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Inhibition of Large-Conductance Calcium-Activated Potassium Channel by 2-Methoxyestradiol in Cultured Vascular Endothelial (HUV-EC-C) Cells

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Abstract. 2-Methoxyestradiol, an endogenous metabolite of 17β-estradiol, is known to have antitumor and antiangiogenic actions. The effects of 2-methoxyestradiol on ionic currents were investigated in an endothelial cell line (HUV-EC-C) originally derived from human umbilical vein. In the whole-cell patch-clamp configuration, 2-methoxyestradiol (0.3–30 μM) reversibly suppressed the amplitude of K^+ outward currents. The IC_{50} value of the 2-methoxyestradiol-induced decrease in outward current was 3 μM. Evans blue (30 μM) or niflumic acid (30 μM), but not diazoxide (30 μM), reversed the 2-methoxyestradiol-induced decrease in outward current. In the inside-out configuration, application of 2-methoxyestradiol (3 μM) to the bath did not modify the single-channel conductance of large-conductance Ca^{2+}-activated K^+ (BK_{Ca}) channels; however, it did suppress the channel activity. 2-Methoxyestradiol (3 μM) produced a shift in the activation curve of BK_{Ca} channels to more positive potentials. Kinetic studies showed that the 2-methoxyestradiol-induced inhibition of BK_{Ca} channels is primarily mediated by a decrease in the number of long-lived openings. 2-Methoxyestradiol-induced inhibition of the channel activity was potentiated by membrane stretch. In contrast, neither 17β-estradiol (10 μM) nor estriol (10 μM) affected BK_{Ca} channel activity, whereas 2-hydroxyestradiol (10 μM) slightly suppressed it. Under current-clamp condition, 2-methoxyestradiol (10 μM) caused membrane depolarization and Evans blue (30 μM) reversed 2-methoxyestradiol-induced depolarization. The present study provides evidence that 2-methoxyestradiol can suppress the activity of BK_{Ca} channels in endothelial cells. These effects of 2-methoxyestradiol on ionic currents may contribute to its effects on functional activity of endothelial cells.

Key words: 2-Methoxyestradiol — Endothelial cells — BK_{Ca} channels — Membrane potential — Patch-clamping technique

Introduction

2-Methoxyestradiol is known to be an endogenous and active metabolite of 17β-estradiol. Recent studies have demonstrated that this compound may not only inhibit mammary carcinogenesis (Pribluda & Green, 1998; Zhu & Conney, 1998; Tsutsui et al., 2000) but also exert a variety of other actions. These effects include the inhibition of the neovascularization in solid tumor (Fotsis et al., 1994; Klauber et al., 1997; Banerjeei et al., 2000), the induction of apoptotic endothelial cell death (Yue et al., 1997; Tsukamoto et al., 1998), and the induction of microtubule disruption (Cushman et al., 1995; Hamel et al., 1996). In addition, 2-methoxyestradiol was recently found to have anti-proliferative effects on vascular endothelial cells and on angiotumor cell lines (Josefsson & Tarkowski, 1997; Reiser et al., 1998; Lippert et al., 2000). However, none of the studies have thus far demonstrated the underlying mechanism of actions of estrogen metabolites including 2-methoxyestradiol and 2-hydroxyestradiol, on ionic currents in endothelial cells.

Large-conductance Ca^{2+}-activated K^+ (BK_{Ca}) channels differ from most of other K^+ channels in that their activation is under dual control, i.e., switched on by either depolarization or by an increase in intracellular Ca^{2+}. BK_{Ca} channels present in smooth myocytes comprise two dissimilar subunits, i.e., a pore-forming α-subunit and a regulatory β-subunit that forms a modulatory

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site sensitive to intracellular Ca^{2+} (Vogalis et al., 1996; Tanaka et al., 1997). Recent studies showed that there is a lack of β-subunits expressed in vascular endothelial cells (Papassotiriou et al., 2000). More importantly, the activity of BK_{Ca} channels expressed in endothelial cells plays a role in modulating some of endothelial function. For example, the activity of Ca^{2+}-activated K^+ channels present in vascular endothelial cells can control K^+ efflux and affect the K^+ concentration in myoendothelial gap junctions (Edwards et al., 1998; Bény, 1999). Increased BK_{Ca} channel activity has been noted to facilitate the release of nitric oxide from endothelial cells that have been stimulated by application of shear stress on cell membrane (Hoyer et al., 1994; Busse & Fleming, 1998). A previous report also showed an increase in the expression pattern and activity of endothelial Ca^{2+}-activated K^+ channels existing in endothelium from patients with colonic adenocarcinoma, suggesting that a disease-related alteration in the expression pattern of Ca^{2+}-activated K^+ channels in cancer patients could be associated with endothelial function during angiogenesis (Köhler et al., 2000). In addition, it has been reported that BK_{Ca} channels expressed in endothelial cells induced an elevated Ca^{2+} entry (Kamouchi, Droogmans & Nilius, 1999).

Previous observations at our laboratory have shown that BK_{Ca} channels were expressed in human vascular endothelial cells (Wu et al., 1999; Wu, Li & Lo, 2000). The K^+ selectivity, single-channel conductance, voltage-dependence, and pharmacological properties of these channels are similar to those of BK_{Ca} channels, as reported previously in other types of endothelial cells (Nilius, Viana & Droogmans, 1997). Therefore, the purpose of this study was (1) to determine whether 2-methoxyestradiol affects Ca^{2+}-activated K^+ currents in cultured endothelial cells of human umbilical veins; (2) to examine the effect of 2-methoxyestradiol on BK_{Ca} channel activity, (3) to address the issue whether 2-methoxyestradiol affects the kinetic properties of BK_{Ca} channels, and (4) to study whether membrane stretch modifies the 2-methoxyestradiol-induced effects on BK_{Ca} channel activity. The results indicate that, unlike 17β-estradiol, 2-methoxyestradiol can directly suppress BK_{Ca} channels in vascular endothelial cells.

Materials and Methods

Cell Preparation

The clonal strain HUV-EC-C cell line, an endothelial cell line originally derived from the vein of a normal human umbilical cord, was obtained from Culture Collection and Research Center (CCRC-60016; Hsinchu, Taiwan) (Wu et al., 2000b). Endothelial cells were grown in monolayer culture in 50-ml plastic culture flasks at 37°C in a humidified environment containing 5% CO_2/95% air. Cells were maintained in 5 ml of Ham's F-12K nutrient medium supplemented with 10% fetal bovine serum (v/v), 100 μg/ml heparin, and 30 to 50 μg/ml endothelial cell growth supplement. Cells were passaged once a week, and a new stock line was generated from frozen cells (frozen in 10% dimethyl sulfoxide in cultured medium) every 3 months. The experiments were performed after 5 or 6 days of subcultivation (60 to 80% confluence).

