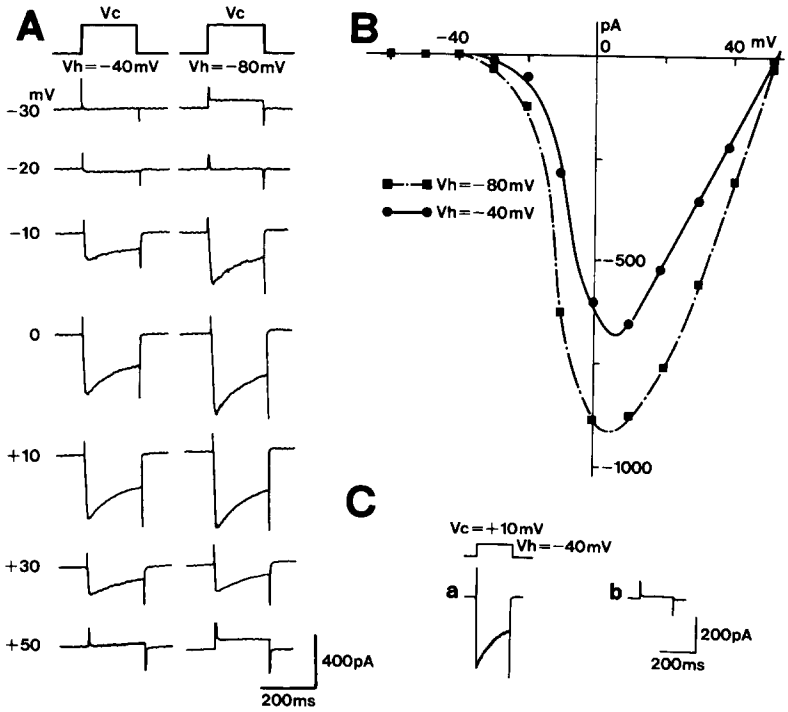


Fig. 1. Voltage-dependent $Ca^{2+}$ currents in aortic smooth muscle cells (A7r5). (A) The cell was held at −40 mV or −80 mV, and the command voltage pulses (200 ms in duration) to various membrane potentials were applied at 0.2 Hz. The patch pipette contained CsCl-internal solution, and 5mM $Ba^{2+}$ was added to the bath solution. The original current traces at a holding potential of −40 mV and −80 mV are shown at each command voltage steps. (B) The current-voltage relationships of $Ca^{2+}$ currents at the holding potential of −80 mV (closed square) and −40 mV (closed circle) after the leakage currents were subtracted. (C) Effects of nifedipine on $Ca^{2+}$ currents. The cell was held at −40 mV and the command voltage pulse to +0 mV were applied. The current traces are shown in control (a) and in the presence of nifedipine (10 μM, b).

1991; Marks and Jones, 1992). Fig. 1A showed the voltage-dependent $Ca^{2+}$ current in the A7r5 cells. To eliminate $K^+$ currents, the pipette was filled with the $Cs^+$-internal solution, and the bathing solution contained 5 mM $Ba^{2+}$ in place of $Ca^{2+}$. During the depolarizing steps from both −80 mV and −40 mV, the inward currents were elicited, and its current-voltage relationship of the peak inward current is shown in Fig. 1B. This inward current developed at potentials positive to −30 mV and reached a maximal amplitude at near +0 mV. The inward currents at any command voltage pulses was dramatically enhanced by Bay K 8644, but blocked by $Cd^{2+}$ (1 mM) or nifedipine (10 μM) (Fig. 1C), indicating that the inward current actually flowed through the L-type $Ca^{2+}$ channel. Therefore, we investigated the effects of 17β-estradiol and the related hormones on the $I_{Ba}$ through the L-type $Ca^{2+}$ channel as indicated in Fig. 2A. The cell was held at −40 mV and the command voltage pulses to +0 mV were applied at 0.2 Hz. Ethanol at doses lower than 0.3% did not affect $I_{Ba}$ significantly, but 17β-estradiol (30 μM) reversibly reduced the amplitude of $I_{Ba}$ (Fig. 2A, upper trace). Diethylstilbestrol (30 μM), a synthetic estrogen, almost completely abolished $I_{Ba}$ (Fig. 2A, lower trace). Fig. 2B shows the effects of 17β-estradiol (10 μM) on the current-voltage relation of $I_{Ba}$. $I_{Ba}$ evoked by depolarizing command steps from −40 mV was reduced by 17β-estradiol (10 μM) (Fig. 2B). The amplitude of the peak inward current in the absence and presence of 17β-estradiol was plotted at each command potentials in Fig. 2C. 17β-estradiol consistently reduced the amplitude of the inward cur-

