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RESEARCH PAPER

(+/−)-Naringenin as large conductance Ca^{2+}-activated K^+ (BK_{Ca}) channel opener in vascular smooth muscle cells

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Background and purpose. The aim of this study was to investigate, in vascular smooth muscle cells, the mechanical and electrophysiological effects of (+/−)-naringenin.
Experimental approach. Aorta ring preparations and single tail artery myocytes were employed for functional and patch-clamp experiments, respectively.
Key results. (+/−)-Naringenin induced concentration-dependent relaxation in endothelium-denuded rat aortic rings pre-contracted with either 20 mM KCl or noradrenaline (pIC_{50} values of 4.74 and 4.68, respectively). Tetraethylammonium, iberiotoxin, 4-aminopyridine and 60 mM KCl antagonised (+/−)-naringenin-induced vasorelaxation, while glibenclamide did not produce any significant antagonism. Naringin [(+/−)-naringenin 7-β-neohesperidoside] caused a concentration-dependent relaxation of rings pre-contracted with 20 mM KCl, although its potency and efficacy were significantly lower than those of (+/−)-naringenin. In rat tail artery myocytes, (+/−)-naringenin increased large conductance Ca^{2+}-activated K^+ (BK_{Ca}) currents in a concentration-dependent manner; this stimulation was iberiotoxin-sensitive and fully reversible upon drug wash-out. (+/−)-Naringenin accelerated the activation kinetics of BK_{Ca} current, shifted, by 22 mV, the voltage dependence of the activation curve to more negative potentials, and decreased the slope of activation. (+/−)-Naringenin-induced stimulation of BK_{Ca} current was insensitive either to changes in the intracellular Ca^{2+} concentration or to the presence, in the pipette solution, of the fast Ca^{2+} chelator BAPTA. However, such stimulation was diminished when the K^+ gradient across the membrane was reduced.
Conclusions and Implications. The vasorelaxant effect of the naturally-occurring flavonoid (+/−)-naringenin on endothelium-denuded vessels was due to the activation of BK_{Ca} channels in myocytes.
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Keywords: Large conductance Ca^{2+}-activated K^+ channels; (+/−)-naringenin; naringin; rat aorta ring; rat tail artery myocyte; whole-cell patch-clamp

Abbreviations: BK_{Ca} channels, large conductance Ca^{2+}-activated K^+ channels; V_h, holding potential

Introduction

Large conductance Ca^{2+}-activated K^+ (BK_{Ca}) channels respond to changes in intracellular Ca^{2+} (e.g. they are effectors of Ca^{2+} sparks, originating from the submembrane sarcoplasmic reticulum) and regulate membrane potential thus playing an important role in the control of myogenic tone in vascular smooth muscle (Nelson and Quayle, 1995). Furthermore, the voltage- and/or Ca^{2+}-dependent activation of BK_{Ca} channels account for their role as negative feedback mechanisms to limit depolarization and vasoconstriction. A reduced expression of BK_{Ca} channels in aged coronary arteries, for example, leads both to a decreased vasodilating capacity and to an increased risk of coronary spasm and myocardial ischaemia in older people (Marijic *et al.*, 2001). Moreover, a reduced expression of the BK_{Ca} channel β_1 subunit associated with genetic, borderline or severe hypertension, reduces the activity of these channels by decreasing their sensitivity to physiological changes in cytosolic Ca^{2+} concentration (Amberg and Santana, 2003). Therefore, the pharmacological activation of BK_{Ca} channels is considered as a rational therapeutic approach to improve

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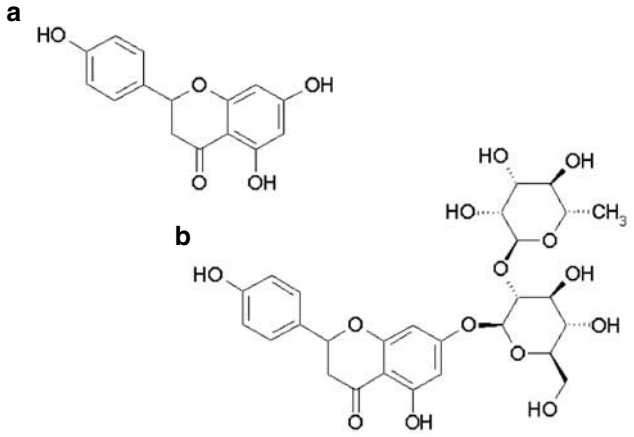

Figure 1 Chemical structures (**a**) of (+/−)-naringenin (4′,5,7-trihydroxy-flavanone) and (**b**) of its 7-β-neohesperidoside, naringin.

the impaired vasodilatatory capacity and to treat certain cardiovascular diseases.

In a previous study, the activation of BK_{Ca} channels was proposed to explain, at least in part, the vasodilatory action of some natural compounds belonging to the chemical class of flavones/flavanones (Calderone *et al*., 2004). The electrophysiological profile of apigenin and kaempferol as openers of BK_{Ca} channels was demonstrated in *Xenopus* oocytes expressing BK_{Ca} channels and a structure-activity relationship study has suggested that the pharmacophoric moiety common to these flavonoids is similar to that of NS004, a well-characterized synthetic activator of BK_{Ca} channels (Li *et al*., 1997).

The aim of this study was to investigate the effects of the *Citrus* flavonoid (+/−)-naringenin (Figure 1) on vascular functions *in vitro*, by comparing its mechanical and electrophysiological actions in rings of rat aorta and single tail artery myocytes, respectively. The data presented demonstrate that (+/−)-naringenin was able to stimulate BK_{Ca} current, reversibly and independently of increased intracellular Ca^{2+}, thus pointing to a novel mechanism of vasodilation performed by this natural compound.

