Evidence for the stimulatory effect of resveratrol on Ca\(^{2+}\)-activated K\(^{+}\) current in vascular endothelial cells

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Abstract

Objective: Resveratrol, a natural phytoalexin compound, is present in grapes and wine, and it can produce vasorelaxation. However, little is known of its mechanisms of action on ionic currents in endothelial cells. Methods: The effect of resveratrol on Ca\(^{2+}\)-activated K\(^{+}\) currents in an endothelial cell line (HUV-EC-C) originally derived from human umbilical vein was investigated with the aid of the patch-clamp technique. Results: In the whole-cell configuration, resveratrol reversibly increased the amplitude of K\(^{+}\) outward currents. The increase in outward current caused by resveratrol was greatly inhibited by iberiotoxin (200 nM) or paxilline (1 \(\mu\)M), but not by glibenclamide (10 \(\mu\)M), tamoxifen (10 \(\mu\)M), or \(\beta\)-bungarotoxin (200 nM). Thus, this outward current is believed to be Ca\(^{2+}\)-activated K\(^{+}\) current (\(I_{KCa}\)). In the inside-out configuration, bath application of resveratrol (30 \(\mu\)M) caused no change in the single-channel conductance, but increased the activity of large-conductance Ca\(^{2+}\)-activated K\(^{+}\) (BK\(_{Ca}\)) channels. Resveratrol enhanced the channel activity in a concentration-dependent manner. The EC\(_{50}\) value for resveratrol-induced channel activity was 20 \(\mu\)M. The resveratrol-stimulated increase in the channel activity was independent of internal Ca\(^{2+}\). Resveratrol (30 \(\mu\)M) also shifted the activation curve of BK\(_{Ca}\) channels to less positive membrane potentials. The change in the kinetic behavior of BK\(_{Ca}\) channels caused by resveratrol in these cells is due to an increase in mean open time and a decrease in mean closed time. In a pancreatic islet endothelial cell line (MS1), resveratrol (30 \(\mu\)M) also increased the activity of intermediate-conductance K\(_{Ca}\) channels. Conclusions: These results provide evidence that in addition to the presence of antioxidative activity, resveratrol can also stimulate K\(_{Ca}\) channels in endothelial cells. The direct Ca\(^{2+}\) stimulation of these K\(_{Ca}\) channels by resveratrol may be responsible for its effect on the functional activities of endothelial cells. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: Endothelial function; K-channel

1. Introduction

Vascular endothelial cells that are situated at the interface between blood and the muscular media of the vessel play an important role in local control of vascular tone and the regulation of exchange between blood and tissue. Because these cells lack voltage-dependent Ca\(^{2+}\) channels, membrane hyperpolarization can lead to an increase in Ca\(^{2+}\) influx, which may subsequently activate endothelial nitric oxide synthase [1,2]. Several substances, including substance P and acetylcholine can relax the vascular smooth muscle in an endothelium-dependent manner [3]. These effects are not be fully explained by the release of prostacyclin or nitric oxide and believed to be due to the fact that the fluctuation of extracellular K\(^{+}\) caused by these substances is responsible for the endothelium-dependent vasodilation [4]. Recent studies have also shown that the vascular tone can be modulated by either the change of K\(^{+}\) concentration in myoendothelial gap junctions [4,5] or electrotonic spreading of membrane potential [6].

Resveratrol is a naturally occurring phytoalexin present in grapes and wine. It has been demonstrated to produce a variety of actions. These effects include the inhibition of arachidonate metabolism in leukocytes and platelets [7,8],...
the suppression of tumor necrosis factor-α-induced ICAM-1, VCAM-1 and tissue factor gene expression in endothelial cells [9], the inhibition of adhesion of U937 monocytoid cells to endothelial cells [9,10], the reduced activity of peroxidase of prostagland H synthase-1 [11], a cell cycle arrest at S/G2 phase transition and an apoptotic cell death in HL-60 cells [12,13], the inhibition of cyclooxygenase 2 activity and cell growth in mammary epithelial cells [14,15] and leukocyte function [16], and antioxidative activity in rat hepatocytes [17]. In addition, resveratrol was found to attenuate antigen-induced contractions in guinea-pig tracheal smooth muscle [18], phenylephrine-induced pressor response in isolated rat aorta [19] and histamine- or fluoride-induced vasoconstriction in isolated porcine coronary artery [20]. A previous report also showed that the vasorelaxant action of resveratrol was in part mediated by the release of nitric oxide from the endothelium, since the inhibitor of nitric oxide synthase can block its relaxant action [19]. However, none of the studies have thus far demonstrated the underlying mechanism of actions of resveratrol on ionic currents in endothelial cells, even though resveratrol may affect vascular tone and the functional activity of endothelial cells [9,10,19–21].