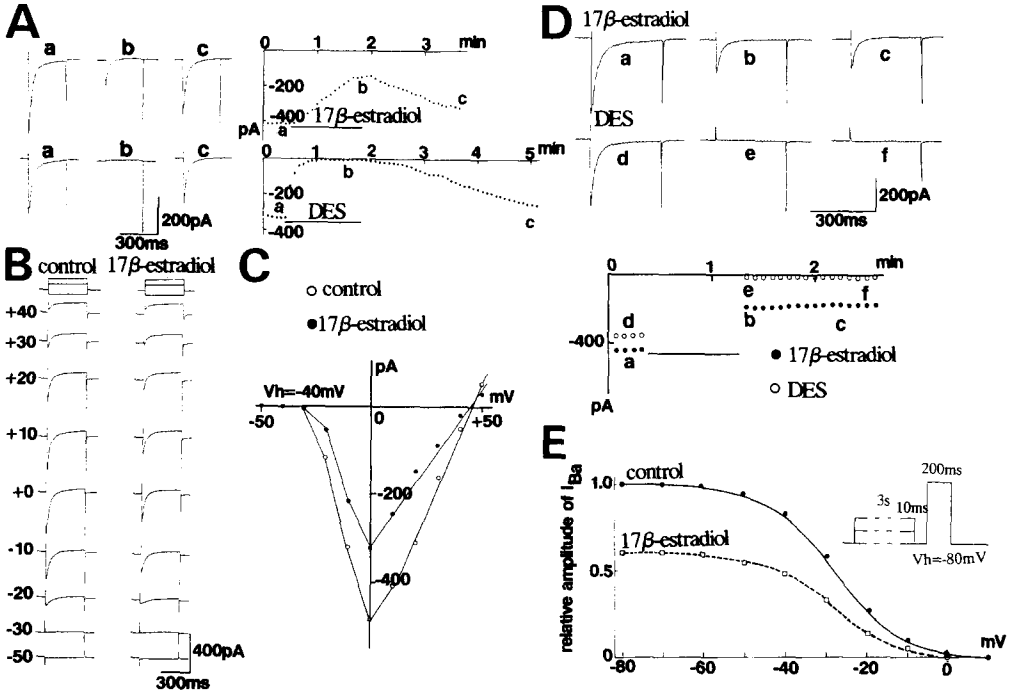


Fig. 2. Effects of estrogens on the voltage-dependent L-type $Ca^{2+}$ current ($I_{Ca}$) in aortic smooth muscle cells. (A) Effects of estrogens on $I_{Ca}$. The cells were held at −40 mV, and command voltage pulses to +0 mV were applied at 0.2 Hz. 17β-Estradiol (30 μM, upper part) or diethylstilbestrol (30 μM, lower part) was applied. The time courses of the alterations of $I_{Ca}$ amplitude are indicated in each right part. The drug sequences are also shown. (B) and (C) The current-voltage relationships of $I_{Ca}$ in the absence and presence of 17β-estradiol. The cell was held at −40 mV, and the original current traces elicited by depolarizing steps are indicated in the absence and presence of 17β-estradiol (10 μM) in (B). The current-voltage relationships of $I_{Ca}$ after the leakage currents were subtracted are shown in control (open circle) and in the presence of 17β-estradiol (closed circle) in (C). (D) Use-dependent inhibition of $I_{Ca}$ by estrogens. The cells were held at −40 mV, and command voltage steps to +0 mV (300 ms in duration) were applied at 0.2 Hz. The protocols perfusing 17β-estradiol (30 μM) or diethylstilbestrol (30 μM) are indicated by the bar in the lower part. The current traces obtained at the times indicated by a–f in the lower part are shown. The time courses of the $I_{Ca}$ amplitude during perfusion of each hormone are indicated in lower part. After having stopped the voltage steps, 17β-estradiol (upper part) or diethylstilbestrol (lower part) was added to the bath solution. After 90 s of pause in hormone-containing solution, repetitive depolarization pulses to +0 mV at 0.2 Hz were reapplied. Under the application of hormones, the amplitude of $I_{Ca}$ evoked by the first voltage step after pause was already suppressed (b,e), and remained constant during the repetitive depolarizing pulses (c,f). (E) Quasi-steady-state inactivation curve of $I_{Ca}$ in the absence and presence of 17β-estradiol. The quasi-steady-state inactivation parameters ($f_\infty$) of $I_{Ca}$ were obtained using double-pulse protocol in the control and under the application of 17β-estradiol (10 μM). The conditioning voltage steps (3 s in duration) to various membrane potentials between −70 and +30 mV were applied from a holding potential of −80 mV. At 10 ms after each conditioning pulse, a test pulse to +10 mV (200 ms in duration) was applied to evoke $I_{Ca}$. The relative amplitude of $I_{Ca}$ in response to the test pulse is plotted at each membrane potential of the conditioning pulses. The relationships between the membrane potential and $f_\infty$ in the control and under the application of 17β-estradiol were fit by the Boltzmann equation.

