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RESEARCH PAPER

Kaempferol stimulates large conductance Ca^{2+}-activated K^+ (BK_{Ca}) channels in human umbilical vein endothelial cells via a cAMP/PKA-dependent pathway

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Background and purpose: Kaempferol has been shown to possess a vasodilator effect but its mechanism of action remains unclear. In this study, experiments were carried out to study the effect of kaempferol on K^+ channels in endothelial cells.
Experimental approach: K^+ channel activities in human umbilical vein endothelial cells (HUVECs) were studied by conventional whole cell and cell-attached patch-clamp electrophysiology.
Key results: Kaempferol stimulated an outward-rectifying current in HUVECs in a dose-dependent manner with an EC_{50} value of $2.5 \pm 0.02\,\mu M$. This kaempferol-induced current was abolished by large conductance Ca^{2+}-activated K^+ (BK_{Ca}) channel blockers, such as iberiotoxin (IbTX) and charybdotoxin (ChTX), whereas the small conductance Ca^{2+}-activated K^+ (SK_{Ca}) channel blocker, apamin, and the voltage-dependent K^+ (K_V) channel blocker, 4-aminopyridine, had no effect. Cell-attached patches demonstrated that kaempferol increased the open probability of Bk_{Ca} channels in HUVECs. Clamping intracellular Ca^{2+} did not prevent kaempferol-induced increases in outward current. In addition, the kaempferol-induced current was diminished by the adenylyl cyclase inhibitor SQ22536, the cAMP antagonist Rp-8-Br-cAMP and the PKA inhibitor KT5720, but was not affected by the guanylyl cyclase inhibitor ODQ, the cGMP antagonist Rp-8-Br-cGMP and the PKG inhibitor KT5823. The activation of BK_{Ca} channels by kaempferol caused membrane hyperpolarization of HUVECs.
Conclusion and implications: These results demonstrate that kaempferol activates the opening of BK_{Ca} channels in HUVECs via a cAMP/PKA-dependent pathway, resulting in membrane hyperpolarization. This mechanism may partly account for the vasodilator effects of kaempferol.
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Keywords: kaempferol; potassium channels; endothelial cells; cAMP; vasodilation

Abbreviations: BK_{Ca}, large-conductance Ca^{2+}-activated K^+; ChTX, charybdotoxin; HUVECs, human umbilical vein endothelial cells; IbTX, iberiotoxin

Introduction

Kaempferol, a flavonoid present in diet and plants, modulates vascular functions, including inhibition of platelet-activating factor (Zang *et al*., 2004) and protection of endothelial cells against injury by linoleic acid hydroperoxide (Kaneko and Baba, 1999). Kaempferol also relaxes rat aortic rings (Padilla *et al*., 2005). Part of the kaempferol-induced relaxation of vascular tissue is dependent on the integrity of the endothelial cell layer as the effect of the compound is significantly diminished when the endothelium is removed. However, species difference exists. Thus, Xu *et al*. (2006) reported that the relaxation effect of kaempferol on porcine coronary artery was endothelium-independent.

Endothelial cell function is strongly affected by changes of intracellular Ca^{2+} concentrations ($[Ca^{2+}]_i$) but endothelial cells do not contain voltage-dependent Ca^{2+} channels. Changes of $[Ca^{2+}]_i$ in endothelial cells are mainly due to Ca^{2+} release from internal stores or through Ca^{2+} influx across the cell membrane, which depends on the membrane potential. Potassium channels regulate membrane potential and thereby influence $[Ca^{2+}]_i$. The major types of K^+ channels in endothelial cells are large-conductance Ca^{2+}-activated (BK_{Ca}), intermediate-conductance Ca^{2+}-activated, small-conductance Ca^{2+}-activated channels and voltage-dependent K^+ channels (Kestler *et al*., 1998; Dittrich and Daut, 1999; Fan and Walsh, 1999; Gluais *et al*., 2005; Gillham *et al*., 2007). Growing evidence indicates that

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change of K^+ channel activity is associated with endothelial cell proliferation and synthesis of vasoactive substances such as nitric oxide (Nilius and Wohlrab, 1992; Wiecha *et al*., 1998; Kuhlmann *et al*., 2004).