Therefore, the objective of the present study was to determine the effect of resveratrol on whole-cell K+ outward currents in cultured endothelial cells of human umbilical veins, and study whether resveratrol can affect the activity and gating of large-conductance Ca2+-activated K+ (BKCa) channels. Previous observations at our laboratory have shown that BKCa channels, which are voltage- and Ca2+-sensitive, were expressed in these cells [22]. The K+ selectivity, unitary conductance, voltage-dependence and pharmacological properties of these channels is similar to those of BKCa channels described previously in other endothelial cells [2,23]. By comparison, the effect of resveratrol on intermediate-conductance KCa channels expressed in MS1 pancreatic endothelial cells was also examined. The present study clearly indicates that resveratrol can enhance the activity of KCa channels in endothelial cells. The effect of resveratrol on these channels in endothelial cells may be a mechanism underlying its vasorelaxant effects.

2. Methods

2.1. Cell culture

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) [22]. Endothelial cells were grown in monolayer culture in 50 ml plastic culture flasks at 37°C in a humidified environment containing 5% CO2/95% air. Cells were maintained in 5 ml Ham’s F-12K nutrient media supplemented with 10% fetal bovine serum, heparin 100 µg/ml and 30–50 µg/ml endothelial cell growth supplement (ECGS). Cells underwent passage once a week, and a new stock line was generated from frozen cells (frozen in 10% dimethyl sulfoxide in culture medium) every 3 months. The experiments were performed after 5 or 6 days of subcultivation (60–80% confluence).

MS1 cell line, a pancreatic islet endothelial cell line, was also obtained from Culture Collection and Research Center [(CCRC-60150], Hsinchu, Taiwan). MS1 cells were maintained in monolayer culture in DMEM medium supplemented with 4 mM L-glutamine, containing 1.5 g/l sodium bicarbonate, 4.5 g/l glucose, 1.0 mM sodium pyruvate and 5% heat-inactivated bovine serum at 37°C in 5% CO2-containing humidified air.

2.2. Electrophysiological measurements

Immediately before each experiment, cells were dissociated and an aliquot of cell suspension was transferred to a recording chamber positioned on the stage of an inverted phase-contrast microscope (Diaphot-200; Nikon, Tokyo, Japan). Cells were bathed at room temperature (20–25°C) with normal Tyrode’s solution. The patch pipettes were made from borosilicated glass capillary tubes (Kimax-51; Kimble Product, Vineland, NJ, USA) using a vertical two-step patch-electrode puller (PB-7; Narishige, Tokyo, Japan) and the tips were fire-polished with a microforge (MF-83; Narishige). The resistance of the patch pipette was 3–5 MΩ when it was immersed in normal Tyrode’s solution. Ionic currents were recorded with glass pipettes in whole-cell or inside-out configuration of the patch-clamp technique, using an RK-400 amplifier (Biologic) [22,24]. All potentials were corrected for liquid junction potential that developed at the tip of the pipette when the composition of pipette solution was different from that in the bath solution. Tested drugs were applied by perfusion or added to the bath to obtain the final concentrations indicated.

2.3. Data recording and analysis

The signals consisting of voltage and current tracings were monitored with a digital storage oscilloscope (model 1602; Gould, Valley View, OH, USA) and on-line recorded in a digital audio tape recorder (model 1204; Biologic). Subsequently, the stored data were fed back and digitized at 5–10 kHz with a Digidata 1200 analog-to-digital device (Axon Instruments, Foster City, CA, USA) interfaced to a Labem computer (Taipei, Taiwan) by using Clampex module of pClamp 6.03 software package (Axon Instruments). Ionic currents were analyzed off-line with Clampfit subroutine of pClamp or pClamp module of Origin software (Microcal, Northampton, MA, USA).

Single-channel currents were analyzed with Fetchan and Pstat subroutines in pClamp software (Axon instruments).
Multigaussian adjustments of the amplitude distributions between channels were used to determine unitary currents. The functional independence between channels was verified by comparing the observed stationary probabilities with the values calculated according to the binomial law. The number of active channels in the patch N was counted at the end of each experiment by perfusing a solution with 100 μM Ca²⁺ and then used to normalize opening probability at each potential. The opening probabilities (Pₒ) were evaluated using an iterative process to minimize the χ² calculated with a sufficiently large number of independent observations.