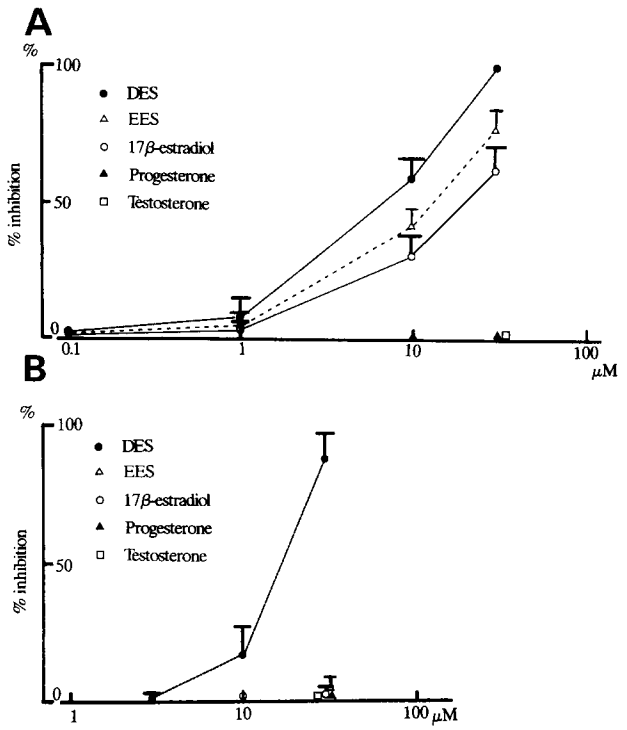


Fig. 3. (A) Concentration-dependent inhibition of $I_{Ca}$ by estrogens and the related hormones. The relationship between the concentration of estrogens (17β-estradiol, diethylstilbestrol (DES), ethynylestradiol (EES)), progesterone, and testosterone and the percent inhibition of $I_{Ca}$ is shown. The cell was held at −40 mV, and command voltage steps to +0 mV were applied at 0.2 Hz. Various concentrations of these hormones were applied. The amplitude of the peak of $I_{Ca}$ after the application of these hormones was compared with the control value. The percent inhibition of these hormones on $I_{Ca}$ is plotted. Mean ± S.D. is indicated ($n$ = 4–5 in each case). (B) Concentration-dependent inhibition of endothelin-1-evoked non-selective cation currents by estrogens and the related hormones. The relationship between the concentration of estrogens (17β-estradiol, diethylstilbestrol (DES), ethynylestradiol (EES)), progesterone, and testosterone and the percent inhibition of non-selective cation currents ($I_{N.S.}$) evoked by endothelin-1 is shown. The cells were held at −40 mV. After endothelin-1 (100 nM) activated $I_{N.S.}$, the various concentrations of these hormones were applied. The amplitude of the $I_{N.S.}$ induced by endothelin-1 in the presence of these hormones was compared with the control value in the absence of hormones. The percent inhibition of these hormones on $I_{N.S.}$ is plotted. Mean ± S.D. is indicated ($n$ = 4 in each case).

rent at each potential without affecting the shape of the current-voltage relationship of $I_{Ba}$. The reversal potential of $I_{Ba}$ also did not change significantly under the application of 17β-estradiol. Similarly, diethylstilbestrol (10 μM) or ethynylestradiol (10 μM) inhibited $I_{Ba}$. On the other hand, progesterone or testosterone (30 μM) was totally ineffective.

Fig. 3A indicates the relationship between the concentration of estrogens and the related sex hormones and the percent inhibition of $I_{Ba}$ at +0 mV from a holding potential of −40 mV. 17β-Estradiol and synthetic estrogens, diethylstilbestrol and ethynylestradiol, (0.1–30 μM) suppressed $I_{Ba}$ in a concentration-dependent manner. The potency of the inhibitory effect on $I_{Ba}$ among estrogens was 17β-estradiol < ethinylestradiol < diethylstilbestrol, which was somewhat similar to that to the estrogen receptors. On the other hand, both testosterone and progesterone (30 μM) failed to inhibit $I_{Ba}$. These results indicate that estrogens specifically have a $Ca^{2+}$-antagonist effect in vascular smooth muscle cells as previously proposed (Collins et al., 1993).

To characterize the mode of estrogen inhibition of $I_{Ba}$, we examined the use dependence of the effect of 17β-estradiol on $I_{Ba}$ as indicated in Fig. 2D. The command voltage steps from −40 mV to +0 mV (200 ms in duration) were applied at 0.2 Hz. The amplitude of $I_{Ba}$ remained stable in the control $Ba^{2+}$-bathing solution (Fig. 2Da,d). The depolarizing pulses were stopped and 17β-estradiol (upper traces in D) or diethylstilbestrol (lower traces in D) (30 μM) were applied to the cells. The relative amplitude of $I_{Ba}$ with respect to that before application of each estrogen was plotted in the lower part of Fig. 2D. Under the application of 30 μM 17β-estradiol, the amplitude of $I_{Ba}$ evoked by the first voltage step after a 90 s pause was already suppressed by −60% (Fig. 2Db). During the repetitive pulse stimuli, the amplitude of $I_{Ba}$ remained constant (Fig. 2Dc). 30 μM diethylstilbestrol also suppressed the amplitude of $I_{Ba}$ evoked by the first stimuli almost completely (Fig. 2De), which remained constant during the repetitive pulse stimuli (Fig. 2Df). These results suggest that estrogen inhibition of $I_{Ba}$ was not use-dependent.