Electrophysiological Measurements

Immediately before each experiment, the cells were dissociated, and an aliquot of cell suspension was placed into a recording chamber affixed to the stage of an inverted phase-contrast microscope (Diaphot-200; Nikon, Tokyo, Japan). Cells were bathed at room temperature (20–25°C) in normal Tyrode's solution containing 1.8 mM $CaCl_2$. The whole-cell or inside-out recordings were performed by the standard "giga-seal" patch-clamp technique, using a patch-clamp amplifier (RK-400; Bio-Logic, Claix, France) (Hamill et al., 1981; Wu et al., 2000b). Patch pipettes (3 to 5 MΩ in bathing solution) were made from borosilicated glass capillary tubes (Kimble Products, Vineland, NJ) using a two-step pipette puller (PB-7; Narishige Scientific, Tokyo, Japan), and the tips were heat-polished with a microforge (MF-83; Narishige).

The signals, consisting of voltage and current tracings, were displayed on a digital storage oscilloscope (model 1602; Gould, Valley View, OH) and on-line recorded in a digital audiotape recorder (model 1204; Bio-Logic). After the experiments, the data were fed back and stored in a Pentium III-grade computer (Lemel, Taipei, Taiwan) at 10 kHz through an analog/digital interface (Digidata 1320A; Axon Instruments, Foster City, CA) using the Clampex subroutine of the pCLAMP 8.02 software (Axon Instruments). Current traces were filtered using a cut-off frequency of 1 kHz for plotting.

The stretch of a membrane patch was elicited by applying negative pressure to the back end of the patch pipette through the suction port of the pipette holder. The pressure level was established by monitoring the height of weight in a U-shaped tube. One end of the tube was at room air pressure, and the other end that was connected to the pipette was at the desired pressure. Change in pressure was produced with a manually operated valve system that switched the pipette holder between the U tube and room air.

Single-Channel Analysis

Single-channel currents were analyzed using Fetchan and Pstat subroutines in the pCLAMP 8.02 software (Axon Instruments). The single-channel conductance was calculated by a linear regression using mean values of the current amplitudes measured at different voltages. To determine the effect of 2-methoxyestradiol on the activation curve of BK_{Ca} channels, the ramp pulses from +20 to +120 mV with a duration of 1 sec were digitally applied with the aid of a programmable stimulator (SMP-311; Bio-Logic). This made it more efficient to measure single-channel conductance or channel activation (Carl & Sanders, 1990; Wu, Li & Chiang, 2000a). The activation curves were calculated by averaging current amplitudes in response to 20 voltage ramps and dividing each point of the mean current by the unitary amplitude of each potential after each leakage component was corrected. To count the number N of active channels in the patch, a high K^+ solution with 100 μM Ca^{2+} was perfused at the end of the experiments. The number was then used to normalize the opening probability obtained at each potential. The relationships between the membrane potentials and opening probability of BK_{Ca} channels with or without the application of 2-methoxyestradiol (3 μM) were plotted and fitted with the Boltz-

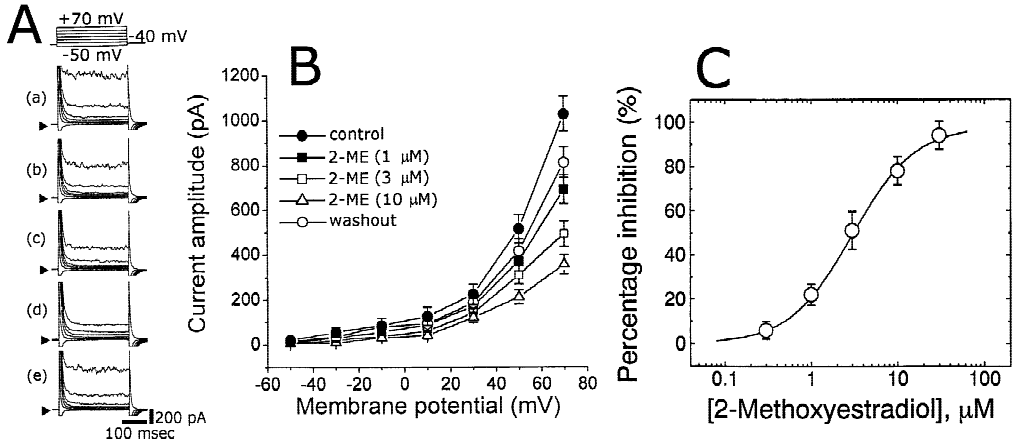

Fig. 1. Inhibitory effect of 2-methoxyestradiol on K^+ outward current (I_K) in cultured endothelial cells of human umbilical veins (HUV-EC-C). (*A*) Superimposed current traces in control, during the exposure to 2-methoxyestradiol (1, 3 and 10 μM) and after washout of 2-methoxyestradiol. The cells, bathed in normal Tyrode's solution containing 1.8 mM $CaCl_2$ were held at −40 mV and voltage pulses from −50 to +70 mV in 20-mV increments were delivered. The uppermost part indicates the voltage protocol. Arrow heads denote the zero-current level. (*a*): control; (*b*) 1 μM 2-methoxyestradiol; (*c*) 3 μM 2-methoxyestradiol; (*d*): 10 μM 2-methoxyestradiol; (*e*): washout. (*B*) Averaged *I-V* relations of I_K measured at the end of voltage pulses in control (●), during exposure to 1 μM (■), 3 μM (□), and 10 μM (△) 2-methoxyestradiol (2-ME), and washout of 2-methoxyestradiol (○) (mean ± SEM; n = 6–10 for each point). (*C*) Concentration-dependent inhibition of I_K by 2-methoxyestradiol. The relation between the percent inhibition of K^+ outward current and the concentration of 2-methoxyestradiol is illustrated. Each cell was depolarized from −40 to +50 mV with a duration of 300 msec. Various concentrations of 2-methoxyestradiol (0.3–30 μM) were applied. The amplitude of outward current during the exposure of cells to 2-methoxyestradiol was compared with the control value (i.e., in the absence of 2-methoxyestradiol). Each point represents mean ± SEM (n = 5–9). The percent inhibition of 2-methoxyestradiol on I_K was plotted. The smooth line represents the best fit to the Hill equation as described in Materials and Methods. The values of IC_{50} and maximally inhibited percentage of K^+ outward current in the presence of 2-methoxyestradiol were 3 μM and 98%, respectively. The Hill coefficient was 1.2.

mann equation using a nonlinear regression analysis: relative $N \cdot P_o = n/\{1 + \exp[-(V - V_{1/2})/k]\}$, where m = the maximal $N \cdot P_o$ level, V = the membrane potential in mV, $V_{1/2}$ = the voltage at which there is half-maximal activation, k = the slope factor of the activation curve.