Open- or closed-time distribution was log-binned using the method of McManus et al. [25]. When the square root of the number of events in a bin was plotted against the open or closed lifetime distribution appeared as a clear peak with the respective time constant falling in the vicinity of the distribution peak [26]. The all-point amplitude histogram was obtained from a continuous recording of 30 s or 1 min and fitted with the Gaussian distribution function using a least-squares method with the aid of Origin software (Microcal).

To estimate all transition rates between states, the single-channel data was idealized and converted to ASCII format, and then used to determine single-channel kinetic parameters by a maximum likelihood algorithm [27]. The highest log likelihood was obtained with the gating scheme: C₂ ⇔ C₁ ⇔ O₁ ⇔ O₂. All channel data were modeled according to this scheme. Simulated single-channel data were also obtained using the determined transition rates.

To calculate stimulatory effect of resveratrol on the activity of BKca channels, the holding potential was set at +80 mV and the bath solution contained 0.1 μM Ca²⁺. The concentration-dependent relation of resveratrol on the activity of BKca channels was fitted with the Hill equation. That is,

\[
\text{Percentage increase} = \frac{(E_{\text{max}} \times [\text{drug}])^n}{(E_{C_{50}}^n + [\text{drug}])^n}
\]

where [drug] represents the concentration of resveratrol; EC₅₀ and n are the concentration required for a 50% stimulation and Hill coefficient, respectively; and Eₘₐₓ is resveratrol-induced maximal stimulation of BKca channels.

All values are reported as means±S.E. Student’s paired or unpaired t test was used for the statistical analyses. To further clarify the statistical difference among the two or four treatment groups, analyses of variance with Duncan’s multiple-range test for multiple comparison were also performed. Differences between values were considered significant when P<0.05 or P<0.01.

2.4. Drugs and solutions

3,4',5-Trihydroxy-trans-stilbene (resveratrol) was purchased from Sigma Chemical (St. Louis, MO, USA). 3,3',4,5,7-Pentahydroxyflavone (quercetin) was obtained from Aldrich (Milwaukee, WI, USA). Paxilline was purchased from Biomol (Plymouth meeting, PA, USA). Iberiotoxin, glibenclamide and ionomycin were purchased from Research Biochemicals (Natick, MA, USA). β-Bungarotoxin was a kind gift of Dr. Long-Sen Chang, Institute of Biomedical Sciences, National Sun Yat-sen University, Kaohsiung, Taiwan. Tissue culture media, t-glutamine, penicillin-streptomycin, fungizone, and trypsin were obtained from Life Technologies (Grand Island, NY, USA). Endothelial cell growth supplement was purchased from Upstate Biotechnology (Lake Placid, NY, USA). All other chemicals were commercially available and of reagent grade. The composition of normal Tyrode’s solution was as follows (in mM): NaCl 136.5, KCl 5.4, CaCl₂ 1.8, 2 mM MgCl₂ 0.53, glucose 5.5 and Hepes–NaOH buffer 5.5 (pH 7.4). In experiments recording K⁺ current or membrane potential, the potential pipettes were filled with the solution (in mM): Kₐ-spartate 130, KCl 20, KH₂PO₄ 1, MgCl₂ 1, EGTA 0.1, Na₂ATP 3, Na₂GTP 0.1 and Hepes–KOH buffer 5 (pH 7.2). In single-channel recording, high K⁺-bathing solution contained (nM): KC1 145, MgCl₂ 0.53 and Hepes–KOH buffer 5 (pH 7.2). The pipette solution contained (nM): KC1 145, MgCl₂ 2 and Hepes–KOH buffer 5 (pH 7.4). Free Ca²⁺ concentration was calculated assuming a dissociation constant for EGTA and Ca²⁺ at pH 7.2 of 10⁻⁷ M [28].

3. Results

3.1. Effect of resveratrol on whole-cell K⁺ outward current (Iₓ) in cultured endothelial cells of human umbilical veins (HUV-EC-C)

In these experiments, the cells were bathed in normal Tyrode’s solution containing 1.8 mM CaCl₂, and pipette solution contained a low concentration (0.1 mM) of EGTA and 3 mM ATP. As shown in Fig. 1A, when the cell was depolarized from −40 mV to various potentials ranging from −30 to +70 mV with 20 mV increments, a family of K⁺ outward currents were elicited. Within 1 min of exposing the cells to resveratrol (30 μM), the amplitude of outward currents was increased throughout the entire range of voltage-clamp step. For example, when the voltage pulses from −40 to +70 mV were applied, resveratrol (30 μM) significantly increased the current amplitude from 193±82 to 594±121 pA (P<0.05, n=9). This stimulatory effect was readily reversed on the washout of resveratrol. Fig. 1B illustrates the averaged current–voltage relations for these currents in the absence and presence of resveratrol (30 μM).