The present study was carried out to examine the effects of kaempferol on K^+ channels in endothelial cells. The results may give a clue to the mechanism by which this flavonoid relaxes blood vessels.

Methods

Cell preparation

Human umbilical vein endothelial cells (HUVECs) were obtained from American Type Culture Collection (Manassas, VA, USA) and cultured in Hams F-12k nutrient medium supplemented with 10% fetal bovine serum (v/v), 100 μg mL^{-1} heparin and 10 μg mL^{-1} endothelial cell growth factor at 37 °C in 95% air-5% CO_2. The medium was changed every other day.

Electrophysiological measurement

The cells were continuously superfused with a bath solution containing (in mM) 120 NaCl, 25 $NaHCO_3$, 5.5 glucose, 4.76 KCl, 1.18 $MgSO_4$, 1.18 NaH_2PO_4, 1.25 $CaCl_2$, with pH adjusted to 7.4 using 5 M NaOH. Patch pipettes (tip resistance, 3–5 MΩ) were pulled from glass capillary tubes, fire polished and filled with an internal solution containing (in mM) 35 KCl, 90 K-gluconate, 10 NaCl, 10 HEPES and 10 1,2-bis (2-aminophenoxy) ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid (BAPTA), with pH adjusted to 7.2 using 5 M KOH. GTP (0.5 mM) was added to provide a substrate for the signal transduction pathways. MgATP (5 mM) was included to inhibit ATP-sensitive K^+ currents and provide a substrate for energy-dependent processes. In certain experiments, $[Ca^{2+}]_i$ was adjusted to 75, 250 or 500 nM by the administration to the intracellular solution of 3, 6.6 or 7.7 nM $CaCl_2$, respectively, in the presence of 10 mM BAPTA. Seal resistances ranged from 1 to 10 GΩ after seal formation. Whole-cell currents were recorded with an Axopatch-1D amplifier (Axon Instruments, Sunnyvale, CA, USA) in voltage-clamp mode. Voltage-clamp protocols were applied with pClamp 9.0 software (Axon Instruments). Data were filtered at 5 kHz, digitized with a Digidata 1200 analog-to-digital converter (Axon Instruments) and analysed with Clampfit 9.0 software (Axon Instruments). Cell capacitance was calculated from the area under the capacitive current elicited by a 10-mV hyperpolarizing pulse starting at a holding potential of −70 mV. Whole-cell current was normalized to cell capacitance and is expressed as picoamperes per picofarad. Series resistance was always less than 15 MΩ. Capacitance subtraction and series resistance compensation (80%) were used in all recordings. Whole-cell currents were recorded with an Axopatch 200 A amplifier (Axon Instruments) in voltage-clamp mode. Membrane currents were activated by depolarizing pulses of 800 ms from a holding potential of −70 mV to test potentials ranging from −70 to +70 mV in +10-mV step increments. Every voltage pulse lasted 2000 ms and the time between voltage pulse was 500 ms in which no recording took place. These current–voltage (I–V) relationship measurements were made under control conditions. Afterwards, cells were either washed or incubated with kaempferol plus pharmacological agents for a further 3 min and another measurement was obtained. Membrane potential measurements were recorded under current-clamp mode, with the current equal to zero. External solution was changed with a perfusion system (Warner Instruments Inc., Holliston, MA, USA) with a multibarrel pipette connected to a common orifice positioned 100–200 μm from the cells studied. The solution flow rate was 0.5 mL min^{-1}. Only one barrel was used for perfusion at any given time. To achieve fast solution changes, a barrel with small dead volume was used. Complete solution change was achieved in less than 1 sec. All experiments were conducted at room temperature (22–25 °C).