Fig. 2E illustrates the quasi-steady-state inactivation ($f_\infty$) curve of $I_{Ba}$ in the absence and presence of 17β-estradiol (10 μM). The double pulse protocol (see under Methods) was used. The test pulse (200 ms in duration) to +10 mV from the holding potential of −80 mV was preceded by 3 s conditioning pulses to various membrane potential. The relationships between the membrane potential and the $f_\infty$ value in the absence and presence of 17β-estradiol were fit by the following Boltzman equation using the least square method:

$$f_\infty(V) = f_{\infty.max}/\{1 + \exp[(V-a)/b]\}$$

where $f_{\infty.max}$ = the maximal value of $f_\infty$ (in the control condition, the value of $f_{\infty.max} = 1$), $V$ = the membrane potential in mV, $a$ = the membrane potential at 1/2 $f_{\infty.max}$ and $b$ = the slope factor. In the absence of 17β-estradiol, $f_{\infty.max} = 1$, $a = -35.3$ mV, $b = 9.8$ mV. In the presence of 17β-estradiol (10 μM), $f_{\infty.max} = 0.6$, $a = -38.0$ mV, $b = 10.2$ mV. Thus, 17β-estradiol reduced the maximal conductance of $I_{Ba}$ (0.64 ± 0.08, $n$ = 4) with only a slight shift of the curve to more negative (−4 ± 2 mV, $n$ = 4), and did not change the

slope factor significantly (9.6 ± 1.0 mV ($n = 4$) in control and 9.8 ± 1.3 mV ($n = 4$) in the presence of 17β-estradiol).

### 3.2. Effects of estrogens on $Ca^{2+}$-permeable non-selective cation currents evoked by vasopressin or endothelin-1 in aortic smooth muscle cells

The above results indicate that estrogens may regulate vascular tone by inhibiting the voltage-dependent L-type $Ca^{2+}$ current. In aortic smooth muscle cells and other vascular smooth muscle cells, receptor-activated $Ca^{2+}$ entry seems to be mediated by $Ca^{2+}$-permeable non-selective cation channels (Benham and Tsien, 1987; Ruegg et al., 1989; Wallnofer et al., 1989; Amedee et al., 1990; Shimpson et al., 1990; Loirand et al., 1991; Wang and Large, 1991). Therefore, to investigate whether estrogens selectively inhibit the voltage-dependent $Ca^{2+}$ currents, the effects of estrogens and the related hormones on receptor-operated $Ca^{2+}$-permeable non-selective cation channels were also investigated. With CsCl in the pipettes, the cell was held at −40 mV. When vasopressin (100 nM) was added to the normal Tyrode solution (Fig. 4A), the inward current with a noise was elicited. The current-voltage relationships of the vasopressin-induced current were examined with the ramp voltage steps. With 140 mM $Na^+$ in the extracellular solution, the current-voltage relationships of the current reversed at −2 ± 3 mV ($n = 12$, Fig. 4A). The reversal potential ($E_{rev}$) of the current was unaffected by decreasing $[Cl^-]_o$ from 140 mM to 0 mM (Fig. 4Bc) or $[Cl^-]_i$ from 140 mM to 0 mM (Fig. 4Bb). These results suggest that vasopressin mainly activates a non-selective cation channel, but not