To determine the nature of Iₓ in these cells, the effects of tamoxifen, quercetin, iberiotoxin and paxilline on Iₓ were examined and compared. As illustrated in Fig. 2B, quercetin (30 μM), another polyphenolic compound, did
not affect the amplitude of $I_K$. Tamoxifen (10 μM), which is an antagonist of estrogen receptors, also caused no effect on $I_K$. However, iberiotoxin (200 nM) or paxilline (1 μM) was found to suppress $I_K$ significantly. Paxilline, an indole alkaloid isolated from *Penicillium paxilli*, was previously reported to be a potent inhibitor of large-conductance Ca$^{2+}$-activated K$^+$ (BK$_{Ca}$) channels [29].

The effects of various K$^+$ channel blockers, including glibenclamide, iberiotoxin, paxilline and β-bungarotoxin, on resveratrol-stimulated $I_K$ in these cells were also compared. β-Bungarotoxin was found to be a blocker of voltage-dependent K$^+$ outward current [30]. As shown in Fig. 2C, neither glibenclamide (10 μM) nor β-bungarotoxin (200 nM) affected the resveratrol-mediated increase in the amplitude of $I_K$ significantly. Tamoxifen (10 μM) also had no effect on resveratrol-stimulated $I_K$. However, iberiotoxin (200 nM) or paxilline (1 μM) was able to

![Fig. 1. Stimulatory effect of resveratrol on K$^+$ outward current ($I_K$) in cultured endothelial cells of human umbilical veins (HUV-EC-C). (A) Current traces in the absence (left) and presence (right) of 30 μM resveratrol (Res). The upper part in (A) indicates the voltage protocol. Arrows indicate the zero current level. (B) The averaged current–voltage relations of outward currents in control (filled circles), after the application of resveratrol (open circles) and after the washout of resveratrol (open squares) (mean±S.E.; n=8 ± 12 for each point).](https://academic.oup.com/cardiovascres/article-abstract/45/4/1035/299073/464/103529073?煌点'on 2019-04-16)

![Fig. 2. Comparison between the effect of resveratrol and those of other related compounds on the amplitude of $I_K$. Each cell was held at −40 mV, and the voltage pulses to +50 mV (300 ms in duration) were applied. (A) Original current traces showing the effect of resveratrol (Res) and tamoxifen (Tam) on $I_K$ in human umbilical vascular endothelial cells. The label 'a' indicates the control and label 'b' indicates the samples that were recorded after application of 10 μM resveratrol (Res) or 10 μM tamoxifen (Tam). (B) Bar graph showing the comparison between the effect of resveratrol (Res; 10 μM) and those of tamoxifen (Tam; 10 μM), quercetin (Quer; 30 μM), and iberiotoxin (Iber; 200 nM) on $I_K$. The amplitude of $I_K$ in the control was considered to be 1.0 and the relative amplitude of increased $I_K$ after application of each agent was plotted. Each point represents mean±S.E. (n=5–9). (C) Bar graph showing the effect of various compounds on the resveratrol-induced increase in $I_K$. The magnitude of the increased $I_K$ by the presence of resveratrol (10 μM) alone was taken as 1.0 and the relative amplitude of increased $I_K$ after application of resveratrol plus each agent was then plotted. Each point represents mean±S.E. (n=5–8). Res: resveratrol (10 μM); Tam: tamoxifen (10 μM); Quer: quercetin (30 μM); Iber: iberiotoxin (200 nM); Pax: paxilline (1 μM); Glib: glibenclamide (10 μM); βBT: β-bungarotoxin (200 nM).](https://academic.oup.com/cardiovascres/article-abstract/45/4/1035/299073/464/103529073?煌点'on 2019-04-16)
suppress the increase of $I_k$ caused by resveratrol. These results indicate that the observed increase in outward current by resveratrol is due to its selective stimulation of $I_k$ that is sensitive to iberiotoxin or paxilline.

3.2. Effect of iberiotoxin and paxilline on the resveratrol-induced change in resting membrane potential in umbilical vascular endothelial cells.