Single-channel membrane currents were measured by means of cell-attached patches. HUVECs were maintained in a bath solution containing (in mM) K^+ gluconate 130, KCl 10, $MgCl_2$ 0.5, D-glucose 5.5, HEPES 10, $CaCl_2$ 1.5, pH 7.3 (with KOH). The pipette solution contained (in mM) K^+ aspartate 110, KCl 30, HEPES 5, $MgCl_2$ 1, EGTA 0.1; pH = 7.3 (with KOH). Records were obtained before (control) and after 3 min of exposure to kaempferol, with a sample duration of 30 s. The role of BK_{Ca} channels in kaempferol effect was analysed by further incubating the cells with kaempferol plus iberiotoxin (IbTX) for 2 min and records were obtained subsequently. Analysis of the unitary currents was performed with pClamp 9.0 software (Axon Instruments). Current amplitude histograms based on the analysis of current amplitudes in single-channel records provided the clearest demonstration of multiple current levels. The total number of functional channels (*N*) in a patch was estimated by observing the number of peaks in current amplitude histograms. NP_o, the product of the number of channels times the open probability, was used as a measure of the activity of channels within a patch. NP_o was calculated as the relative area under an all-point amplitude histogram and expressed as follows:

$$NP_o = \frac{\sum_{i=1}^{N} i \cdot Ai}{\sum_{i=1}^{N} Ai}$$

Where *A* is the area under the Gaussian curve, *N* is the total number of observable channels in a patch, *i* is the number of channels and P_o is the open probability of an individual channel in a patch. If channels open independently of one another and the exact number of channels in a patch is known, then the open probablility of a single channel (P_o) can be calculated by dividing NP_o by the number of channels.

Statistical analysis

Data were expressed as means ± s.e.mean. Curves were fitted by Boltzmann equations. Paired Student *t*-test and ANOVA were used for the statistical evaluation of differences among means. $P<0.05$ was considered to indicate statistically significant differences.

Drugs

All chemicals were purchased from Sigma-Aldrich (St Louis, MO, USA). Cell culture media and supplements were from Invitrogen (Grand Island, NY, USA). The stock solution of kaempferol was dissolved in ethanol, KT5720 ((9*S*,10*S*,12*R*)-2,3,9,10,11,12-hexahydro-10-hydroxy-9-methyl-1-oxo-9,12-epoxy-1*H*-diindolo[1,2,3-fg:3′,2′,1′-kl]pyrrolo[3,4-i][1,6]benzodiazocine-10-carboxylic acid hexyl ester), ODQ (1*H*-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one) and KT5823 ((9*S*,10*R*,12*R*)-2,3,9,10,11,12-hexahydro-10-methoxy-2,9-dimethyl-1-oxo-9,12-epoxy-1*H*-diindolo[1,2,3-fg:3′,2′,1′-kl]-pyrrolo[3,4-i][1,6]benzodiazocine-10-carboxylic acid, methyl ester) were dissolved in dimethyl sulphoxide; SQ22536 (9-(tetrahydro-2-furanyl)-9*H*-purin-6-amine 9-THF-Ade), Rp-8-Br-cAMP, Rp-8-Br-cGMP, IbTX, charybdotoxin (ChTX), apamin and 4-aminopyridine were dissolved in water. The final concentration of solvents in the bath solution was always less than or equal to 0.1%, and this bath concentration of solvents had no significant effect on ion-channel activity in HUVECs (data not shown).

Results

Kaempferol increases outward potassium K^+ in HUVECs

In the present study, the average cell capacitance of HUVECs was 35.2 ± 2.8 pF ($n = 70$). Step depolarizations from a holding potential of -70 mV to test potentials between -70 and $+70$ mV elicited a voltage-dependent outward current (Figures 1a and b). Kaempferol increased the outward current. This effect occurred in less than 10 s and reached maximum in 30 s. The kaempferol-induced current was readily reversed upon washout for 5 min (Figures 1a and b). The effects of kaempferol on the outward current were dose-dependent, with an EC_{50} value of 2.5 ± 0.2 μM ($n = 5$; Figure 1c).