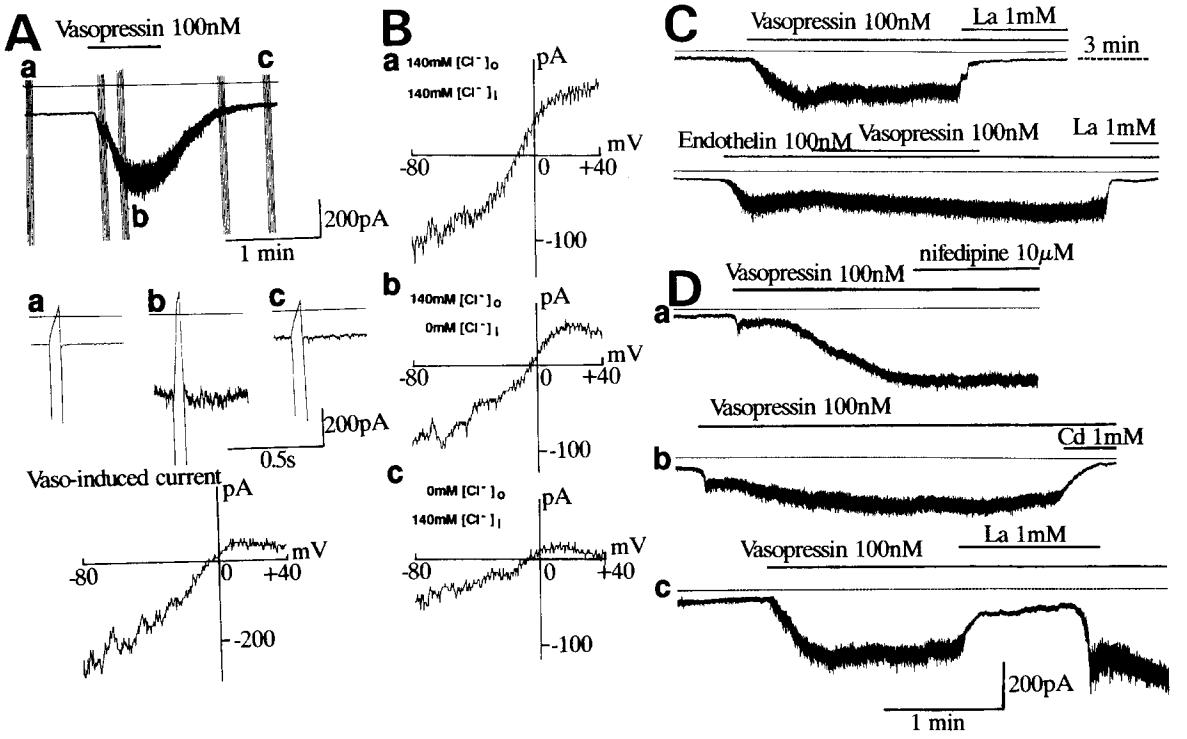


Fig. 4. (A) and (B) Activation of a $Ca^{2+}$-permeable non-selective cation channel by vasopressin in aortic smooth muscle cells. (A) Activation of an inward current by vasopressin. The cell was held at −40 mV. The patch pipette contained CsCl-internal solution. Ramp voltage pulses from −80 mV to +40 mV (100 ms in duration) were applied before, during and after the application of vasopressin (100 nM). The current traces of the ramp pulses (a–c) were recorded at the times indicated in the upper part of the trace. The zero current level denotes dotted lines. The current-voltage relationships of the subtraction current from (b) to (a) are shown in the lower part of panel A. (B) Effect of extracellular or intracellular $Cl^-$ concentration on the reversal potential of the vasopressin-induced current. Extracellular or intracellular $Cl^-$ was replaced by aspartic acid. The typical current-voltage relationships of the vasopressin-induced current are indicated in a–c. (C) Activation of non-selective cation currents by both vasopressin and endothelin-1. The cell was held at −40 mV, and the patch pipette contained CsCl-internal solution. Vasopressin (100 nM) activated the non-selective cation currents. After washout of vasopressin, the increased current returned to a control level. The application of endothelin-1 (100 nM) also activated the current, but the additional application of vasopressin (100 nM) failed to activate it furthermore. (D) Effect of nifedipine (a), $Cd^{2+}$ (b) or $La^{3+}$ (c) on vasopressin-activated non-selective cation currents in aortic smooth muscle cells. The cells were held at −40 mV, and the patch pipettes contained CsCl-internal solution. Note that $Cd^{2+}$ (1 mM) or $La^{3+}$ (1 mM) completely abolished the current. The zero current level is shown by dotted lines.

$Cl^-$ current in this condition, which was compatible with the previous papers (Van Renterghem et al., 1988b; Krautwurst et al., 1994). In addition, even when extracellular $Na^+$ was replaced totally by $Ca^{2+}$, vasopressin still activated the current, which suggests that $Ca^{2+}$ is also the charge carrier of the vasopressin-induced inward current. As shown in Fig. 4C, endothelin-1 also activated non-selective cation currents. After endothelin-1 (100 nM) activated the current, the additional application of vasopressin (100 nM) could not induce the current furthermore, suggesting that both vasopressin and endothelin-1 activate the same population of $Ca^{2+}$-permeable non-selective cation channels in aortic smooth muscle cells. The current was not affected by nifedipine (10 $\mu$M, Fig. 4Da), but blocked by $Cd^{2+}$ (1 mM, Fig. 4Db) or $La^{3+}$ (1 mM, Fig. 4Dc).