The effect of resveratrol on membrane potential in the absence and presence of iberiotoxin or paxilline was also studied. This type of endothelial cells had a resting membrane potential of $-35 \pm 8$ mV ($n=16$) under the current-clamp condition. As shown in Fig. 3, resveratrol caused a membrane hyperpolarization and the further application of iberiotoxin or paxilline was found to antagonize resveratrol-induced membrane hyperpolarization. During the exposure to resveratrol (30 μM), the cell was hyperpolarized to $-49 \pm 7$ mV from a control value of $-35 \pm 6$ mV ($P<0.05$, $n=12$). In continued presence of resveratrol (30 μM), iberiotoxin (200 nM) depolarized the cells from $-48 \pm 8$ to $-37 \pm 7$ mV ($P<0.05$, $n=6$). The application of paxilline (1 μM) in continued presence of resveratrol also caused a significant change in the resting membrane potential from $-48 \pm 9$ to $-36 \pm 7$ mV ($P<0.05$, $n=5$). Similarly, the presence of paxilline (1 μM) can reverse membrane hyperpolarization caused by ionomycin (1 μM), a $Ca^{2+}$ ionophore (data not shown). Thus, the blockers of BK$_{Ca}$ channels can counteract resveratrol-induced membrane hyperpolarization in these cells.

3.3. Stimulatory effect of resveratrol on BK$_{Ca}$ channels in umbilical vascular endothelial cells.

To further elucidate the effect of resveratrol on ionic current, its effects on the activity of BK$_{Ca}$ channels were studied. The single-channel recording with an inside-out configuration was performed in symmetrical $K^+$ (145 mM) concentration. The bath solution contained 0.1 μM Ca$^{2+}$ and the holding potential was set at +80 mV. As shown in Fig. 4, when resveratrol (30 μM) was applied to the intracellular surface of membrane patch, the activity of channel openings was significantly increased. The opening probability of the channel at +80 mV in the absence of resveratrol was found to be 0.043±0.003 ($n=8$). The addition of resveratrol (30 μM) significantly increased the activity to 0.221±0.015 ($P<0.01$, $n=7$). However, the amplitude of single-channel current was not changed by the presence of resveratrol (Fig. 4). When resveratrol was washed out, channel activity almost returned to the control level (0.045±0.004, $n=6$). The relationship between the concentration of resveratrol (30 μM) and the opening probability of BK$_{Ca}$ channels was then constructed and plotted (Fig. 5A). After the curve was fitted to Hill equation, the values of EC$_{50}$ and maximally stimulated activity in the presence of resveratrol were 20 μM and 0.32, respectively. The Hill coefficient was found to be 1.1. Thus, it is clear that the presence of resveratrol can increase the activity of BK$_{Ca}$ channels in a concentration-dependent manner.

3.4. Effect of internal Ca$^{2+}$ concentration on resveratrol-stimulated the activity of BK$_{Ca}$ channels.

Whether the enhanced activity of BK$_{Ca}$ channels produced by resveratrol is related to the level of internal Ca$^{2+}$ concentration was further examined. In this series of experiments, when an excised membrane patch was formed, various concentrations of Ca$^{2+}$ in the bath solution in the absence and presence of resveratrol (30 μM) were applied. As shown in Fig. 5B, at a given concentration of resveratrol (30 μM), the magnitude of resveratrol-stimulated activity of BK$_{Ca}$ channels was increased as internal Ca$^{2+}$ was elevated. However, resveratrol caused an in-
3.5. Lack of effect of resveratrol on single-channel conductance of BK_{Ca} channels

The effect of resveratrol on BK_{Ca} at various membrane potentials was also studied. In an inside-out configuration, cells were bathed in symmetrical K^+ concentration and the bath solution contained 0.1 μM Ca^{2+}. Fig. 6 illustrates current–voltage relations of BK_{Ca} channels with or without bath application of resveratrol (30 μM). The single-channel conductance of BK_{Ca} channels in control was 176±7 pS (n=12) with a reversal potential of 0±2 mV (n=12). The value of single-channel conductance was not significantly different from that (177±8 pS, P>0.05, n=9) measured in the presence of resveratrol (30 μM). These results indicated that resveratrol caused no change in single-channel conductance, but enhanced the activity of BK_{Ca} channels in these cells.

3.6. Effect of resveratrol on the activation curve of BK_{Ca} channels

Fig. 6C shows the activation curve of BK_{Ca} channels in the absence and presence of resveratrol (30 μM). The relationships between membrane potentials and opening probability of BK_{Ca} channels with or without the application of resveratrol (30 μM) were plotted and well fit by Boltzmann equation using a non-linear regression analysis with the aid of Origin 5.0 (Microcal). That is:

\[ N_P = n/\left(1 + \exp\left[-(V - a)/b\right]\right) \]

where N is the number of channels in the patch, n is the maximal N_P level, V is the membrane potential in mV, a is the membrane potential for half-maximal activation and b is the slope factor of the activation curve. In control, n=0.091±0.005, a=78±6 mV and b=10.6±0.8 mV (n=7), whereas in the presence of resveratrol (30 μM), n=0.282±0.013, a=59±5 mV and b=10.4±0.9 mV (n=7). These results indicated that resveratrol not only caused a 3.1-fold increase in the maximal opening probability of BK_{Ca} channels, but also significantly shifted the activation curve to less positive membrane potential by 17 mV. However, there was no significant difference in the slope (i.e., b value) of activation curve between the absence and presence of resveratrol. Therefore, resveratrol can enhance the activity of BK_{Ca} channels in a voltage-dependent fashion in these cells.