Kaempferol activates BK_{Ca} channels in HUVECs

The kaempferol-induced current in HUVECs was abolished by the BK_{Ca} channel blockers IbTX (IbTX, 100 nM) and ChTX (100 nM) (Figure 2a). However, the small-conductance Ca^{2+}-activated channel blocker apamin (1 μM) and the voltage-dependent K^+ channel blocker 4-aminopyridine (100 μM) had no effect on the inhibition of the kaempferol-induced current (Figure 2b). The results were the same when a lower dose of kaempferol (1 μM) was used (data not shown). The ability of kaempferol to activate BK_{Ca} channels was further assessed by conducting cell-attached patches. Channels with conductance of 170 ± 7 pS were detected in HUVECs (Figure 3a). The channel activity was minimal under control conditions but the openings of the channels were increased by kaempferol (Figure 3b). All-point amplitude histograms showed that there was one active channel (two peaks in the histogram corresponding to 0 and 1 channels) open at the beginning of recording (Figure 3c), whereas two active channels were open (three peaks in the histogram corresponding to 0, 1 and 2 channels) after exposure of the cells to 10 μM kaempferol (Figure 3c). NP_o (number of channels × open probability; see Methods) was increased significantly after treatment with kaempferol (0.088 ± 0.006 and 0.343 ± 0.017 for control and kaempferol-treated cells, respectively; $n = 5$). Kaempferol also increased the mean open time constant from 13 to 43 ms. The kaempferol-induced increase in channel activity was inhibited by IbTX (Figures 3b and c).

Kaempferol increases BK_{Ca} current independently of changes in $[Ca^{2+}]_i$

Clamping the $[Ca^{2+}]_i$ at 75 nM by adding BAPTA (10 mM) and $CaCl_2$ (4 mM) to the intracellular solution significantly increased the outward current when compared with the current produced

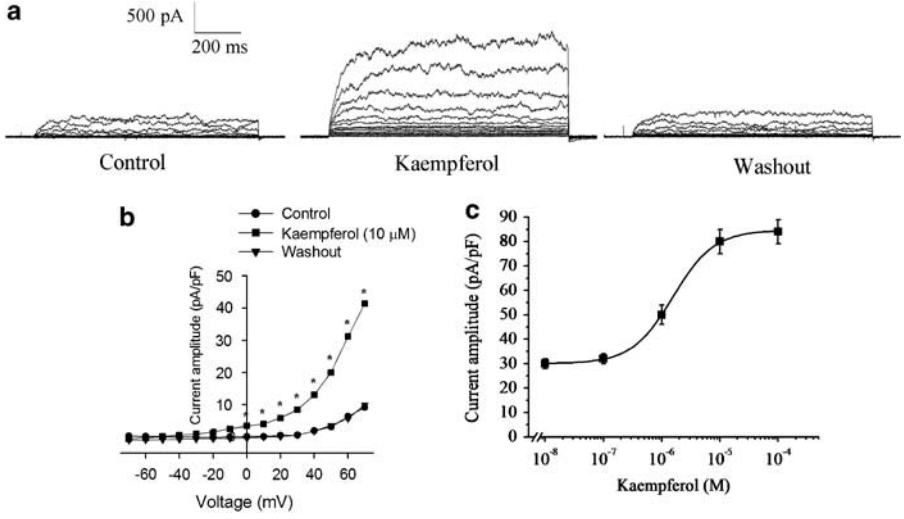

Figure 1 Effect of kaempferol on whole-cell current in HUVECs. (**a**) Sample traces of whole-cell currents produced by stepwise depolarization, in steps of 10 mV, from -70 to $+70$ mV with a holding potential of -70 mV. Currents were recorded under control conditions, in the presence of kaempferol (10 μM) and after the washing out of kaempferol. (**b**) Current–voltage relationship for steady-state current (holding potential = -70 mV) under control conditions, in the presence of 10 μM kaempferol and after the washing out of kaempferol. (**c**) Dose–response curve for kaempferol. Steady currents at $+70$ mV were measured in the presence of various concentrations of kaempferol. Values are the mean ± s.e.mean ($n = 5$). *$P < 0.05$ versus the control group. HUVECs, human umbilical vein endothelial cells.