Methods

Functional test

The investigation conforms with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). Compounds were tested on isolated rings of rat thoracic aorta to assess their possible vasodilating effect. Rats (male Sprague–Dawley, 350±50 g) were killed by cervical dislocation under light ether anaesthesia and exsanguinated. The aortae were immediately excised and freed of connective tissues. The endothelial layer was removed by gently rubbing the intimal surface of the vessel with a hypodermic needle. Aorta rings (5 mm wide) were suspended, under a preload of 20 mN, in 10 ml organ baths, containing a Tyrode solution (see below), thermostated at 37°C and continuously gassed with a mixture of O_2 (95%) and CO_2 (5%). Changes in tension were recorded by means of an isometric transducer (Grass FTO3), connected with a preamplifier (Buxco Electronics Inc., NC, USA) and with a software of data acquisition (BIOPAC Systems Inc., CA, USA, MP 100). After an equilibration period of 60 min, the absence of functional endothelium was assessed with ACh (10 μM) in rings precontracted with noradrenaline (1 μM). A relaxation <10% of the noradrenaline-induced contraction was considered to demonstrate lack of the endothelial layer. Between 30 and 40 min after the endothelium removal had been confirmed, rings were contracted with 20 mM KCl and when the contraction reached a plateau, cumulative (1–100 μM) concentrations of (+/−)-naringenin or naringin (Figure 1) were added. For a further characterization of the mechanism of (+/−)-naringenin, cumulative concentrations of this flavonoid were also added to the bath chamber of aortic rings precontracted with noradrenaline (1 μM). Some experiments were performed on 20 mM KCl-contracted rings in the presence of the K^+ channel blockers tetraethylammonium (10 mM), iberiotoxin (200 nM), 4-aminopyridine (3 mM) or glibenclamide (1 μM). These blockers were added into the organ bath when the contractile effect induced by 20 mM KCl reached the plateau and were allowed to equilibrate with tissues for 20 min, before the addition of the flavonoid. Furthermore, in order to evaluate the influence of marked depolarization, (+/−)-naringenin and naringin were tested on aorta rings precontracted with 60 mM KCl.

The vasorelaxant efficacy was evaluated as the maximal vasorelaxant response evoked by the highest concentration of the flavonoids, expressed as the percentage of the tone induced by the contractile agent. Potency was expressed as pIC_{50}, calculated as negative logarithm of the molar concentration of flavonoid, evoking half reduction of the tone induced by the contractile agent. pIC_{50} values could not be calculated when the efficacy parameter was lower than 50%.

Electrophysiological tests

Cell isolation procedure. Smooth muscle cells were isolated by enzymatic treatment of rat tail artery excised from animals under general anaesthesia induced with a mixture of Ketavet (0.3 mg kg^{-1} Gellini, Italy) and Rompum (0.08 mg kg^{-1} Bayer, Germany), decapitated and exsanguinated. The tail was immediately removed, cleaned of skin and placed in physiological salt solution (see below for composition). The tail main artery was dissected free, removing the connective tissue. A small piece (1 cm) was cut out at about 2–3 cm from the base of the tail, longitudinally opened, washed in physiological salt solution at 4°C and stored overnight at 4°C in 1 ml of enzyme solution (see below) containing 1.5 mg papain, 0.4 mg DL-dithiothreitol and 1.6 mg BSA. The day after, the vessel was incubated for 5–15 min at 37°C in the above-mentioned solution, gently bubbled with a 95% O_2–5% CO_2 gas mixture. The tissue was carefully washed with external solution (see below) and single smooth muscle cells were then obtained by gentle agitation with a Pasteur pipette, until the solution became cloudy. Cells were then stored at 4°C in 1 ml recording solution containing 2 mg BSA and used

within 10 h after isolation. An aliquot of the cell suspension (1–2 μl) was transferred to a small recording chamber (250 μl) mounted on the stage of an inverted phase-contrast microscope (TE300, Nikon, Japan) and monitored with a video camera (JVC TK-1280E).

Cells, characterized by an elongated shape (20–30 μm in width, 100–150 μm in length), after adhesion to the glass bottom of the chamber (10 min), were continuously superfused with the recording solution, at a flow rate of 500 μl min^{-1}, using a peristaltic pump (LKB 2132, Bromma, Sweden). Electrophysiological responses were tested at room temperature (22–24°C) only in those cells that were phase dense.

Whole-cell patch-clamp recording. Conventional whole-cell patch-clamp method (Hamill *et al*., 1981) was employed to voltage-clamp smooth muscle cells. Recording electrodes were pulled from borosilicate glass capillaries (WPI, Berlin, Germany) and fire-polished to give a pipette resistance of 2–5 MΩ when filled with the internal solution (see below). A low-noise, high-performance Axopatch 200B (Axon Instruments, Union City, USA) patch-clamp amplifier, driven by an IBM computer in conjunction with an A/D, D/A board (1322A series interface, Axon Instruments, USA) was used to generate and apply voltage pulses to the clamped cells and record the corresponding membrane currents. Current signals, after compensation for whole-cell capacitance, series resistance and liquid junction potential, were low-pass filtered at 1 kHz and digitized at 3 kHz before being stored on the computer hard disk. BK_{Ca} current was measured over a range of test potentials (500 ms) from −20 to 100 mV from a holding potential (V_h) of −40 mV. Data were collected once the current amplitude had been stabilized (usually 8–10 min after the whole-cell configuration had been obtained). BK_{Ca} current did not run down during the following 30–40 min under these conditions.

The BK_{Ca} current amplitude varied considerably depending on intracellular Ca^{2+} concentration and voltage. When highly activated, the current would incur a significant voltage sensing error if not compensated. To minimize this error, internal K^+ was lowered from 90 to 14 mM (see below) and the activation curves were derived from the current–voltage relationship measured over a range of test potentials (1500 ms) from −60 to 140 mV from a V_h of −40 mV. Conductance (G) was calculated from the equation $G = I_K/(E_m - E_K)$, where I_K is the peak current, E_m is the membrane potential and E_K is the equilibrium potential for K^+ (−21.4 mV, as estimated with the Nernst equation). G_{max} is the maximal K^+ conductance (calculated at potentials ⩾120 mV). The ratio G/G_{max} was plotted against the membrane potential and fitted to the Boltzmann equation.

Current values were corrected for leakage using 1 mM tetraethylammonium, which was assumed to block completely BK_{Ca} currents (see Results section).

Acquisition and analysis of data were accomplished using pClamp 9.0.2.018 software (Axon Instruments, USA). The current–voltage relationships were calculated on the basis of the values recorded during the last 200 ms of each test pulse (leakage corrected).