Open lifetime distribution of BK_{Ca} channels measured before and after the addition of 2-methoxyestradiol was fitted with a logarithmically scaled bin width using the method of McManus, Blatz & Magleby (1987). When the square root of the number of events in a bin is plotted against the open lifetime, each component of the open lifetime distribution appears as a clear peak and the respective time constant falls in the vicinity of this peak.

To calculate the percentage inhibition of 2-methoxyestradiol on K^+ outward current (I_K), each cell was depolarized from −40 to +50 mV, and current amplitudes obtained in the presence of 2-methoxyestradiol were compared with the control value. The concentration of 2-methoxyestradiol required to inhibit 50% of current amplitude was determined using a Hill function, $y = E_{max}/\{1 + (IC_{50}{}^n/[C]^n)\}$, where [*C*] is the concentration of 2-methoxyestradiol; IC_{50} and n are the half-maximal concentration of 2-methoxyestradiol required to inhibit I_K and Hill coefficient, respectively; and E_{max} is 2-methoxyestradiol-induced maximal inhibition of current amplitude.

To determine the effect of 2-methoxyestradiol on the stretch-stimulated BK_{Ca} channels, the relationships between the channel activity and the level of negative pressure obtained in the absence and presence of 2-methoxyestradiol were fitted with the Boltzmann function of the form: relative $N \cdot P_o = m/\{1 + \exp[P_{1/2} - P)]/k\}$, where m is the maximum relative $N \cdot P_o$, k is the slope factor of the stretch-induced activation, and $P_{1/2}$ is the pressure at which there is half-maximal activation.

All values are reported as means ± SEM. The paired or unpaired Student's *t*-test and ANOVA with a least-significance difference method for multiple comparison were used for the statistical evaluation of differences among means. Differences between the values were considered statistically significant when P was <0.05.

Drugs and Solutions

2-Methoxyestradiol, 2-hydroxyestradiol, 17β-estradiol, estriol, Evans blue, and diazoxide were purchased from Sigma (St. Louis, MO). Niflumic acid, 8-bromo-cyclic AMP, A23187, NS1619 (1,3-dihydro-1-[2-hydroxy-5-(trifluoromethyl)phenyl-5-(trifluoromethyl)-2H-benzimidazol-2-one] and iberiotoxin were obtained from Research Biochemicals (Natick, MA). Paxilline was purchased from Biomol (Plymouth Meeting, PA). Tissue culture media, L-glutamine, penicillin-streptomycin, fungizone, and trypsin were obtained from Life Technologies (Grand Island, NY). Endothelial cell growth supplement was purchased from Upstate Biotechnology (Lake Placid, NY). All other chemicals were of analytical grade. The twice-distilled water that had been de-ionized through a Millipore-Q system was used in all experiments. The composition of normal Tyrode's solution was (in mM): NaCl 136.5, KCl 5.4, $CaCl_2$ 1.8, $MgCl_2$ 0.53, glucose 5.5, and HEPES-NaOH buffer 5.5 (pH 7.4). To record K^+ currents or membrane potential, the pipettes were filled with (in mM): K-aspartate 130, KCl 20, $MgCl_2$ 1, EGTA 0.1, Na_2ATP 3, Na_2GTP 0.1, and HEPES-KOH 5 (pH 7.2). For inside-out patch-clamp recording, the high K^+ bathing solution contained (mM): KCl 145, $MgCl_2$ 0.53 and HEPES-KOH buffer 5 (pH 7.4), and the pipette solution contained (mM): KCl 145, $MgCl_2$ 2, and HEPES-KOH buffer 5 (pH 7.2). The value of free Ca^{2+} concentration was calculated assuming the dissociation constant for EGTA and Ca^{2+} (at pH 7.2) at 0.1 μM.

Results

Inhibitory Effect of 2-Methoxyestradiol on K^+ Outward Current (I_K) in Cultured Endothelial Cells of Umbilical Veins (HUV-EC-C)

The whole-cell configuration of the patch-clamp technique was performed to investigate the effect of 2-methoxyestradiol on ionic currents in these cells. In these experiments, cells were bathed in normal Tyrode's solution containing 1.8 mM $CaCl_2$ and pipette solution contained a low concentration (0.1 mM) of EGTA and 3 mM ATP. As shown in Fig. 1*A*, when the cell was held at the level of −40 mV, the voltage pulses from −50 to +70 mV with 20-mV increments elicited a family of outward currents. The amplitudes of these outward currents were increased with greater depolarization. Within 1 min of exposing the cells to 2-methoxyestradiol, the amplitude of outward currents was reduced throughout the entire range of voltage-clamp steps (Fig. 1). For example, with a voltage step from −40 to +50 mV, 1, 3 and 10 μM 2-methoxyestradiol significantly decreased the current amplitude to 370 ± 45, 308 ± 40 and 210 ± 32 pA, respectively, from a control value of 513 ± 65 pA (n = 8). This inhibitory effect was readily reversed on the washout of 2-methoxyestradiol. The average current-voltage (*I-V*) relations of these currents in the absence and presence of 2-methoxyestradiol are illustrated in Fig. 1*B*. Fig. 1*C* shows the relationships between the concentration of 2-methoxyestradiol and the percent inhibition of I_K. The half-maximal concentration required for the inhibitory effect of 2-methoxyestradiol on I_K was 3 μM, and 30 μM 2-methoxyestradiol almost completely suppressed the current amplitude. These results indicated that 2-methoxyestradiol had a depressant effect on I_K in a concentration-dependent manner in these cells.