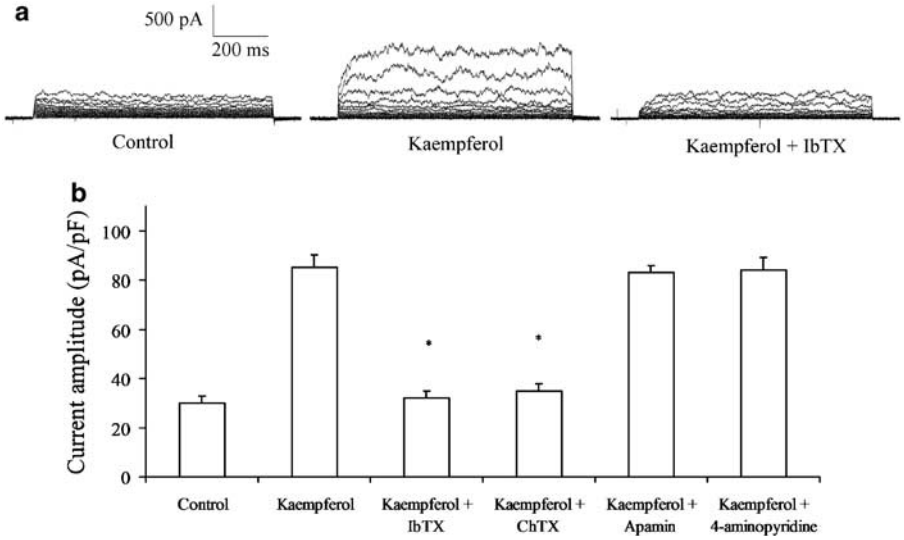

Figure 2 Effects of K^+ channel inhibitors on kaempferol-induced current in HUVECs. (**a**) Sample traces of whole-cell currents were studied at different voltages generated by stepwise depolarization, in steps of 10 mV, from −70 to +70 mV with a holding potential of −70 mV. Currents were recorded under control conditions and stimulation by kaempferol (10 μM) in the absence or presence of IbTX (0.1 μM). (**b**) Effects of IbTX (100 nM), ChTX (100 nM), apamin (1 μM) and 4-aminopyridine (100 μM) on the kaempferol-induced current (steady currents measured at +70 mV). Values are the mean ± s.e.mean ($n=5$). *$P<0.05$ versus the control group. ChTX, charybdotoxin; HUVECs, human umbilical vein endothelial cells; IbTX, iberiotoxin.