Solutions

The following solutions have been used. *Tyrode solution* (in mM): NaCl 136.8, KCl 2.95, $CaCl_2$ 1.80, $MgSO_4 \cdot 7\ H_2O$ 1.05, NaH_2PO_4 0.41, $NaHCO_3$ 11.9, glucose 5.5, pH 7.4; *modified Tyrode solution* containing either 20 mM or 60 mM KCl were made iso-osmotic by subtracting equimolar amounts of NaCl. *Physiological salt solution* (in mM): NaCl 145, KCl 4.5, NaH_2PO_4 1.2, $MgSO_4$ 1.0, Na_2EDTA 0.025, HEPES 5, $CaCl_2$ 0.1, pH 7.3. *Enzyme solution* (in mM): NaCl 110, KCl 5, $MgCl_2$ 2, $CaCl_2$ 0.16, NaHEPES 10, $NaHCO_3$ 10, KH_2PO_4 0.5, NaH_2PO_4 0.5, glucose 10, Na_2EDTA 0.49, taurine 10, pH 7. *External solution* (in mM): NaCl 140, KCl 6, glucose 10, taurine 10, Na-pyruvate 5, $MgCl_2$ 1.2, $CaCl_2$ 0.1, pH 7.4. *Internal solution* (in mM): KCl 90, NaCl 10, HEPES 10, EGTA 10, $MgCl_2$ 1, $CaCl_2$ 6.41 (pCa 7.0), pH 7.4; in some experiments, pCa was either decreased to 6.6 or increased to 7.3 by changing the $CaCl_2$ concentration; for current activation measurements, KCl was reduced to 14 mM by equimolar substitution with choline-chloride; furthermore, in another series of experiments, EGTA was replaced by an equimolar concentration of BAPTA. *Recording solution* (in mM): NaCl 145, KCl 6, glucose 10, HEPES 10, Na-pyruvate 5, $MgCl_2$ 1.2, $CaCl_2$ 0.1, nicardipine 0.003, pH 7.4.

Free Ca^{2+} concentrations were calculated using the computer programme EqCal (BioSoft, Cambridge, UK) by taking into account pH and Mg^{2+} concentration, as described by Fabiato and Fabiato (1979).

Statistical analysis

Data are reported as means ± s.e.m.; n (indicated in parentheses) represents the number of aorta rings or tail artery myocytes, respectively, isolated from at least three animals. Analysis of data were accomplished using GraphPad Prism version 4.03 (GraphPad Software, USA). Statistical analyses and significance as measured by the Student's *t*-test for either paired or unpaired samples (two-tail) as well as by ANOVA followed either by Bonferroni or by Dunnett's post-test were obtained using GraphPad InStat version 3.06 (GraphPad Software, USA). In all comparisons, $P<0.05$ was considered significant.

Materials

The chemicals used were: noradrenaline, ACh, papain, DL-dithiothreitol, BSA, iberiotoxin, (+/−)-naringenin, naringin, EGTA, BAPTA, taurine, nicardipine, 4-aminopyridine, tetraethylammonium chloride, choline-chloride and glibenclamide (Sigma Chimica, Italy).

Nicardipine, dissolved directly in ethanol, and both (+/−)-naringenin and naringin, dissolved in dimethylsulphoxide, were diluted at least 1000 times with the recording solution, before use. The resulting concentrations of dimethylsulphoxide and ethanol (below 0.1 %) failed to alter the responses of the preparations (data not shown).

Results

Vasorelaxant effect of (+/−)-naringenin and naringin on aortic rings

(+/−)-Naringenin induced an almost complete relaxation of endothelium-denuded rat aortic rings precontracted with

20 mM KCl (efficacy = 95.3 ± 1.7%; pIC_{50} = 4.74 ± 0.03; n = 10 for all results in Figure 2a) (Figure 2a and c). Vasodilator activity evoked by (+/−)-naringenin on noradrenaline-contracted rings (efficacy = 100% in all the experiments; pIC_{50} = 4.68 ± 0.03) was almost completely comparable to that observed in 20 mM KCl-contracted ones. The vasodilating effects of (+/−)-naringenin on 20 mM KCl-contracted rings were significantly reduced in the presence of 10 mM tetraethylammonium (efficacy = 22.7 ± 13.1%), 200 nM iberiotoxin (efficacy = 91.8 ± 1.7%; pIC_{50} = 4.36 ± 0.05; Figure 2a and d), and 3 mM 4-aminopyridine (efficacy = 84.5 ± 5.5%; pIC_{50} = 4.26 ± 0.02), as well as in aorta rings precontracted with 60 mM KCl instead of 20 mM KCl (efficacy = 41.8 ± 11.5%). Conversely, glibenclamide did not affect the concentration–response curve to (+/−)-naringenin (efficacy = 89.1 ± 2.4%; pIC_{50} = 4.55 ± 0.04). Naringin showed lower, albeit significant, vasorelaxant properties as compared to (+/−)-naringenin (efficacy = 56.9 ± 6.7%; pIC_{50} = 4.04 ± 0.04; n = 10; P < 0.05) (Figure 2b). At high depolarization (60 mM KCl), naringin failed to exhibit any significant vasorelaxing effect.

Effects of (+/−)-naringenin and naringin on BK_{Ca} currents of tail artery myocytes

In myocytes derived from rat tail arteries, the application of 500-ms voltage steps from a V_h of −40 mV to test potentials in the range −20 to 100 mV evoked iberiotoxin- and tetraethylammonium-sensitive, large, noninactivating and fluctuating outward currents (BK_{Ca} currents). Iberiotoxin (100 nM), a specific blocker of BK_{Ca} channels (Wei *et al*., 2005), significantly inhibited these currents (e.g. by 85.4 ± 2.2% at 70 mV, n = 5). Moreover, the addition of tetraethylammonium, at 1 mM concentration, proven to be very selective for BK_{Ca} channels (Nelson and Quayle, 1995), produced a comparable decrease of the currents (81.4 ± 2.5% at 70 mV, n = 5). Thus, under the conditions used in these experiments, the outward current mostly consisted of BK_{Ca} current (see also below).