Comparison of the Effect of 2-Methoxyestradiol on the Amplitude of I_K in the Absence and Presence of Evans Blue, Niflumic Acid or Diazoxide

We examined whether the inhibitory effect of 2-methoxyestradiol on I_K can be altered by the presence of Evans blue, niflumic acid, or diazoxide. Evans blue and niflumic acid were reported to enhance BK_{Ca} channel activity, whereas diazoxide was known to be an opener of ATP-sensitive K^+ channels (Wu et al., 1999; Li et al., 2000). As shown in Fig. 2, when the cells were depolarized from −40 to +50 mV, 2-methoxyestradiol (10 μM) or iberitoxin (200 nM) significantly suppressed the amplitude of I_K. In addition, Evans blue (10 μM) or niflumic acid (10 μM) significantly reversed 2-methoxyestradiol-induced inhibition of I_K. When cells were depolarized from −40 to +50 mV, a significant

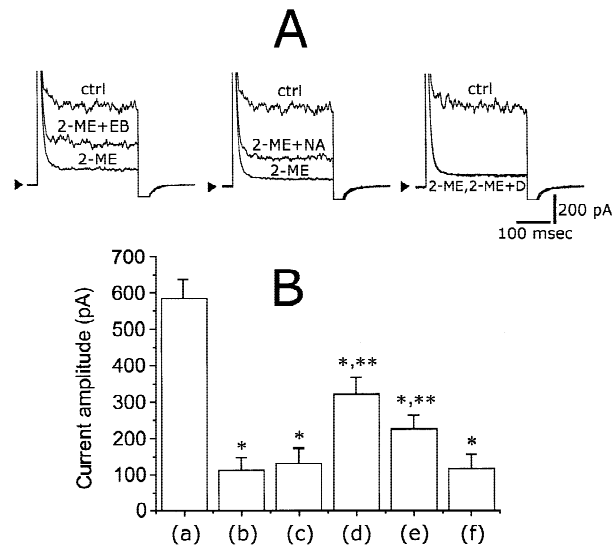

Fig. 2. Effect of 2-methoxyestradiol on the amplitude of I_K in the absence and presence of Evans blue, niflumic acid and diazoxide. Each cell was depolarized from −40 to +50 mV (300 msec in duration). (*A*) Original current traces showing the effect of 2-methoxyestradiol (2-ME; 10 μM) with and without addition of Evans blue (EB; 10 μM), niflumic acid (NA; 10 μM), or diazoxide (D: 10 μM). (*B*) Bar graph showing the effect of 2-methoxyestradiol on the amplitude of I_K in the absence and presence of Evans blue, niflumic acid, or diazoxide. (*a*): control; (*b*): 2-methoxyestradiol (10 μM); (*c*): iberiotoxin (200 nM); (*d*): 2-methoxyestradiol (10 μM) plus Evans blue (10 μM); (*e*): 2-methoxyestradiol (10 μM) plus niflumic acid (10 μM); (*f*): 2-methoxyestradiol (10 μM) plus diazoxide (30 μM). Each point represents mean ± SEM (n = 5–8). *Significant difference from control group. **Significant difference ($P < 0.05$) from 2-methoxyestradiol alone group.

difference in the current amplitude between 2-methoxyestradiol alone and 2-methoxyestradiol plus Evans blue was observed (113 ± 23 pA, n = 7, *vs.* 368 ± 34 pA, n = 7). However, there was no significant difference in the amplitude of I_K between 2-methoxyestradiol (10 μM) alone and 2-methoxyestradiol plus diazoxide (20 μM) (112 ± 36 pA, n = 6, *vs.* 114 ± 32 pA, n = 6). In addition, NS1619 (30 μM), a blocker of BK_{Ca} channels, reversed the 2-methoxyestradiol-induced increase in I_K. These results suggest that these currents suppressed by 2-methoxyestradiol are Ca^{2+}-activated K^+ currents that can be stimulated by Evans blue or niflumic acid (Wu et al., 2000).

Effect of 2-Methoxyestradiol on BK_{Ca} Channels in Inside-out Patches

To determine the inhibitory effect of 2-methoxyestradiol on I_K, single-channel experiments with an inside-out configuration were also performed. Interestingly, in this configuration, bath application of 2-methoxyestradiol (3 μM) was found to suppress BK_{Ca} channel activity. As shown in Fig. 3, when the patch pipette contained 1 μM

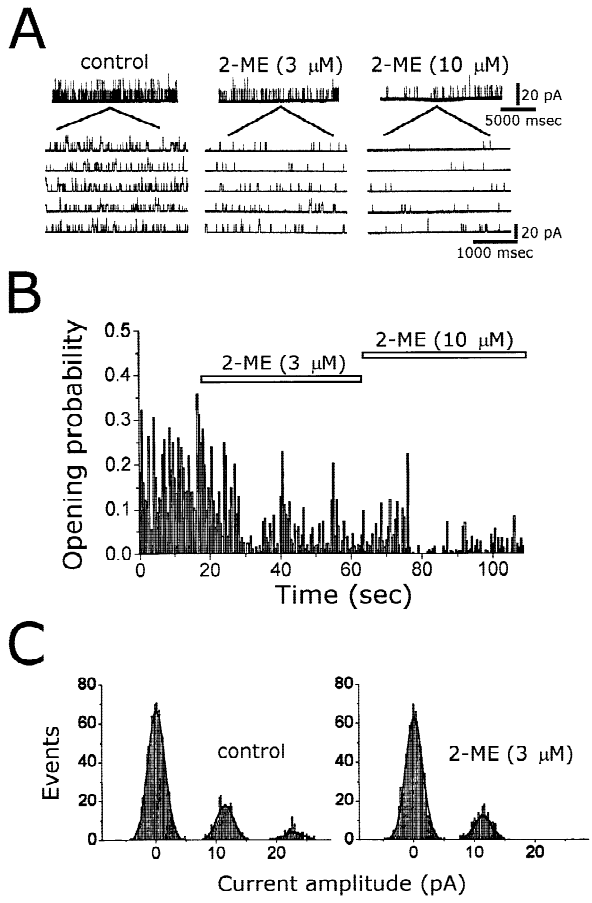

Fig. 3. Effect of 2-methoxyestradiol on BK_{Ca} channel activity recorded from an inside-out patch of human vascular endothelial (HUV-EC-C) cells. The experiments were conducted with a symmetrical K^+ concentration (145 mM). The bath solution contained 1 μM Ca^{2+} and the membrane potential was held at +80 mV. (*A*) Original current trace showing the change in the activity of BK_{Ca} channels after addition of 2-methoxyestradiol (2-ME). Channel openings are shown as an upward deflection. The lower parts show the current traces obtained in expanded time scale. Note that there are a few, brief openings in the presence of 2-methoxyestradiol. (*B*) Opening probability for BK_{Ca} channel activity shown in panel A plotted against time of recording. Bin width is 0.5 sec. Horizontal bars shown in the panel indicate the application of 2-methoxyestradiol (3 and 10 μM). (*C*) Amplitude histograms measured in the absence or presence of 2-methoxyestradiol (3 μM). All points shown in the amplitude histograms were fitted by one or more Gaussian distribution. The closed state corresponds to the peak at 0 pA.