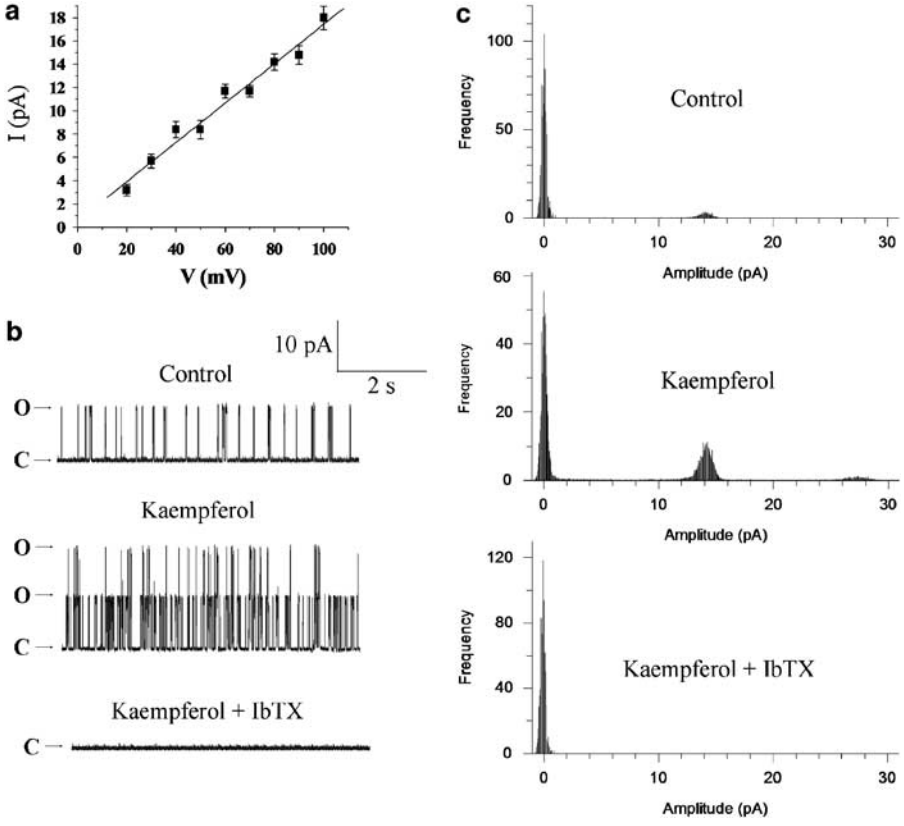

Figure 3 Effect of IbTX on the unitary outward currents induced by kaempferol. (**a**) Current–voltage relationship of the unitary outward current measured in cell-attached patches. Data points were expressed as mean ± s.e.mean ($n=5$). By using linear regression, a single-channel slope conductance of 170 ± 7 pS was calculated. (**b**) Sample traces of unitary currents measured in cell-attached patches at a holding potential of +70 mV. Unitary currents were measured in control condition, and after 1-min perfusion with kaempferol (10 μM), in the absence or presence of IbTX (100 nM). (**c**) All-point histogram corresponding to the records in **(b)**. IbTX, iberiotoxin.

in the presence of BAPTA alone (Figure 4). Clamping the $[Ca^{2+}]_i$ at 250 or 500 nM further increased the outward current produced by a +100-mV step. Despite the $[Ca^{2+}]_i$ being clamped at various concentrations, kaempferol (10 μM) still increased the outward current produced by a +100-mV step.

cAMP/PKA signalling is involved in activation of the BK_{Ca} channels by kaempferol

The signal transduction pathway by which BK_{Ca} channels in HUVECs were activated by kaempferol was examined next. Figures 5a and b show that the AC inhibitor SQ22536 (200 μM), the cAMP antagonist Rp-8-Br-cAMP (40 μM) and the PKA inhibitor KT5720 (20 μM) antagonized the kaempferol-induced current. By contrast, the GC inhibitor ODQ (10 μM), the cGMP antagonist Rp-8-Br-cGMP (40 μM) and the PKG inhibitor KT5720 (20 μM) did not antagonize the kaempferol-induced current (Figure 5b).

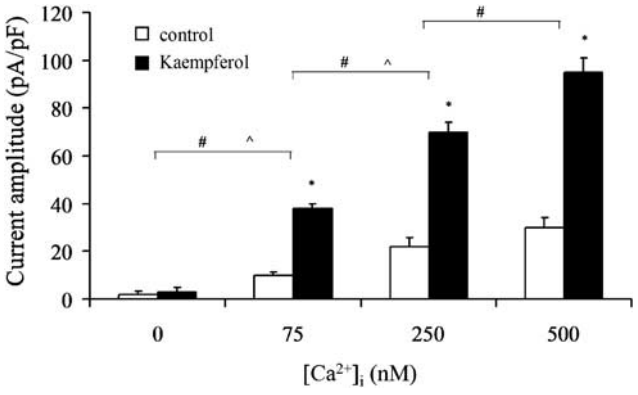

Figure 4 Effects of $[Ca^{2+}]_i$ on the effect of kaempferol-induced outward current. The amplitude of outward current evoked by a single voltage step (from −70 to +70 mV) was measured under control conditions and dosing stimulation by kaempferol (10 μM), with the $[Ca^{2+}]_i$ clamped at indicated levels. In all groups, 10 mM BAPTA was included in the pipette solution. Values are the mean ± s.e.mean ($n=5$). *$P<0.05$ kaempferol-treated group versus the control groups; ^$P<0.05$ comparison among the control groups with different $[Ca^{2+}]_i$; #$P<0.05$ comparison among the kaempferol-treated group with different $[Ca^{2+}]_i$. BAPTA, 1,2-bis (2-aminophenoxy) ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid.