Figure 3a shows a typical recording of BK_{Ca} currents elicited with clamp pulses in the range between −20 and 100 mV from a V_h of −40 mV under control conditions and after the addition of 30 μM (+/−)-naringenin as well as (+/−)-naringenin plus 100 nM iberiotoxin. The current–voltage relationships show that (+/−)-naringenin increased the BK_{Ca} current in a concentration-dependent manner (Figure 3b and c).

Over a range of voltages from 20 to 100 mV the percent increase in current by 1–30 μM (+/−)-naringenin was the same (data not shown), whereas that by 100 μM (+/−)-naringenin exhibited a bell-shaped pattern (e.g. 194% at 20 mV, 258% at 50 mV and 148% at 100 mV), indicating that stimulation by (+/−)-naringenin was not voltage-dependent at concentrations < 100 μM.

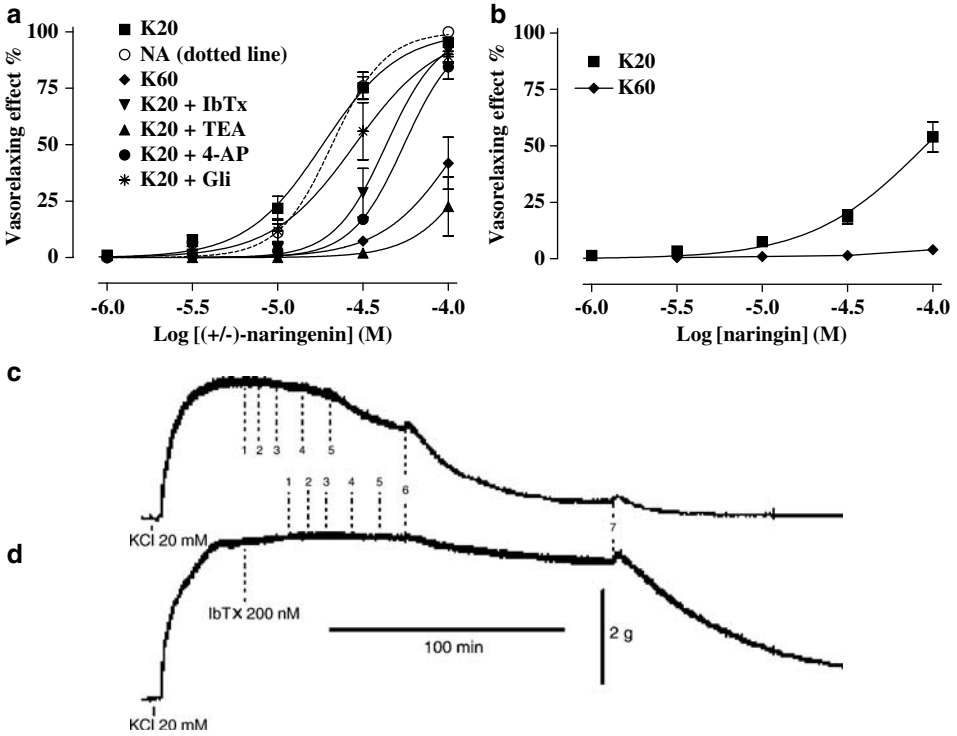

Figure 2 Effects of (+/−)-naringenin and naringin on rat aortic rings. Concentration–response curves for (**a**) (+/−)-naringenin and (**b**) naringin on 1 μM noradrenaline (NA)-, 60 mM KCl (K60)- or 20 mM KCl (K20)-contracted vessels as well as on 20 mM KCl-contracted vessels in the presence of 200 nM iberiotoxin (K20 + IbTx), 10 mM tetraethylammonium (K20 + TEA), 3 mM 4-aminopyridine (K20 + 4-AP) or 1 μM glibenclamide (K20 + Gli). Vasorelaxant effects are expressed as the percentage of the contractile tone induced by 20 mM KCl (K20, IbTx, TEA, 4-AP and Gli), noradrenaline (NA) or 60 mM KCl (K60). Data points are means ± s.e.m. (n = 10, (+/−)-naringenin and n = 8, naringin). (**c**, **d**) Representative original traces of the vasorelaxant effects of (+/−)-naringenin, recorded on aortic rings precontracted with 20 mM KCl, in the absence (**c**) or in the presence (**d**) of 200 nM iberiotoxin. The flavonoid was added cumulatively at the concentrations 100 nM (1), 300 nM (2), 1 μM (3), 3 μM (4), 10 μM (5), 30 μM (6) and 100 μM (7). The traces were obtained by a computerized acquisition system (software BIOPAC systems, MP100).