Ca^{2+} and the potential was held at +80 mV, bath application of 2-methoxyestradiol caused a drastic decrease in channel activity. The opening probability of BK_{Ca} channels was significantly decreased from a control value of 0.226 ± 0.012 (n = 8) to 0.114 ± 0.007 (n = 8) and 0.022 ± 0.004 (n = 7) after the addition of 3 and 10 μM 2-methoxyestradiol, respectively. The channel activity was significantly increased to 0.203 ± 0.007 (n = 5) after the washout of 2-methoxyestradiol. However, there was no significant difference in the amplitude of unitary current between the absence and presence of 2-methoxyestradiol (Fig. 3*C*). These results indicate that 2-methoxyestradiol reversibly suppresses BK_{Ca} channel activity in these cells. It is also possible that the 2-methoxyestradiol-mediated decrease in the activity of BK_{Ca} channels does not require the presence of cytoplasmic factors and is mainly due to the result of direct binding to the channel.

In order to testify whether the effects of 2-methoxyestradiol shown at very positive potentials have, indeed, some impact at the level of reasonable membrane potential, additional experiments, in which the holding potential was set at −20 mV, were conducted with an inside-out configuration. Patch pipettes were filled with 5.5 mM KCl, and bath medium contained 145 mM KCl and 1 μM Ca^{2+}. 2-Methoxyestradiol (10 μM) applied intracellularly was also found to decrease channel activity from 0.212 ± 0.008 to 0.032 ± 0.004 (n = 5).

Effect of 2-Methoxyestradiol on the Activation Curve of BK_{CA} Channels

Figure 4*A* shows the activation curve of BK_{Ca} channels in the absence and presence of 2-methoxyestradiol (3 μM). In these experiments, the activation curves of BK_{Ca} channels were obtained with the aid of the voltage ramp protocols. The ramp pulses were delivered from +20 to +120 mV with a duration of 1 sec. The plots of opening probability of BK_{Ca} channels as a function of membrane potential were constructed and fitted with the Boltzmann equation as described under Materials and Methods. In control m = 0.99 ± 0.04, $V_{1/2}$ = 76.1 ± 1.8 mV, and k = 11.2 ± 0.7 mV (n = 7), whereas in the presence of 2-methoxyestradiol (3 μM), m = 0.45 ± 0.03, $V_{1/2}$ = 86.8 ± 2.1 mV, and k = 11.4 ± 0.7 mV (n = 6). Thus, the presence of 2-methoxyestradiol (3 μM) not only produced a reduction in the maximal opening probability of BK_{Ca} channels, but also shifted the activation curve of these channels to a more positive potential by approximately 10 mV. In contrast, no significant effect of 2-methoxyestradiol on the slope (i.e., k) of the activation curve was found. These results indicate that 2-methoxyestradiol suppresses the activity of BK_{Ca} channels in a voltage-dependent fashion in these cells.

Lack of Effect of 2-Methoxyestradiol on Single-Channel Conductance of BK_{CA} Channels

We examined whether 2-methoxyestradiol affects the single-channel conductance of BK_{Ca} channels. To construct the plot of current amplitude as a function of membrane potential, the voltage ramp pulses from 0 to +80 mV with a duration of 1 sec were applied at a rate of 0.1 Hz. Fig. 4*B* illustrates the *I-V* relationships of BK_{Ca}

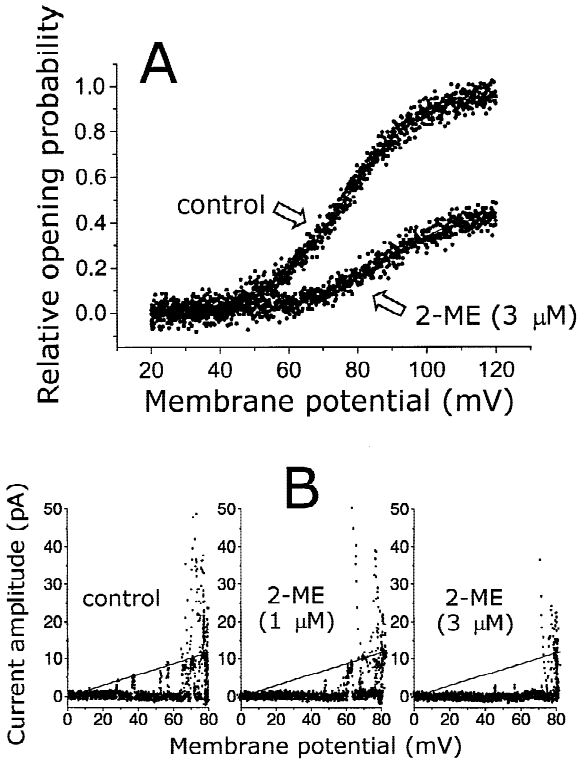

Fig. 4. Voltage-dependency of 2-methoxyestradiol on BK_{Ca} channels in human vascular endothelial cells. These experiments were conducted with a symmetrical K^+ concentration (145 mM). Under the inside-out configuration, the holding potential was +60 mV and bath medium contained 1 μM Ca^{2+}. (*A*) Effect of 2-methoxyestradiol (2-ME) on the activation curve of BK_{Ca} channels. The activation curves were obtained by use of the ramp pulses that were from +20 to +120 mV with a duration of 1 sec. The smooth lines showed Boltzmann fits of the data yielding a $V_{1/2}$ of 76 mV for control and 87 mV when the detached patch was intracellularly exposed to 3 μM 2-methoxyestradiol (2-ME). (*B*) Lack of effect of 2-methoxyestradiol on the single-channel conductance of BK_{Ca} channels. Under a symmetrical K^+ condition, the holding potential was +60 mV in an inside-out configuration and bath solutions contained 1 μM Ca^{2+}. The voltage ramp pulses from 0 to +80 mV with a duration of 1 sec were used to measure single-channel conductance. The straight lines with a reversal potential of 0 mV represent the *I-V* relationships of BK_{Ca} channels in the absence or presence of 2-methoxyestradiol (2-ME; 1 and 3 μM).