3.7. The effect of resveratrol on kinetic behavior of BK_{Ca} channels in human umbilical vascular endothelial cells

The effect of resveratrol on the kinetic behavior of BK_{Ca} channels was analyzed. As shown in Fig. 7A, in an excised inside-out patch of control cells (i.e., in the absence of resveratrol), open- and closed-time histograms of BK_{Ca} channels were fitted to the following equations:

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Closed time histogram: \[ T_{closed} = T_{max} \exp(-T_{max}/\tau_{closed}) \]

Where \( T_{max} \) is the maximum time, \( \tau_{open} \) and \( \tau_{closed} \) are the time constants for open and closed states, respectively.

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where N is the number of channels in the patch, n is the maximal N_P level, V is the membrane potential in mV, a is the membrane potential for half-maximal activation and b is the slope factor of the activation curve. In control, n=0.091±0.005, a=78±6 mV and b=10.6±0.8 mV (n=7), whereas in the presence of resveratrol (30 μM), n=0.282±0.013, a=59±5 mV and b=10.4±0.9 mV (n=7). These results indicated that resveratrol not only caused a 3.1-fold increase in the maximal opening probability of BK_{Ca} channels, but also significantly shifted the activation curve to less positive membrane potential by 17 mV. However, there was no significant difference in the slope (i.e., b value) of activation curve between the absence and presence of resveratrol. Therefore, resveratrol can enhance the activity of BK_{Ca} channels in a voltage-dependent fashion in these cells.
Fig. 5. The concentration- and Ca\(^{2+}\)-dependent effect of resveratrol-induced increase in BK\(_{ca}\) channels in human umbilical vein endothelial cells. (A) The original current trace with bath application of 1, 10, 30 and 100 \(\mu\)M resveratrol. The experiments were conducted with symmetrical K\(^+\) concentrations. Under the inside-out configuration, the holding potential was set at +80 mV and the bath solution contained 0.1 \(\mu\)M Ca\(^{2+}\). Channel openings are shown as an upward deflection. (B) The concentration-response curve for resveratrol-induced stimulation of BK\(_{ca}\) channels. The smooth curve was fitted with Hill equation. The EC\(_{50}\) value and maximal opening probability of BK\(_{ca}\) channels were 20 \(\mu\)M and 0.32, respectively. The Hill coefficient was 1.1. Each point represents mean±S.E. \((n=5–9)\). (C) Bar graph showing the various concentrations of Ca\(^{2+}\) in the bath before and during exposure to resveratrol (30 \(\mu\)M).

Fig. 6. The effect of resveratrol on current–voltage relation of BK\(_{ca}\) channels in human umbilical vascular endothelial cells. The experiments in an excised membrane patch were conducted in symmetrical K\(^+\) concentration in which the bath solution contained 0.1 \(\mu\)M Ca\(^{2+}\). (A) The activity of BK\(_{ca}\) channels before (left) and after (right) bath application of 30 \(\mu\)M resveratrol (Res). Unitary channel currents were recorded at various membrane potentials denoted by the left side of each current trace. Upward deflections are the opening events of the channel. (B) The current–voltage relations of BK\(_{ca}\) channels in the absence (filled circles) and presence (open circles) of resveratrol (30 \(\mu\)M). Each point represents mean±S.E. \((n=5–7)\). Of note, single-channel conductance in the absence and presence of resveratrol is nearly identical. (C) The relationship between opening probability of BK\(_{ca}\) channels and membrane potential in the absence (filled circles) and presence (open circles) of resveratrol (30 \(\mu\)M).
channels at the level of +80 mV can be fitted by a two-exponential curve. The time constants for the fast and slow components of open-time histogram were 5.4±0.8 and 16.4±2.6 ms (n=5), respectively, whereas those in closed-time histogram were 5.5±0.5 and 125.5±6.8 ms (n=5). Resveratrol (30 μM) increased the fast and slow time constants of the open state to 8.7±1.5 and 35.6±2.8 ms (P<0.05, n=5). In addition, resveratrol can decrease the mean closed time to 2.8±0.9 and 42.1±3.2 ms (P<0.05, n=5). It is clear that the stimulatory effect of resveratrol on the activity of BKCa channels may be explained by a lengthening of the open and a shortening of the closed times.