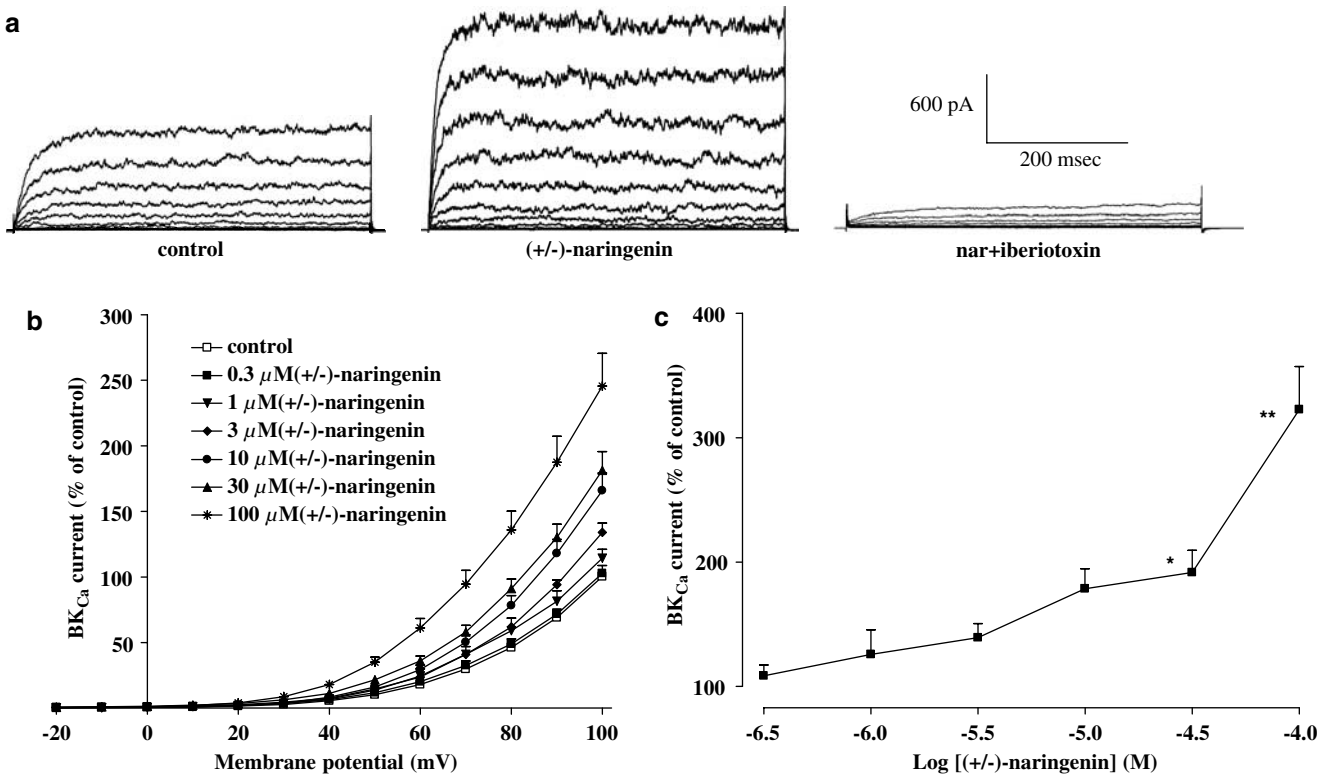

Figure 3 Effects of (+/−)-naringenin on BK_{Ca} currents of rat tail artery myocytes. (**a**) Original recordings of conventional whole-cell BK_{Ca} currents elicited with 500-ms voltage steps from a V_h of −40 mV to test potentials in the range −20 to 100 mV, measured in the absence (control) and in the presence of (+/−)-naringenin (30 μM) or (+/−)-naringenin plus 100 nM iberiotoxin (nar + iberiotoxin). (**b**) Current–voltage relationships constructed before the addition of (control) and in the presence of various concentrations of (+/−)-naringenin. On the ordinate scale, response, representing the iberiotoxin-sensitive component of the whole-cell current, is reported as the percentage of the current recorded at 100 mV under control conditions. Data points are means ± s.e.m. ($n = 3$–20). (**c**) Concentration–effect relationship of (+/−)-naringenin on the current measured during the depolarizing pulse to 70 mV from a V_h of −40 mV. On the ordinate scale, response is reported as the percentage of control. Data points are means ± s.e.m. ($n = 3$–20). *$P < 0.05$, **$P < 0.01$, Dunnett's *post-test*.

To verify that the current recorded in the presence of (+/−)-naringenin is predominantly carried by BK_{Ca} channels, we assessed the inhibitory effects of iberiotoxin. Indeed, in the five cells tested, 100 nM iberiotoxin reduced the (+/−)-naringenin-stimulated current at 70 mV to 11% of control values (see as an example Figure 3a).

Figure 4 shows the time course of the effects of (+/−)-naringenin on the current recorded from V_h −40 mV to a test potential of 70 mV. After BK_{Ca} current had reached a steady value, the addition to the bath solution of 100 μM (+/−)-naringenin produced a gradual increase of the current that reached a plateau in about 2 min. Noticeably, (+/−)-naringenin-induced stimulation of the current was completely reversible upon drug wash-out.

The increase in BK_{Ca} current recorded at 70 mV by 30 μM naringin (180 ± 22.7%, $n = 5$) was comparable to that induced by 30 μM (+/−)-naringenin under the same experimental conditions (200 ± 16.7%, $n = 10$).

Effects of (+/−)-naringenin on BK_{Ca} activation kinetic

Evidence that the macroscopic K^+ current is essentially carried by a homogeneous population of K^+ channels was provided by the observation that the rising phase of the currents evoked by depolarizing pulses ⩾50 mV were well fitted by single exponential functions (Figure 5a). Figure 5b plots the activation τ (τ_{act}) as a function of voltage in the absence (control) or presence of 30 μM (+/−)-naringenin. The τ_{act}, which appeared to be voltage-dependent, was reduced significantly by the flavonoid at all membrane voltages.

Effect of (+/−)-naringenin on BK_{Ca} channels voltage sensitivity of activation

In order to reduce the voltage-sensing errors, given the large outward currents evoked at high voltages in these cells, the internal K^+ concentration was decreased to 14 mM. This produced currents always lower than 5.2 nA, thus allowing accurate measurements of the entire BK_{Ca} current–voltage relationships along with the associated activation characteristics. Figure 6a shows the effect of (+/−)-naringenin on the current–voltage relationship. Interestingly, at low intracellular K^+ concentrations, the percent increase in current by 100 μM (+/−)-naringenin was reduced by about 50% at all voltages as compared to that recorded at 90 mM intracellular K^+ concentration (see Figure 3b). The activation curves of BK_{Ca} channels, calculated as percentage of maximum

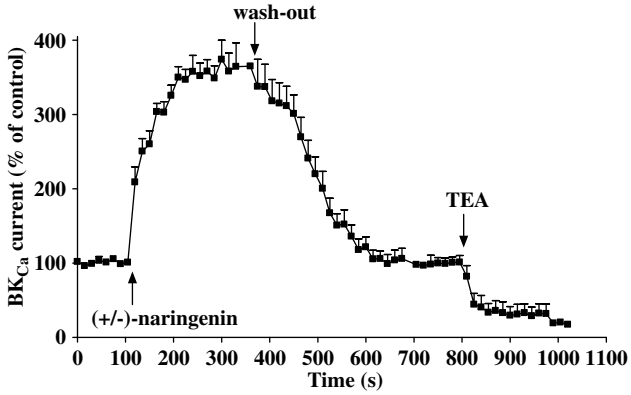

Figure 4 Time course of BK_{Ca} current stimulation induced by (+/−)-naringenin in rat tail artery myocytes. (+/−)-Naringenin (100 μM) was applied, at the time indicated by the arrow, and currents were recorded during a typical depolarization from −40 to 70 mV applied every 15 s and subsequently normalized towards the current recorded just before (+/−)-naringenin addition. Wash-out of drug allows for complete recovery of the current recorded before (+/−)-naringenin addition. BK_{Ca} current suppression by 1 mM tetraethylammonium (TEA) is also shown. Data points are means±s.e.m. ($n=5$).

conductance vs membrane voltage from the current–voltage relationships in Figure 6a, were fitted to the Boltzmann equation (Figure 6b). (+/−)-Naringenin reduced both the 50% activation potential (from 75.7±9.4 to 53.6±6.1 mV, $n=5$; $P<0.01$, Student's *t*-test for paired samples) and the slope factor (from 18.7±1.5 to 15.3±1.2 mV; $P<0.01$).