Fig. 5 illustrates the effects of estrogens and the related hormones on the $Ca^{2+}$-permeable non-selective cation currents evoked by vasopressin and endothelin-1. The cells were held at −40 mV and command voltage steps to +0 mV were applied at 0.2 Hz. When vasopressin (100 nM, Fig. 5A) or endothelin-1 (100 nM, Fig. 5B) was applied into the bathing solution, the holding current increased in the inward direction by activating non-selective cation currents. During the command pulses, the voltage-dependent L-type $Ca^{2+}$ currents were elicited, but vasopressin (100 nM) or endothelin-1 (100 nM) rather depressed $I_{Ba}$ (Fig. 5Ab,

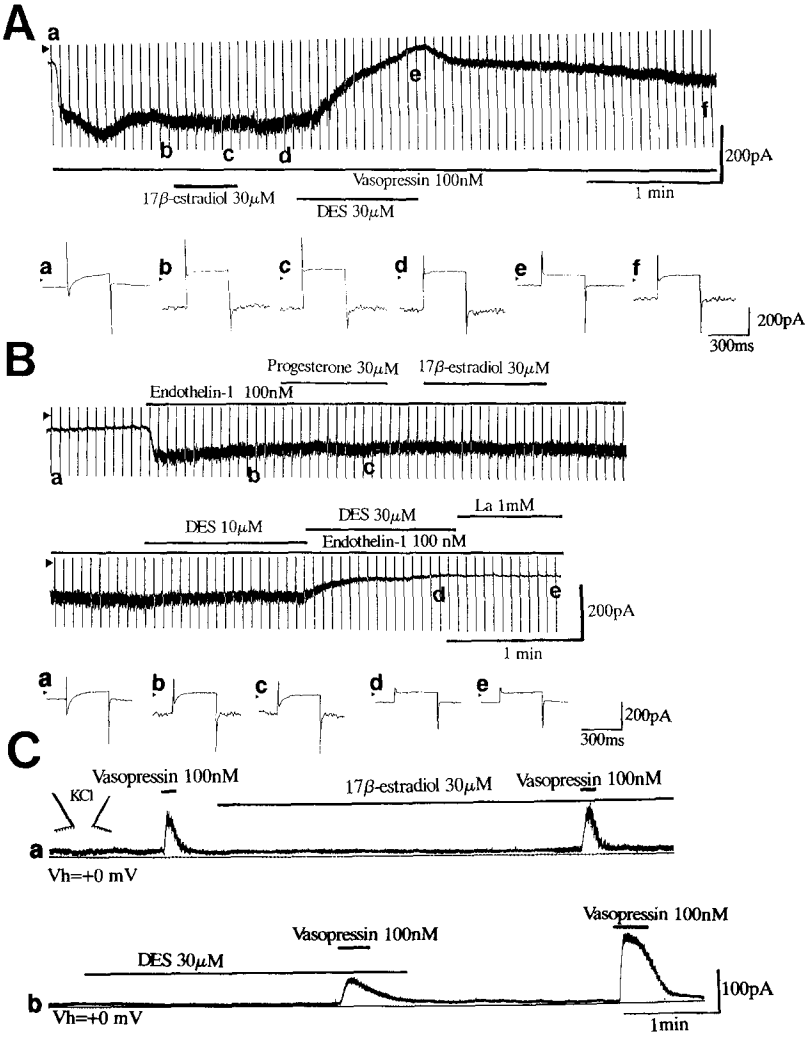


Fig. 5. (A) and (B) Effects of estrogens and the related hormones on $Ca^{2+}$-permeable non-selective cation currents evoked by vasopressin or endothelin-1 in aortic smooth muscle cells. The cells were held at −40 mV, and command voltage pulses to +0 mV were applied at 0.2 Hz. In A and B, vasopressin (100 nM) or endothelin-1 (100 nM) was applied. The continuous current recordings are shown in the upper part of panels A and B. The current traces in the lower part of panels A and B were recorded at the times indicated in the upper part. (C) Effect of 17β-estradiol or diethylstilbestrol on membrane currents and $K^+$ currents elicited by vasopressin. The cell was held at +0 mV, and the holding current was continuously recorded. The patch pipette contained KCl-internal solution. Note that 17β-estradiol (30 μM) failed to affect the holding currents and $K^+$ currents induced by vasopressin (100 nM) at all, but diethylstilbestrol (30 μM) inhibited the $K^+$ currents evoked by vasopressin. The zero current level is shown by dotted lines.

Fig. 5Bb). 17β-Estradiol (30 μM) (Fig. 5Ac) or progesterone (30 μM) (Fig. 5Bc) failed to inhibit the non-selective cation currents evoked by vasopressin or endothelin-1. But, 17β-estradiol inhibited $I_{Ba}$ significantly. On the other hand, diethylstilbestrol (30 μM) markedly inhibited the non-selective cation currents evoked by vasopressin (Fig. 5Ae) or endothelin-1 (Fig. 5Bd) as well as $I_{Ba}$ in a reversible manner (Fig. 5Af). Fig. 3B shows the relationship between the concentration of estrogens and the related hormones and the percent inhibition of the non-selective cation currents evoked by endothelin-1. 17β-Estradiol, ethynylestradiol, testosterone, and progesterone (30 μM) did not inhibit the non-selective cation currents significantly, while diethylstilbestrol (1–30 μM) inhibited it in a concentration-dependent manner. Similarly, diethylstilbestrol inhibited the non-selective cation current evoked by vasopressin, while 17β-estradiol, ethynylestradiol, progesterone or testosterone did not inhibit it significantly.