Kaempferol causes membrane hyperpolarization of HUVECs through the activation of BK_{Ca} channels

The membrane potential of HUVECs was −50.38 ± 1.14 mV ($n=5$). Kaempferol (10 μM) produced a hyperpolarization (membrane potential: −57.12 ± 2.43 mV, $n=5$, $P<0.05$ vs control). In the presence of IbTX, kaempferol did not affect the membrane potential (data not shown).

Discussion

The type of Ca^{2+}-activated K^+ channel expressed in endothelial cells varies with species and tissue and may be influenced by the cell culture conditions (Kestler *et al.*, 1998). Although BK_{Ca} channels are absent from the endothelium in certain vascular beds (Kohler *et al.*, 2000; Bychkov *et al.*, 2002; Gauthier *et al.*, 2002), their existence has been well documented in the endothelium of the human aorta (Jow and Numann, 1999) and cerebral microvasculature (Jow and Numann, 2000), pig and rat coronary arteries (Baron *et al.*, 1997; Bang *et al.*, 1999; Frieden *et al.*, 1999), and in HUVECs (Kestler *et al.*, 1998; Begg *et al.*, 2003). In the present study, kaempferol dose-dependently stimulated an outward current in HUVECs (Figure 1). The flavonoid increased the open probability as well as the number of opened channels in cell-attached patches (Figure 3). Pharmacologically, IbTX and ChTX, the selective BK_{Ca} channel inhibitors, totally blocked the kaempferol-induced

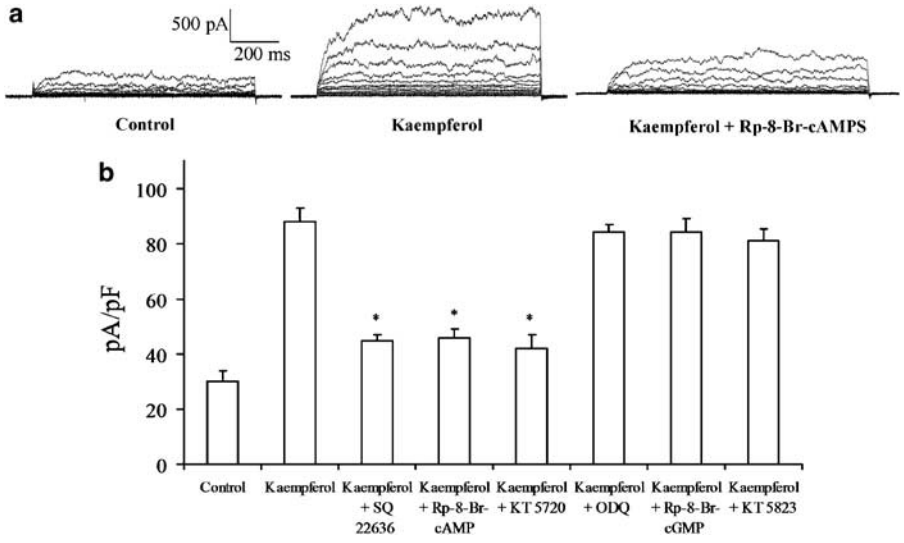

Figure 5 Effects of inhibition of cAMP-dependent or cGMP-dependent pathways on kaempferol-induced current in HUVECs. (**a**) Sample traces of whole-cell currents measured under control conditions and stimulation by kaempferol (10 μM) in the absence or presence of Rp-8-Br-cAMPS (40 μM). (**b**) Effects of inhibitors of cAMP-dependent (40 μM Rp-8-Br-cAMPS, 200 μM SQ22536 and 20 μM KT5720) and cGMP-dependent (10 μM ODQ, 40 μM Rp-8-Br-cGMPS and 20 μM KT5823) pathways on the kaempferol-induced current (steady currents measured at +70 mV). Values are the mean ± s.e.mean ($n=5$). *$P<0.05$ versus the kaempferol-treated group. HUVECs, human umbilical vein endothelial cells.

current (Figures 2 and 3). On the other hand, apamin, a small-conductance Ca^{2+}-activated channel blocker, and 4-aminopyridine, a voltage-dependent K^+ channel blocker, had no effect. Taken in conjunction, all these results suggest that kaempferol activates BK_{Ca} channels in HUVECs. To our knowledge, this is the first description of such an effect of kaempferol on BK_{Ca} channels.