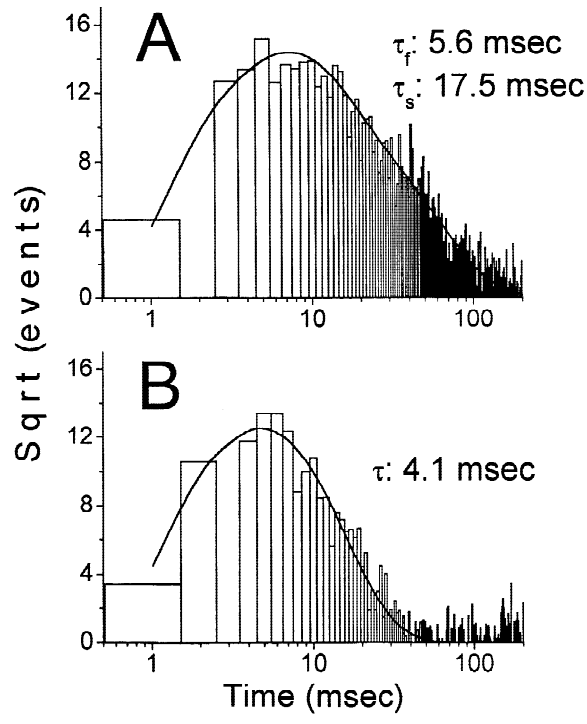

Fig. 5. Open-time histograms of BK_{Ca} channels in the absence and presence of 2-methoxyestradiol. The mean open-time histograms of BK_{Ca} channels were obtained before (*A*) and after (*B*) the addition of 2-methoxyestradiol (3 μM). Under a symmetrical K^+ (145 mM) condition, the holding potential was +80 mV in an inside-out configuration and the bath solution contained 1 μM Ca^{2+}. Data were obtained from a measurement of 676 channel openings with a total recording time of 1 min in the control (*A*), whereas data obtained during the exposure to 3 μM 2-methoxyestradiol (*B*) were measured from 521 channel openings with a total recording time of 2 min. Open-time histograms were fitted by a one- or two-exponential function. The values of time constants are shown in each panel. Note that the abscissa and ordinate show the logarithm of apparent open time (msec) and the square root of the number of events ($n^{1/2}$), respectively.

channels in the absence and presence of 2-methoxyestradiol (1 and 3 μM). The single-channel conductance of BK_{Ca} channels calculated from the linear *I-V* relationship in control (i.e., in the absence of 2-methoxyestradiol) was 145 ± 7 pS ($n = 11$), with a reversal potential of 0 ± 2 mV ($n = 11$). The value of single-channel conductance for these channels was found to be similar to that reported previously (Wu et al., 1999, 2000; Li, Chen & Wu, 2000); however, it did not significantly differ from that (144 ± 7 pS; $n = 10$) measured in the presence of 2-methoxyestradiol (3 μM). Thus, it is clear that the single-channel currents inhibited by 2-methoxyestradiol displayed no decrease in single-channel conductance.

Effect of 2-Methoxyestradiol on Kinetic Behavior of BK_{Ca} Channels

Because it was observed that 2-methoxyestradiol tends to reduce the open-time duration of BK_{Ca} channels, the effect of 2-methoxyestradiol in kinetic properties of BK_{Ca} channels in these cells was further characterized. As shown in Fig. 5*A*, in the absence of 2-methoxyestradiol, the open-time histogram of these channels at +80 mV could be fitted to a two-exponential curve. The time constants for the fast and slow components of the open-time histogram were 5.6 ± 0.7 and 17.5 ± 1.2 msec ($n = 5$). However, the presence of 2-methoxyestradiol (3 μM) was found to decrease the lifetime of the open state. A single-exponential function was sufficient to fit the open-time histogram obtained in the presence of 2-methoxyestradiol (3 μM) (Fig. 5*B*). When the inside-out patches

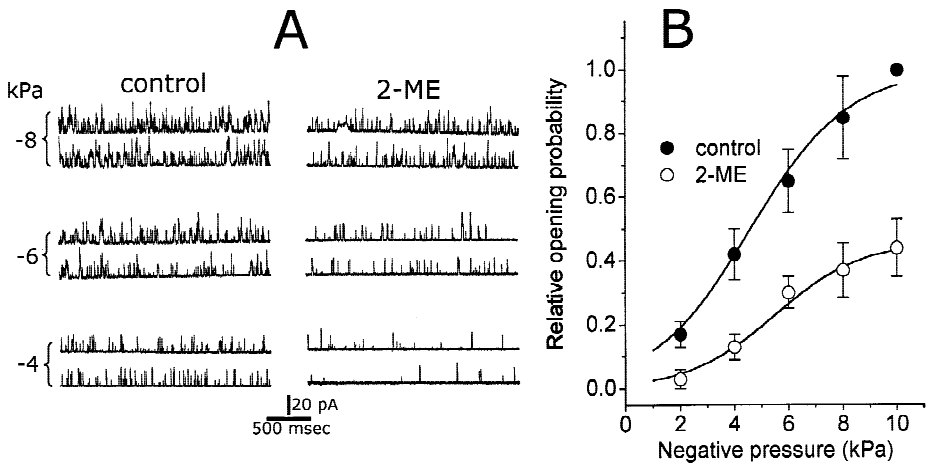

Fig. 6. Effect of membrane stretch on 2-methoxyestradiol-induced inhibition of BK_{Ca} channel activity. (*A*) BK_{Ca} channel activity before (*left*) and after (*right*) bath application of 3 μM 2-methoxyestradiol (2-ME). An inside-out configuration was performed with a holding potential of +60 mV, and bath solution contained 0.1 μM Ca^{2+}. Single-channel currents were recorded when the detached membranes were at the different negative pressures denoted at the left side of each current trace. Upward deflections are the opening events of the channel. (*B*) Relationships between open probability of BK_{Ca} channels and membrane stretch in the absence (●) and presence (○) of 3 μM 2-methoxyestradiol (2-ME). The opening probability of BK_{Ca} channels in the presence of −10 kPa was considered to be 1.0. The smooth lines represent the best fit to the Boltzmann equation as described in Materials and Methods.

were exposed to 2-methoxyestradiol (3 μM) extracellularly, the open-time histogram was fitted by a single-exponential curve with a mean open time of 4.1 ± 0.5 msec ($n = 5$). Thus, the presence of 2-methoxyestradiol can reduce endothelial BK_{Ca} channel activity by decreasing the number of channel openings and the open-time duration.