In dog coronary arterial preparation, it has been reported that diethylstilbestrol hyperpolarized the membrane and reduced input resistance, possibly by an increase in $K^+$ conductance (Harder and Coulson, 1979). Therefore, the effects of 17β-estradiol or diethylstilbestrol on membrane currents were investigated, using the patch pipette filled with KCl-internal solution. As shown in Fig. 5C, the cell was held at +0 mV. Vasopressin (100 nM) or endothelin-1 (100 nM, not shown) increased the holding current in the outward direction, by activating $Ca^{2+}$-dependent $K^+$ currents due to the $Ca^{2+}$ release from the storage sites (Van Renterghem et al., 1988b). 17β-Estradiol (30 μM) failed to affect the holding current significantly, and inhibit the activation of $K^+$ currents by vasopressin (Fig. 5Ca) or endothelin-1. Diethylstilbestrol (30 μM) did not affect the holding current significantly, but markedly reduced the activation of $K^+$ currents by vasopressin (Fig. 5Cb) or endothelin-1. These results suggest that 17β-estradiol did not activate $K^+$ currents and did not affect $Ca^{2+}$ release from the intracellular $Ca^{2+}$ storage sites evoked by vasopressin or endothelin-1.

## 4. Discussion

We have demonstrated that 17β-estradiol and synthetic estrogens, diethylstilbestrol, and ethynylestradiol (0.1–30 μM), inhibited the voltage-dependent L-type $Ca^{2+}$ currents in aortic smooth muscle cells (A7r5 cells). In addition, testosterone and progesterone even at doses of 30 μM failed to inhibit the $Ca^{2+}$ currents, suggesting that estrogens selectively inhibit the voltage-dependent L-type $Ca^{2+}$ current. On the other hand, diethylstilbestrol inhibited $Ca^{2+}$-permeable non-selective cation currents evoked by endothelin-1 or vasopressin in a concentration-dependent manner, while 17β-estradiol, ethinylestradiol, testosterone and progesterone did not inhibit the currents significantly. Thus, it is concluded that 17β-estradiol inhibits the voltage-dependent L-type $Ca^{2+}$ currents, which may contribute to the relaxation effect of estrogens in vascular smooth muscle cells.

### *4.1. Estrogen inhibits the voltage-dependent L-type $Ca^{2+}$ current in vascular smooth muscle cells*

This study provides direct evidence indicating that estrogens inhibit the voltage-dependent L-type $Ca^{2+}$ current ($I_{Ca.L}$) in aortic smooth muscle cells. These results are consistent with the previous findings that 17β-estradiol (0.1–10 μM) inhibited the contraction evoked by high $K^+$ medium in rabbit isolated coronary artery and rat aortic preparation (Jiang et al., 1991), and 4-hydroxylated estrogen reduced the $Ca^{2+}$ uptake in uterine smooth muscle through potential-sensitive channels (Stice et al., 1987a,b). The potency of inhibitory effects on $I_{Ca.L}$ among estrogens were 17β-estradiol < ethinylestradiol < diethylstilbestrol, which was similar to that of estrogens on estrogen receptors. Diethylstilbestrol (30 μM) completely abolished $I_{Ca.L}$. Aortic smooth muscle cells contain cytoplasmic and nuclear estrogen receptors (Horwitz and Horwitz, 1982; Lin and Shain, 1985; Lin et al., 1986; McGill, 1989), but it remains unsettled whether estrogen receptors are involved in the inhibitory effects of estrogen on $I_{Ca.L}$. Further studies are needed to clarify this possibility.

As shown in Fig. 2, 17β-estradiol appeared to reduce the maximal conductance of $I_{Ca.L}$, but it did a very small negative shift in the steady-state inactivation curve. Dihydropyridine $Ca^{2+}$ antagonists like nifedipine, which have affinities for the inactivated state of the channels, cause a distinct negative shift of the steady-state inactivation curve. Therefore, in the presence of nifedipine, an increasing affinity of the drug for channels is characterized by an increasing reduction of current at depolarizing holding potential. On the other hand, such a significant lack of shift suggests that the effect of 17β-estradiol may be voltage-independent, and the extent of current inhibition by 17β-estradiol is not altered by membrane potential. Also, 17β-estradiol and diethylstilbestrol did not exhibit the use-dependence characteristics of D600, verapamil and diltiazem as illustrated in Fig. 2 (Terada et al., 1987; Hering, 1988). Both testosterone and progesterone failed to have a $Ca^{2+}$-antagonistic action like estrogens, suggesting that estrogens specifically inhibit the voltage-dependent L-type $Ca^{2+}$ channels in vascular smooth muscle cells. The concentration of 17β-estradiol to cause inhibition of the $Ca^{2+}$ channels (0.1–30 μM) was much higher than the in vivo concentration of 17β-estradiol

(1–10 nM). However, similar findings have been shown for $Ca^{2+}$ antagonists, for which therapeutic levels in plasma are lower than those required to have effects in vitro (Opie and Singh, 1987). Thus, it is possible that estrogens play a role in regulating vascular tone, by inhibiting the voltage-dependent L-type $Ca^{2+}$ current.