3.8. The effect of resveratrol on the gating mode of BKCa channels

In order to characterize the effect of resveratrol on the mean lifetimes of open or closed states, channel currents were idealized and modeled using a kinetic scheme described above. This gating scheme consists of two open and two closed states. Transition rates between states were derived from the maximum likelihood estimation [27]. The results of kinetic parameters are summarized in Table 1. Fig. 7B shows examples of simulated single-channel data generated using the transition rates that were obtained for BKCa channels activated with or without the addition of resveratrol. It is clear that resveratrol can increase both dissociation constant (K value) and gating rate constant (L value).

3.9. Stimulatory effect of resveratrol on intermediate-conductance Ca2+-activated K+ (IKCa) channels in MS1 endothelial cells derived from the pancreatic islet

In order to determine whether the effect of resveratrol on BKCa channels occurs in other types of endothelial cells, the effect of resveratrol in MS1 pancreatic endothelial cells was also examined. In the inside-out configuration, under symmetrical K+ condition, the activity of IKCa channels in these cells can be observed as reported previously [2,31]. When resveratrol (30 μM) was applied to the bath containing 0.1 μM Ca2+, the channel activity was increased significantly (Fig. 8). However, the amplitude of unitary current measured at the level of +80 mV It is clear that resveratrol can increase both dissociation constant (K value) and gating rate constant (L value).

Table 1

<table>
<thead>
<tr>
<th>Control</th>
<th>Resveratrol (30 μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>L1</td>
<td>0.10±0.02</td>
</tr>
<tr>
<td>L2</td>
<td>0.09±0.02</td>
</tr>
<tr>
<td>K</td>
<td>0.29±0.04</td>
</tr>
</tbody>
</table>

*a The gating scheme used to obtain the transition rates between states was C1 ↔ C2 ↔ O1 ↔ O2. K, equilibrium dissociation constant; L, equilibrium gating constant that equals opening rate constant divided by closing rate constant; 1 and 2, first and second component. (mean±S.E.; n=4).

*P<0.05 versus control.
there was no significant difference in the single-channel conductance between the absence and presence of resveratrol (30 μM) (24.0±0.8 [n=6] versus 24.1±0.9 pS [n=5], P>0.05) (Fig. 8C). These results indicate that resveratrol is capable of stimulating IKCa channels in this type of endothelial cells.

4. Discussion

The major findings in the present study are as follows. First, in cultured endothelial cells of umbilical veins, resveratrol can reversibly increase the amplitude of K⁺ outward current. Second, resveratrol stimulates the activity of large-conductance Ca²⁺-activated K⁺ (BKCa) channels in a concentration- and voltage-dependent manner, but does not change single-channel conductance. Third, a shortening of closed time and a lengthening of open time can explain resveratrol-induced activation of BKCa channels. Fourth, resveratrol can also enhance the activity of intermediate-conductance KCa channels in MS1 endothelial cells. The stimulatory effect of resveratrol on KCa channels will cause membrane hyperpolarization, hence affecting endothelial function.

The EC₅₀ value for resveratrol-induced stimulation of BKCa channels observed in our study was 20 μM. This value is similar to those required to inhibit platelet aggregation [8,9,32], to suppress adhesion of monocytoïd cells [10], and to relax precontracted blood vessels [19,20]. More importantly, the stimulatory effect of resveratrol observed in the present study was found to occur at a concentration achievable in humans [33]. Therefore, BKCa channels expressed in endothelial cells may be a relevant ‘target’ for the action of resveratrol. Since the activation of BKCa channels by resveratrol in endothelial cells can cause membrane hyperpolarization followed by an increase in Ca²⁺ influx [2], the elevated intracellular Ca²⁺ concentration is also capable of producing a positive feedback increase in the activity of BKCa channels. In addition, the increased K⁺ efflux following the activation of BKCa channels caused by resveratrol may produce an increase in K⁺ concentration in myoendothelial space, hence leading to hyperpolarize vascular myocytes and dilate blood vessels [4–6]. Given that the fact that vascular myocytes exhibit the activity of a variety of Ca²⁺-activated K⁺ channels [4–6], resveratrol may also target BKCa channels in these cells. However, it remains to be further determined whether the effect of resveratrol by binding to and stimulating these channels contributes to its preventive role against coronary heart disease [8].