Effect of 2-Methoxyestradiol on the Stretch-Stimulated Channel Activity

Because the activity of endothelial BK_{Ca} channels in response to membrane stretch was detectable (Hoyer et al., 1994), we also investigated the effect of 2-methoxyestradiol on stretch-induced activation of BK_{Ca} channels in these cells. The plots of relative open-probability of BK_{Ca} channels as a function of the pressure applied to the pipette were constructed and fitted with a Boltzmann function as described in Materials and Methods (Fig. 6). In control, $m = 0.97 \pm 0.03$, $P_{1/2} = 4.6 \pm 0.2$ kPa, $k = 1.8 \pm 0.1$ ($n = 5$), whereas in the presence of 2-methoxyestradiol (3 μM), $m = 0.46 \pm 0.02$, $P_{1/2} = 5.5 \pm 0.2$, $k = 1.6 \pm 0.1$ ($n = 4$). Thus, in the presence of 2-methoxyestradiol (3 μM), not only a decrease in the maximal opening probability of BK_{Ca} channels but also a shift of the stretch-induced activation curve to more negative values was found. However, there was no significant effect on the slope (i.e., k) of the stretch-induced activation curve in the presence of 2-methoxyestradiol. These results indicate that 2-methoxyestradiol suppresses the activity of BK_{Ca} channels in a pressure-dependent fashion in these endothelial cells.

Comparison between the Effects of 2-Methoxyestradiol and Those of 17β-Estradiol, Estriol, 2-Hydroxyestradiol, 8-Bromo Cyclic AMP and Paxilline

Effects of 17β-estradiol, estriol, 2-hydroxyestradiol and paxilline on the activity of BK_{Ca} channels in HUV-EC-C endothelial cells were also examined and compared. As shown in Fig. 7, neither 17β-estradiol (10 μM) nor estriol (10 μM) applied intracellularly had any effect on the channel activity, whereas 2-hydroxyestradiol (10 μM) produced a slight inhibition of BK_{Ca} channels by about 22%. 2-Hydroxyestradiol, one of the metabolites of 17β-estradiol, can be degraded to 2-methoxyestradiol via *O*-methylation (Zhu & Conney, 1998). In addition ,8-bromo cyclic AMP (100 μM), a membrane-permeable cyclic AMP analogue that elevates levels of cyclic AMP, did not affect the channel activity significantly. However, paxilline (1 μM), applied intracellularly, was found to be potent in suppressing the channel activity. Paxilline was reported to be a blocker of BK_{Ca} channels (Sanchez & McManus, 1996; Wu et al., 2000). These results indicate that, unlike 2-methoxyestradiol, neither 17β-estradiol nor estriol affected BK_{Ca} channel activity in these cells. BK_{Ca} channels thus appear to be the main component responsible for a decrease in I_K observed in the presence of 2-methoxyestradiol.

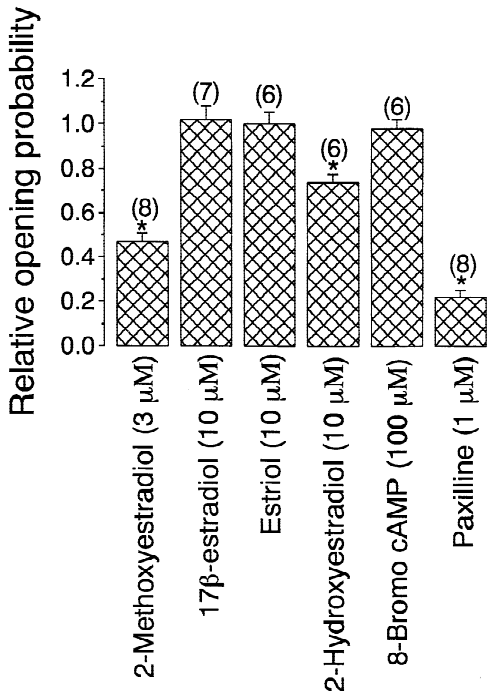

Fig. 7. Comparison between the effect of 2-methoxyestradiol and that of 17β-estradiol, estriol, 2-hydroxyestradiol, 8-bromo cyclic AMP, and paxilline on BK_{Ca} channel activity recorded from the inside-out patch of cultured endothelial cells of human umbilical veins. The potential held at each detached patch was +80 mV, and the bath medium contained 1 μM Ca^{2+}. The channel activity in the absence of each agent was considered to be 1.0, and the relative $N \cdot P_o$ obtained in the presence of each agent was compared and plotted. The parentheses shown on each bar denote the number of cells examined. Mean ± SEM. *Significantly different from controls.

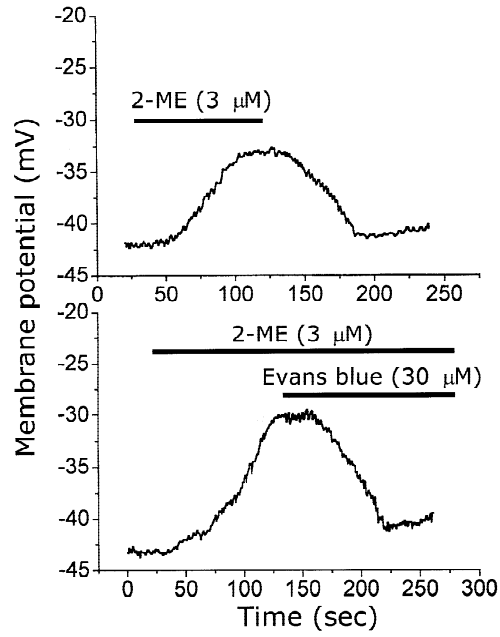

Fig. 8. Effect of 2-methoxyestradiol on membrane potential in human umbilical vascular endothelial cells. The cells were bathed in normal Tyrode's solution containing 1.8 mM $CaCl_2$. The change in resting membrane potential was measured under current-clamp condition. Horizontal bars shown in each panel denote the application of 2-methoxyestradiol (2-ME; 3 μM) or Evans blue (30 μM). Note that the presence of Evans blue can reverse the membrane depolarization caused by 2-methoxyestradiol.

Effect of 2-Methoxyestradiol on Resting Membrane Potential in Umbilical Vascular Endothelial Cells

The effect of 2-methoxyestradiol on membrane potential in the absence and presence of Evans blue was also studied. This type of endothelial cells had a resting membrane potential of −41 ± 7 mV (n = 23) under current-clamp condition. As shown in Fig. 8, 2-methoxyestradiol (3 μM) reversibly caused membrane depolarization. The further application of Evans blue (30 μM) was found to reverse 2-methoxyestradiol-induced membrane depolarization. During the exposure of cells to 2-methoxyestradiol (3 μM), the cells were significantly depolarized to −33 ± 6 mV from a control value of −42 ± 7 mV (n = 8). In continued presence of 2-methoxyestradiol (3 μM), Evans blue (30 μM) hyperpolarized the cells from −29 ± 5 to −38 ± 8 mV (n = 7). The application of niflumic acid (30 μM) also produced a significant change in the resting membrane potential from −40 ± 6 to −49 ± 7 mV (n = 5). NS1619 (30 μM) also hyperpolarized endothelial cells significantly. Similarly, the presence of A23187 (10 μM), a Ca^{2+} ionophore, can reverse membrane depolarization caused by 2-methoxyestradiol (3 μM) (*data not shown*). Thus, the stimulators of BK_{Ca} channels can counteract the 2-methoxyestradiol-induced membrane depolarization in these cells.