Efficacy of (+/−)-naringenin at different intracellular Ca^{2+} concentrations and in the presence of BAPTA

In order to evaluate how free intracellular Ca^{2+} affects the stimulation of BK_{Ca} current by (+/−)-naringenin, measurements were performed at Ca^{2+} concentrations of 50, 100 and 250 nM in the internal solution. Higher Ca^{2+} concentrations were not tested, in order to avoid myocyte contraction. Within the range considered, BK_{Ca} activity was dependent on free Ca^{2+} concentration. In fact, a fivefold change in intracellular Ca^{2+} concentration from 50 to 250 nM induced a significant change in current density recorded at 100 mV from a V_h of −40 mV (9.83±2.71 pA/pF, 50 nM Ca^{2+}, $n=6$, 31.00±6.27 pA/pF, 250 nM Ca^{2+}, $n=6$, $P<0.05$, Student's *t*-test for unpaired samples). As shown in Figure 7, however, no significant changes were observed in the efficacy of 100 μM (+/−)-naringenin when Ca^{2+} concentration varied in the internal solution.

Although present in the internal solution at 10 mM concentration, EGTA may be inadequate to buffer submembrane Ca^{2+} concentrations in such restricted spaces as those between the sarcoplasmic reticulum and the plasma membrane. The effect of (+/−)-naringenin was therefore tested in the presence of the fast Ca^{2+} chelator BAPTA, at 100 nM free Ca^{2+} concentration, in the internal solution. As shown in Figure 7, (+/−)-naringenin stimulation in the presence of BAPTA was not significantly different from that observed in the presence of EGTA.

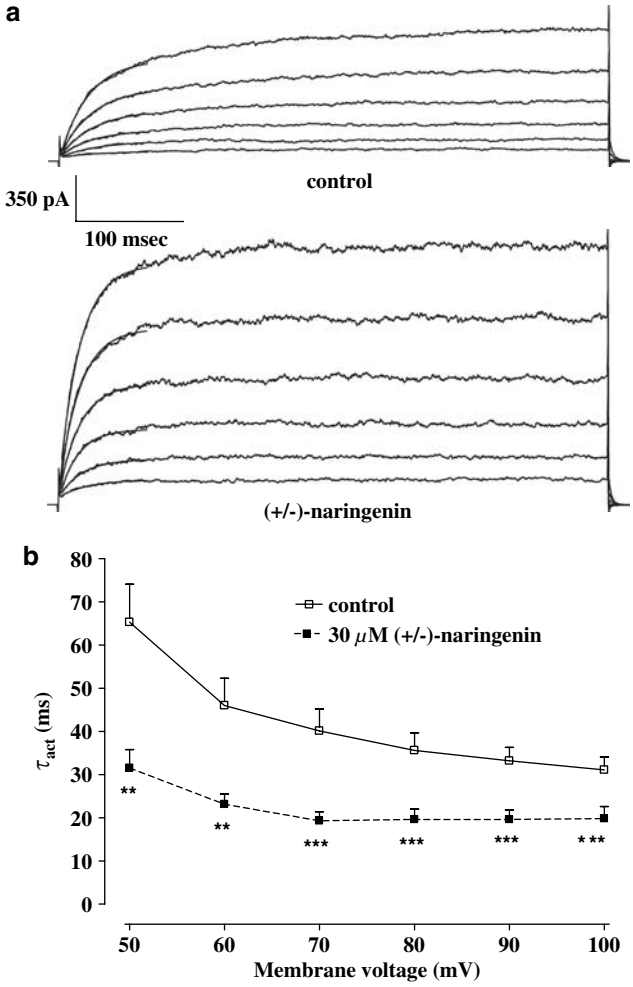

Figure 5 Effects of (+/−)-naringenin on the kinetics of activation of BK_{Ca} channels. (**a**) Original recordings and their curve fit (single exponential functions, smooth lines) of conventional whole-cell BK_{Ca} currents elicited with 500-ms voltage steps from a V_h of −40 mV to test potentials in the range 50–100 mV, measured in the absence (control) and in the presence of (+/−)-naringenin (30 μM). (**b**) Plot of the activation τ (τ_{act}) as a function of membrane potential in the absence (control) or presence of 30 μM (+/−)-naringenin. Data points are means±s.e.m. ($n=9$). **$P<0.01$, ***$P<0.001$, Student's *t* test for paired samples.

Discussion

Flavonoids exert several biological effects, in particular, their well-documented antioxidant activity (Rice-Evans *et al*., 1997; Aviram and Fuhrman, 1998) and vasodilatation (Duarte *et al*., 1993; Fitzpatrick *et al*., 1993; Herrera *et al*., 1996; Fusi *et al*., 2003a, b), which might explain the correlation between their intake in foodstuffs and a lower incidence of cardiovascular diseases in human populations (Hertog *et al*., 1993; Knekt *et al*., 1996). The vasodilatatory activity has been explained by various, and not yet completely understood mechanisms. The BK_{Ca} channel opener profile of apigenin, kaempferol and other related flavonoids, however, has been well documented in *Xenopus* oocytes expressing BK_{Ca} channels (Li *et al*., 1997).