### *4.2. Effects of estrogens on $Ca^{2+}$-permeable non-selective cation currents evoked by endothelin-1 and vasopressin in aortic smooth muscle cells*

In vascular smooth muscle cells, $Ca^{2+}$-permeable non-selective cation channels are known to be involved in $Ca^{2+}$ influx evoked by neurotransmitters (Benham and Tsien, 1987; Ruegg et al., 1989; Wallnofer et al., 1989; Shimpson et al., 1990; Wang and Large, 1991; Amedee et al., 1990). In aortic smooth muscle cells and coronary arterial cells, it has been shown that endothelin activates non-selective cation currents (Van Renterghem et al., 1988a; Chen and Wagoner, 1991). Also, the present study shows that in aortic smooth muscle cells (A7r5 cells), both vasopressin and endothelin activated $Ca^{2+}$-permeable non-selective cation currents. Even when $Ca^{2+}$ was charge-carrier, vasopressin and endothelin still activated non-selective cation currents, suggesting that the channels were $Ca^{2+}$-permeable. Similarly, it has been also reported that in A7r5 cells, vasopressin activated $Ca^{2+}$-permeable non-selective cation currents (Van Renterghem et al., 1988b; Krautwurst et al., 1994). After endothelin-1 (100 nM) activated the non-selective cation currents fully, the additional application of vasopressin (100 nM) could not activate it furthermore, suggesting that both vasopressin and endothelin activate the same population of the channels. Nifedipine failed to inhibit the non-selective cation currents evoked by vasopressin and endothelin, while $La^{3+}$ or $Cd^{2+}$ abolished them. These results are compatible with the previous papers in A7r5 cells and other types of smooth muscle cells (Inoue, 1991; Krautwurst et al., 1994). It has been shown that in rabbit coronary and basilar arterial cells (Jiang et al., 1991; Shay et al., 1993), 17$\beta$-estradiol relaxes the contraction induced by endothelin in an endothelium-independent manner, and antagonized a vasopressin-induced coronary spasm in isolated rabbit heart (Raddino et al., 1986). However, 17$\beta$-estradiol even at doses of 30 $\mu$M failed to suppress the $Ca^{2+}$-permeable non-selective cation currents evoked by both endothelin and vasopressin. These results indicate that the relaxation effects of 17$\beta$-estradiol are not due to the inhibition of receptor-operated $Ca^{2+}$-permeable non-selective cation channels. Also, testosterone and progesterone failed to inhibit them. On the other hand, diethylstilbestrol (1–30 $\mu$M) dose dependently inhibited the $Ca^{2+}$-permeable non-selective cation currents evoked by these substances, suggesting that diethylstilbestrol has additional effects other than $Ca^{2+}$-antagonist effects.

In dog coronary artery preparation, it has been reported that diethylstilbestrol hyperpolarized the membrane and reduced input resistance, possibly by an increase in $K^+$ conductance (Harder and Coulson, 1979). In contrast, 17$\beta$-estradiol or diethylstilbestrol (30 $\mu$M) did not activate $K^+$ currents (Fig. 5C) in aortic smooth muscle cells. In A7r5 cells, it has been reported that vasopressin and endothelin activate charybdotoxin-sensitive $K^+$ currents, possibly by releasing $Ca^{2+}$ from the storage sites (Van Renterghem et al., 1988a,b; Krautwurst et al., 1994). At a holding potential of 0 mV, 17$\beta$-estradiol (30 $\mu$M) did not affect the membrane currents and the $K^+$ currents induced by vasopressin or endothelin (100 nM), which suggests that 17$\beta$-estradiol did not affect $Ca^{2+}$ release from the storage sites evoked by these agonists. From these results, it is unlikely that the relaxation effect of 17$\beta$-estradiol is due to the inhibitory effects of $Ca^{2+}$ release from the storage sites. On the other hand, diethylstilbestrol inhibited the $K^+$ currents induced by vasopressin or endothelin-1. It remains to be determined whether the inhibitory effect of diethylstilbestrol on the $K^+$ currents evoked by vasopressin or endothelin is due to the inhibition of $Ca^{2+}$ release from the storage sites. However, since diethylstilbestrol also inhibited the receptor-operated non-selective cation channels as well as $I_{Ca,L}$, diethylstilbestrol is likely to relax smooth muscle cells by several mechanisms.

The present results indicate that 17$\beta$-estradiol has a $Ca^{2+}$ antagonist effect in vascular smooth muscle cells and fails to affect $Ca^{2+}$-permeable non-selective cation currents evoked by endothelin and vasopressin. The inhibitory effect of 17$\beta$-estradiol on the voltage-dependent L-type $Ca^{2+}$ current seems to contribute to its direct relaxation effect of estrogens on vascular smooth muscle cells.

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