BK_{Ca} channel activity is Ca^{2+}-dependent (Nelson and Quayle, 1995; Cui *et al*., 1997; Moss *et al*., 1999). Consistent with this, the present data show that removal of free intracellular Ca^{2+} by BAPTA significantly reduces the voltage-induced outward current (Figure 4). In cells with $[Ca^{2+}]_i$ clamped, the outward K^+ current was proportional to $[Ca^{2+}]_i$. Although the $[Ca^{2+}]_i$ was clamped at any given level, the outward current could still be increased by kaempferol. This implies that the activation of BK_{Ca} channels by kaempferol is unrelated with the mobilization of intracellular Ca^{2+}. Other intracellular second messengers such as cAMP and cGMP increase the open probability of BK_{Ca} channels by phosphorylation (Torphy, 1994; Han *et al*., 1999; White *et al*., 2000; Barman *et al*., 2003, 2004). In the present study, inhibitors of the cAMP/PKA signal transduction pathway such as SQ22536, Rp-8-Br-cAMP and KT5720 markedly reduced the effect of kaempferol on BK_{Ca} channels (Figure 5). These results are in good agreement with biochemical assays that have revealed that kaempferol increases intracellular cAMP (Revuelta *et al*., 1997, 1999, 2000). By contrast, inhibitors of the cGMP/PKG signal transduction pathway such as ODQ, Rp-8-Br-cGMP and KT5823 did not affect the kaempferol-induced BK_{Ca} current (Figure 5), suggesting that the cGMP/PKG pathway plays a minor or negligible role in the effect of kaempferol. The mechanism by which kaempferol increased cAMP remains obscure but it may have been due to decreased hydrolysis of cAMP, as this flavanoid is known to inhibit phosphodiesterase (Davis, 1984). So far, 11 isoforms of phosphodiesterase have been characterized, showing different specificity for cAMP or cGMP (Boswell-Smith *et al*., 2006). It is plausible that kaempferol acts mainly on those phosphodiesterases that are specific for cAMP.

Kaempferol induces relaxation in arteries with intact endothelium starting at concentrations as low as 10^{-7} M (Padilla *et al*., 2005). It is a potent inhibitor of the myosin light-chain kinase in vascular smooth muscle cells (Hagiwara *et al*., 1988), which might account for the relaxation it causes. A significant percentage of this relaxation is dependent on the endothelial integrity. The mechanism involved is not understood but the antioxidant activity of kaempferol may protect nitric oxide against degradation (Rice-Evans *et al*., 1995). This phenomenon might explain, at least in part, the endothelium-dependent effect of kaempferol. Another possibility is the activation of BK_{Ca} channels, which cause endothelial cell hyperpolarization, as observed in the present study. Activation of BK_{Ca} channels results in membrane hyperpolarization of endothelial cells (Frieden *et al*., 1999; Kamouchi *et al*., 1999; Nilius and Droogmans, 2001), which in turn enhances Ca^{2+} influx and hence elevates $[Ca^{2+}]_i$, leading to the stimulation of synthesis and exocytosis of vasoactive substances (Busse *et al*., 1991; Carter and Pearson, 1992; Nilius *et al*., 1997; Nilius and Droogmans, 2001). This scheme is supported by the finding that inhibition of BK_{Ca} channels interferes with nitric oxide release from endothelial cells (Busse *et al*., 1993). Indeed, flavonoids such as apigenin and quercetin increase endothelial nitric oxide production, via the BK_{Ca} channel-dependent, membrane hyperpolarization-induced, Ca^{2+} influx in endothelial cells (Kuhlmann *et al*., 2005; Erdogan *et al*., 2007).

In conclusion, kaempferol activates BK_{Ca} channels in HUVECs, via a cAMP/PKA-dependent pathway. This activity appears to be one of the possible mechanisms involved in the vasodilator effect of kaempferol.

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Conflict of interest

The authors state no conflict of interest.

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