The single-channel conductance of BKCa channels in umbilical vascular endothelial cells measured with the use of 145 mM K⁺ on both sides of the membrane was 176±7 pS (n=12). This value is similar to those of typical BKCa channels reported in rabbit endothelial cells [23] and Ethy926 endothelial cells [34]. Likewise, the single-chan-
nel conductance of intermediate-conductance Ca$^{2+}$-activated K$^-$ (IK$_{ca}$) channels in MS1 endothelial cells was 27±4 pS (n=6), a value not different from that of IK$_{ca}$ channels previously observed in endothelial cells of rat aorta [31,35]. In our study, we provide evidence that resveratrol-mediated increase in outward currents in umbilical vascular endothelial cells is not due to the change in single-channel amplitude, because the single-channel conductance of BK$_{ca}$ or IK$_{ca}$ channels between the presence and absence of resveratrol did not differ significantly. However, the present results demonstrating resveratrol-induced increase in mean open time and decrease in mean closed time of BK$_{ca}$ channels may account for its increase in whole-cell K$^-$ currents. Moreover, in our study, the single-channel kinetic analysis was also used to deduce the mechanism of resveratrol-induced enhancement of BK$_{ca}$ channel activity. Of interest, not only the dissociation constant (K value), but also the equilibrium gating constant (L value) was increased by the presence of resveratrol. These results thus suggest that resveratrol may interact with the binding and gating of BK$_{ca}$ channels.

Several reports have shown that resveratrol may act to be an estrogen agonist [36±38]. However, our results showed that resveratrol can activate BK$_{ca}$ channels in inside-out patches. Tamoxifen, an antagonist of estrogen receptors, also had no effect on the resveratrol-stimulated I$_K$. Therefore, the resveratrol-induced increase in the activity of BK$_{ca}$ channels observed in the present study did not appear to be an interaction with estrogen receptors, although it is unclear whether the concentration of tamoxifen used in our study is effective in blocking estrogen receptors in these cells. On the other hand, quercetin, another polyphenolic compound present in red wine, was not found to have any effect on the amplitude of I$_K$ in endothelial cells. Resveratrol appears to be an important ingredient that can stimulate K$_{ca}$ channels. A previous report showing that quercetin-mediated vasodilator effect did not require the endothelium [39] may be interpreted to be compatible with our findings. Therefore, there might be different mechanisms by which resveratrol and quercetin produce vasorelaxation [19–21,39].

Previous studies have shown that resveratrol may elevate intracellular cyclic GMP [40] or reduce the formation of 5-lipoxygenase products (e.g., 5-HETE) [7]. However, in an inside-out patch, bath application of resveratrol is capable of stimulating the activity of K$_{ca}$ channels. It thus excludes the possibility that diffusible cytosolic messengers inside the cells (e.g., cyclic GMP or arachidonic acid metabolites) are responsible for resveratrol-stimulated increase in the activity of K$_{ca}$ channels.

In the present experiments of recording whole-cell I$_K$, the pipette solution contained 3 mM ATP, a value that is high enough to block ATP-sensitive K$^-$ (K$_{ATP}$) channels. Furthermore, glibenclamide (10 μM) did not have any effect on resveratrol-induced increase of I$_K$, whereas iberiotoxin (200 nM) or paxilline (1 μM) suppressed it significantly. Thus, the resveratrol-induced I$_K$ is referred to as Ca$^{2+}$-activated K$^-$ current that is sensitive to iberiotoxin or paxilline. Resveratrol-induced increase of I$_K$ shown in the present study is unlikely to be due to the activation of K$_{ATP}$ channels. In fact, in inside-out configuration, when the bath solution contained no ATP, the resveratrol-induced increase in the channel activity was still observed.

The present results showing that the effect of resveratrol on BK$_{ca}$ channels was independent of internal Ca$^{2+}$ suggest that resveratrol may not exert its effect via an increase in the affinity of Ca$^{2+}$ ions for Ca$^{2+}$-binding sites in the membrane. However, resveratrol produced a shift of 17 mV to less positive potential in the activation curve of BK$_{ca}$ channels. Therefore, it is possible that the interaction of resveratrol with BK$_{ca}$ channels is dependent of the level of membrane potential, but not coupled to Ca$^{2+}$ binding sites of the channels.

In summary, the present study provides substantial evidence that resveratrol can activate BK$_{ca}$ and IK$_{ca}$ channels in vascular endothelial cells. If similar results are found in endothelial cells in vivo to those occurring in cells observed in our study, the increase in K$^-$ efflux through resveratrol-induced stimulation of K$_{ca}$ channels in endothelial cells may underlie the mechanism through which resveratrol can produce vasorelaxation in an endothelium-dependent pathway [19,20].

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