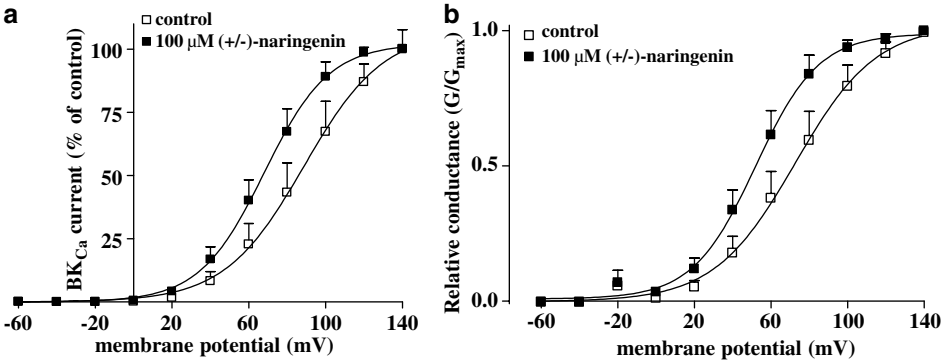

Figure 6 Effects of (+/−)-naringenin on BK_{Ca} current activation curve in rat tail artery myocytes. (**a**) Current–voltage relationships constructed, in the presence of low intracellular K^+ concentration (14 mM), before the addition of (control) and in the presence of 100 μM (+/−)-naringenin. On the ordinate scale, response is reported as the percentage of the current recorded at 140 mV under control conditions. (**b**) Activation curves were obtained directly from the current–voltage relationships of (**a**) and fitted to the Boltzmann equation (see Methods section). Data points represents the mean ± s.e.m. ($n = 5$).

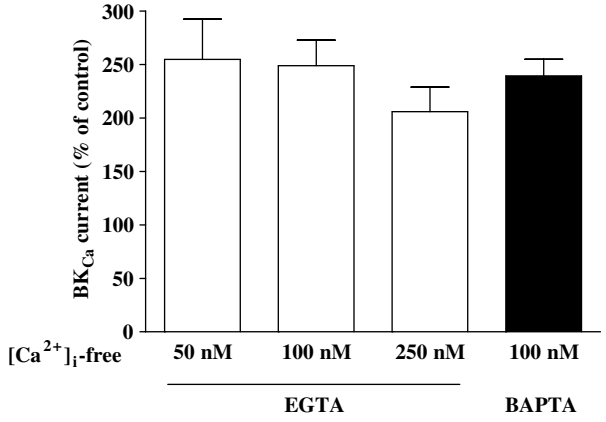

Figure 7 Efficacy of (+/−)-naringenin at various concentrations of intracellular Ca^{2+} as well as in the presence of BAPTA in rat tail artery myocytes. The stimulation by 100 μM (+/−)-naringenin of BK_{Ca} current was monitored in the presence of either EGTA (50, 100 and 250 nM free Ca^{2+}) or BAPTA (100 nM free Ca^{2+} in the internal solution). Currents, recorded during a typical depolarization from −40 to 100 mV, were normalized towards the current recorded just before (+/−)-naringenin application. Columns are means ± s.e.m. ($n = 5$–20).

As (+/−)-naringenin, a flavanone compound abundant in many edible plants of the genus *Citrus*, in particular, is a close structural analogue of apigenin, its potential BK_{Ca} channel stimulating property as well as that of its 7-β-neohesperidoside naringin, were investigated. In endothelium-denuded rat aorta rings, precontracted at a relatively slight depolarization value (20 mM KCl) or with the physiological agonist noradrenaline, (+/−)-naringenin induced an almost complete relaxation. Naringin, however, produced a much lower vasorelaxant effect. This confirms a previous study showing how the glycosilated flavonoids, rhoifolin and hesperidin, have vasorelaxing properties significantly lower than those exhibited by their corresponding aglycones apigenin and hesperetin (Calderone *et al*., 2004). We can conclude that the glycoside moiety reduces the vasorelaxing features of some flavonoids, although in preparations with intact endothelium this is not always observed (Fusi *et al*., 2003b). As the vasorelaxation by K^+ channel activators is strongly inhibited by high (e.g. 60 mM) KCl concentrations, owing to – *inter alia* – the decrease of the chemical K^+ gradient accounting for the outward flow of K^+, (+/−)-naringenin and naringin were also tested on 60 mM KCl precontracted vessels. Indeed, their vasorelaxant activity was markedly depressed at high concentrations of K^+ (60 mM KCl), a finding compatible with the pharmacodynamic pattern typical of the K^+ channel openers (Magnon *et al*., 1998). In a recent study on intact aorta rings, the vasorelaxant effect of (+/−)-naringenin was shown to be mediated, at least in part, by endothelium released nitric oxide (Ajay *et al*., 2003). In the same study, however, at high K^+ concentrations, a marked reduction of (+/−)-naringenin-induced vasorelaxation was also reported, in agreement with the pharmacodynamic profile proposed here. The release of nitric oxide from endothelium has been shown to be promoted by the pharmacological activation of endothelial BK_{Ca} channels (Kuhlmann *et al*., 2004). On the other hand, it is well-known that nitric oxide, in turn, is able to activate BK_{Ca} channels of the vascular smooth muscle cells (Bolotina *et al*., 1994). For these reasons, the model of 'endothelium-denuded' vessels was preferred in the present study as a simpler and more direct means for assessing K^+ channel activation by flavonoids in myocytes.

In the present study, both (+/−)-naringenin and naringin exhibited vasorelaxing properties, apparently due to the activation of K^+ channels; the full characterization of this action, however, has been performed only with (+/−)-naringenin, owing to its greater activity. The endothelium-independent, vasorelaxant action of (+/−)-naringenin, was inhibited by 10 mM tetraethylammonium, a concentration shown to block several types of K^+ channels. Under these conditions, a marked rightward shift of the concentration–response curve, as well as a reduction of the maximum effect of (+/−)-naringenin, were observed. As 10 mM tetraethylammonium cannot be considered a selective blocker of BK_{Ca} channels, the pharmacological analysis was extended to the use of other K^+ channel blocking agents. The fact that glibenclamide did not modify significantly the vasorelaxing effect of (+/−)-naringenin indicates that ATP-sensitive K^+

channels seem not to play a role. On the contrary, the fact that 4-aminopyridine, a blocker of voltage-operated K^+ channels, antagonized (+/−)-naringenin-induced vasorelaxation, suggests a role for these channels in the mechanism of action of the flavonoid. It is worth noting that 4-aminopyridine is capable also of inducing a reversible inhibition of BK_{Ca} currents in freshly isolated rat tail artery myocytes (Petkova-Kirova *et al*., 2000).