Discussion

The results presented here showed that: (1) in HUVEC-C cells, 2-methoxyestradiol can reversibly suppress the amplitude of K^+ outward currents (I_K) in a concentration-dependent manner; (2) 2-methoxyestradiol decreased the activity of BK_{Ca} channels without altering the single-channel conductance; (3) 2-methoxyestradiol inhibited the channel activity in a voltage- and pressure-dependent manner; and (4) the 2-methoxyestradiol-induced reduction in the opening probability may be caused mainly by a decrease in the number of long-lived openings. This inhibitory action may cause membrane depolarization, thus affecting functional activity of endothelial cells, if the 2-methoxyestradiol actions in vivo are the same as those shown in this study.

The IC_{50} value for 2-methoxyestradiol-induced inhibition of BK_{Ca} channels in these vascular endothelial cells was 3 μM. This value is higher than that which

induced apoptosis in bovine pulmonary artery endothelial cells (EC_{50} value = 0.45 μM) or inhibited the migration of these cells (IC_{50} value = 0.71 μM) (Yue et al., 1997). However, the 2-methoxyestradiol concentration used to suppress the growth of HU-VEC-C cells was found to be close to its IC_{50} value for inhibition of BK_{Ca} channels (Reiser et al., 1998). Thus, there seems to be a link between the effects of 2-methoxyestradiol on endothelial cells and its inhibitory effect on BK_{Ca} channel activity.

The data presented in this study could be interpreted as conflicting with previous reports showing that 17β-estradiol enhanced BK_{Ca} channel activity in vascular endothelial cells (Rusko, Li & van Breemen, 1995). However, our results are compatible with recent findings showing a lack of effect of 17β-estradiol on the activity of endothelial BK_{Ca} channels (Papassotiriou et al., 2000; Valverde et al., 2000). This discrepancy could be related to the possibility that there may be different splice variants of the channel or auxiliary subunits expressed in different types of vascular endothelial cells. Nevertheless, because endothelial BK_{Ca} channels reported here may not contain β-subunits, it is possible that the binding of 2-methoxyestradiol to the α-subunits reduces the probability of BK_{Ca} channels to open and that its inhibition of endothelial BK_{Ca} channels is not strictly dependent on the presence of β-subunits.

It has been reported that 2-methoxyestradiol can affect the level of intracellular cyclic AMP in MCF-7 human breast cancer cells (Lottering, Haag & Seegers, 1992). However, the present study showed that 2-methoxyestradiol caused a decrease in endothelial BK_{Ca} channel activity, while a cell-permeable cyclic AMP analogue, 8-bromo-cyclic AMP, had no effect on it. Thus, the 2-methoxyestradiol-induced increase in the channel activity seen in these cells did not seem to result from its effect on the change in the level of intracellular cyclic AMP. On the other hand, 2-hydroxyestradiol was reported to interact with α-adrenergic receptors to suppress insulin release in pancreatic islet cells (Etchegoyen et al., 1998). However, it is unlikely that the 2-hydroxyestradiol- or 2-methoxyestradiol-induced inhibition of I_K seen in these cells is mediated by the binding to α-adrenergic receptors, because the intracellular exposure of the detached patches to 2-methoxyestradiol suppressed BK_{Ca} channel activity.

In our study, 2-methoxyestradiol produced a shift of 15 mV to a positive potential in the activation curve of BK_{Ca} channels. Therefore, 2-methoxyestradiol can suppress channel activity in a voltage-dependent fashion. Additionally, we found that membrane stretch applied in these endothelial cells could potentiate the inhibitory effect on BK_{Ca} channel activity caused by 2-methoxyestradiol. Recent studies have demonstrated that oscillating activity of BK_{Ca} channels appeared to be important for the migration of transformed epithelial cells or glioma cells (Schwab & Oberleithner, 1996; Bordey, Sontheimer & Trouslard, 2000). Unlike 17β-estradiol, 2-methoxyestradiol is also more potent in exerting antiangiogenic activity in vascular endothelial cells (Reiser et al., 1998; Lippert et al., 2000). Thus, it would be of importance to determine whether 2-methoxyestradiol-mediated modulation of BK_{Ca} channels is responsible for the inhibitory effects on the growth of tumor vessels in vivo.

The 2-methoxyestradiol-induced inhibition of I_K in these cells may arise from the different types of single-channel kinetic behavior. Because single-channel conductance was unaffected by 2-methoxyestradiol, the reduced responsiveness of the channel to 2-methoxyestradiol is likely to be secondary to the alterations remote from the pore region of the channel. Moreover, the present results showing that 2-methoxyestradiol applied intracellularly produced a decrease in the number of long-lived openings, resulting in a single open kinetic state, can mainly account for its inhibitory effect on I_K. Taken together, the observations that the application of 2-methoxyestradiol to bath medium in an inside-out membrane patch suppressed endothelial BK_{Ca} channels are in agreement with the results observed in the whole-cell experiments.

In summary, we have presented evidence to demonstrate that 2-methoxyestradiol can directly suppress BK_{Ca} channel activity in vascular endothelial cells. 2-Methoxyestradiol that is endogenously derived from the rapid and sequential degradation of catechol estrogens may also modify the effects of 17β-estradiol or its metabolites on BK_{Ca} channel activity (Zhu & Conney, 1998). BK_{Ca} channels respond directly to shear stress or a number of vasoconstrictors and vasorelaxants (Hoyer et al., 1994; Nilius, Viana & Droogmans, 1997; Busse & Fleming, 1998). Therefore, assuming that 2-methoxyestradiol can exert a significant effect on the in vivo BK_{Ca} channel, even normal homeostatic responses to other agents that affect vascular tone, or responses to change in shear stress or membrane stretch might be compromised. In addition to 17β-estradiol, 2-methoxyestradiol seems to be useful in helping to characterize the properties of BK_{Ca} channels.

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