Finally, the involvement of BK_{Ca} channels was clearly established with the use of the selective BK_{Ca} channel blocker iberiotoxin, which, in fact, caused a significant parallel rightward shift of the concentration–response curve of (+/−)-naringenin, typical of a competitive, reversible antagonist. Although recently the vasorelaxing effect of (+/−)-naringenin was reported not to be influenced by both high depolarization values and by tetraethylammonium (Orallo *et al*., 2005), thus contradicting the involvement of BK_{Ca} channels, the present electrophysiological data clearly confirm the mechanical findings, showing that (+/−)-naringenin significantly increased, in a concentration-dependent manner, the large iberiotoxin-sensitive K^+ outward current. (+/−)-Naringenin-stimulated current was inhibited by both iberiotoxin and tetraethylammonium, the latter used at concentrations specific for BK_{Ca} channels. These data provide compelling evidence that the K^+ current recorded in the presence of (+/−)-naringenin was largely sustained by BK_{Ca} channels. Moreover, this effect of (+/−)-naringenin effect was reversed by wash-out of the drug.

(+/−)-Naringenin-induced current stimulation was comparable to that operated by naringin, although the latter is a much less effective vasorelaxant. This observation suggests that the glycoside moiety, restraining naringenin vasorelaxing activity, plays a key role in the diffusion of the flavonoid into the whole tissue without, however, affecting its modulation of the channel protein in single isolated myocytes.

BK_{Ca} channels are Ca^{2+}- and voltage-activated, although they have been shown to become independent of intracellular Ca^{2+} at concentrations lower than 100 nM, turning into a purely voltage-gated mechanism (Meera *et al*., 1996). However, the data presented here demonstrate that rat tail artery myocyte BK_{Ca} channels are indeed Ca^{2+}-dependent even at concentrations as low as 50 nM. Whether (+/−)-naringenin stimulation of BK_{Ca} channels activity was mediated by the global increase of intracellular Ca^{2+} concentration was assessed by applying various concentrations of Ca^{2+} in the internal solution containing EGTA. (+/−)-Naringenin stimulated activity of BK_{Ca} channels was unaffected by increasing intracellular Ca^{2+} over a wide range of concentrations (50–250 nM). It is widely recognized, however, that a local, sub-plasmalemmal increase in Ca^{2+} concentration, in the close proximity of the internal mouth of the channel, activates BK_{Ca} channels, giving rise to the so-called STOCs (spontaneous transient outward currents; Jaggar *et al*., 1998). These latter currents seem to be elicited by Ca^{2+} sparks released from intracellular Ca^{2+} stores via ryanodine receptors, although a Ca^{2+} influx via Ca_V 1.2 channels may also contribute to the phenomenon. Our electrophysiological recordings, however, were performed in the presence of nicardipine in the recording solution, thus ruling out the latter hypothesis. Furthermore, when the fast Ca^{2+} chelator BAPTA replaced EGTA in the internal solution, (+/−)-naringenin still fully stimulated BK_{Ca} currents. The apparent independence of BK_{Ca} channel stimulation induced by (+/−)-naringenin on global as well as local, intracellular Ca^{2+} concentration might be consistent with a direct effect on the channel protein.

The faster rate of activation of BK_{Ca} channels observed in the presence of (+/−)-naringenin indicates that the flavonoid speeds up the transition from the closed to the open state of the channel, or in some other way modifies its gating mechanisms by interacting directly with the channel protein. The latter hypothesis is supported by the activation curve shift towards more hyperpolarizing potentials observed with (+/−)-naringenin. In addition, (+/−)-naringenin caused a significant change in the slope of the activation curve. These results indicate that (+/−)-naringenin may alter the voltage sensitivity of the channel activation mechanism.

A peculiar feature of stimulation by (+/−)-naringenin is its sensitivity to the intracellular K^+ concentration. When internal K^+ was reduced from 90 to 14 mM, the efficacy of the flavonoid was markedly decreased. A decreased activity, under conditions of symmetrical K^+ concentrations, has already been shown for some blockers of K^+ channels and interpreted to be the effect of the K^+ gradient exerting a modulatory action on the stability of the states, that is, on channel gating (Trequattrini *et al*., 1998).

We can conclude that, among the various mechanisms, which account for the vasorelaxant activity of (+/−)-naringenin, the activation of BK_{Ca} channels seems to be most likely. This appears to be the first observation of the reversible effect of this compound on BK_{Ca} channel in vascular smooth muscle cells. Moreover, preliminary data from this laboratory proved that quercetin, an ubiquitous flavonoid, increases BK_{Ca} currents in vascular myocytes (unpublished observation). Therefore, taken together, all these elements indicate that naturally occurring polyphenols might represent a new class of vascular BK_{Ca} channel openers. This theory provide a molecular mechanism that might explain the lower incidence of cardiovascular diseases associated with a flavonoid-rich diet, distinctive of Mediterranean populations (Hertog *et al*., 1993; Knekt *et al*., 1996), that has been ascribed to the vasorelaxing properties of natural polyphenols. Further experiments, however, are needed in order both to elucidate the direct or indirect mechanism through which (+/−)-naringenin affects channel activity and to clarify the possible involvement of K_{Ca} 1.1 channels.

As a result of their BK_{Ca} channel opening property, (+/−)-naringenin, naringin and, possibly, other structurally related flavonoids, might be considered therapeutic tools for treating patients suffering from both hypertension and impaired vasodilating capacity. Additionally, the flavonoid moiety may represent the template, for the design and synthesis of novel BK_{Ca} channel openers, (+/−)-naringenin being a lead compound in this field. Further development of the naringenin molecule is essential in view of the powerful inhibition of human gut and liver CYP3A4 by ingested naringin and (+/−)-naringenin, which makes their clinical

use almost impossible, owing to the subsequent, marked modification of the kinetics of concomitantly ingested drugs (Wilkinson, 2005).

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Conflict of interest

The authors state no conflict of interest.

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