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ORIGINAL CONTRIBUTION

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Effects of quercetin and rutin on vascular preparations

A comparison between mechanical and electrophysiological phenomena

Summary *Background* Several studies have indicated that quercetin promotes relaxation of vascular smooth muscle both *in vivo* and *in vitro*. However, Saponara et al. [(2002) Br J Pharmacol 135: 1819–1827] have demonstrated that quercetin is an activator of vascular L-type Ca^{2+} channels. *Aim of the study* We investigated the mechanical and electrophysiological properties of quercetin and its rutoside, rutin, in an attempt to clarify how Ca^{2+} channel activation might be related to the myorelaxing activity. *Methods* Aorta ring preparations and single tail artery myocytes were employed for functional and patch-clamp experiments, respectively. *Results* Rutin was found to relax intact rat aorta rings, which had been precontracted with phenylephrine ($pIC_{50} = 5.65 \pm 0.31$) but in contrast had no effect on depolarised (60 mM K^+) preparations or on those from which the endothelium had been removed. Furthermore, rutin did not affect L-type Ca^{2+} current recorded in rat tail artery myocytes. The quercetin-induced relaxation of intact rings precontracted with phenylephrine exhibited two components characterised by 6.23 ± 0.38 and 4.66 ± 0.09 pIC_{50}, respectively. Removal of the endothelium abolished the first component, leaving the second unaltered. Moreover, quercetin was found to relax 60 mM K^+ depolarised rings with a pIC_{50} of 4.59 ± 0.03. The application of quercetin in isolated smooth muscle cells brought about a marked increase of L-type Ca^{2+} current ($pEC_{50} = 5.09 \pm 0.05$). Unlike quercetin, Bay K 8644 contracted aorta rings preincubated with 10, 20 or 30 mM K^+. The myotonic effect of Bay K 8644 was observed both in the absence or presence of 30 μM quercetin. The application of Bay K 8644 (10–100 nM) caused a further significant increase in L-type Ca^{2+} current in rat tail artery myocytes stimulated with 30 μM quercetin. *Conclusions* Quercetin is a naturally occurring L-type Ca^{2+} channel agonist. This effect, however, is overwhelmed by quercetin-induced vasorelaxation taking place *via* pathways which are more relevant than L-type Ca^{2+} influx in the hierarchy of functional competencies.

Key words patch-clamp – quercetin – rutin – rat vascular smooth muscle – wine polyphenols

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Introduction

It is well known that phenols, as hydrogen donors react with lipid radicals and form stable phenoxy radicals acting as effective chain-breaking antioxidants [1]. 3-*t*-Butyl-4-hydroxyanisole (BHA), a synthetic phenolic antioxidant widely used as a stabiliser for fats, oils and lipid-containing foods, has recently been reported to exert an antispasmogenic effect on gastrointestinal and vascular smooth muscle preparations, as have certain other hindered phenols [2–5]. This effect might be at least partly attributed to the inhibition of L-type Ca^{2+} channels [6, 7].

Radical scavengers capable of limiting the Ca^{2+} influx into cells might represent effective drugs in preventing tissue damage from ischemia-reperfusion injury [2].

Food phenolics represent the natural equivalents of synthetic antioxidants. Quercetin, for instance, occurs in our diet either as aglicon or as a gluco/glycosilated

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metabolite, the sugar moiety modulating its effect on the vascular system [8–11]. Furthermore, it modifies eicosanoid biosynthesis (antiprostanoid and anti-inflammatory responses), protects low-density lipoprotein from oxidation (thus preventing atherosclerotic plaque formation), prevents platelet aggregation (antithrombic effects), and promotes relaxation of vascular smooth muscle (antihypertensive effect) [12], while red wine extracts strongly inhibit the synthesis of endothelin-1 [13], a vasoactive peptide that is crucial in the development of coronary atherosclerosis. In addition, oral administration of red wine polyphenolic compounds has recently been shown to produce antihypertensive effects in spontaneously hypertensive rats [14], to decrease blood pressure in normotensive rats [15] as well as to increase nitric oxide production in rat aorta [16]. Furthermore, Martin et al. [17] have recently defined the mechanisms of endothelial NO production caused by wine polyphenols including the increase in intracellular Ca^{2+} and the activation of tyrosine kinases.

During the last two decades, it has been shown that quercetin is capable of inducing both endothelium-dependent [18, 19] and endothelium-independent vasorelaxation *in vitro* [8–10, 20]. Surprisingly enough, however, Saponara et al. [21] have recently demonstrated that quercetin is an activator of vascular L-type Ca^{2+} channels. Since this effect hardly correlates to quercetin-induced vasorelaxation, we investigated the effects of quercetin and its rutoside on vascular functions *in vitro* by comparing their mechanical and electrophysiological actions.

Materials and methods

Aorta rings preparation

Aorta rings (1.5-mm wide) from male Wistar rats (250–350 g) were prepared as described elsewhere [5].

Equilibration period

The rings were allowed to equilibrate for 1 h at a resting tension of 1 g. During this equilibration period, a modified Krebs-Henseleit physiological salt solution (PSS) (see below for composition) was changed every 15 min and the passive tension was re-adjusted to 1 g. Following the equilibration period, the rings were stimulated with 60 mM K^+ PSS until a sustained response was obtained (~15 min) in order to test their contractile capacity. Upon conclusion of a 30-min washout period, the presence of functional endothelium was assessed with 10 μM acetylcholine in rings precontracted with 0.3 μM phenylephrine. Under these conditions, maximal plateau values for active tension of 392.2 ± 20.2 ($n = 18$) (unrubbed rings, with intact endothelium) and 460.2 ± 12.5 mg ($n = 20$; $P < 0.01$) (rubbed rings, without endothelium) were obtained. The rings were then washed and equilibrated for another 30–60-min period before testing the various experimental settings (see below). The control preparations were treated with the vehicle only.

The effects of flavonoids on rat aorta rings

The relaxation produced by quercetin and rutin was assessed in unrubbed or rubbed rings precontracted with a submaximal (90% of maximum) concentration of phenylephrine (0.3 μM) or in rubbed rings precontracted with 60 mM K^+. Cumulative concentration-response curves for quercetin and rutin were made. Each concentration was left in contact with the ring for long enough to allow full development of the effect. Relaxation was then evaluated as a percentage of the initial tension.

In a second series of experiments, the cumulative concentration-response relationship for quercetin or for Bay K 8644 was determined in rubbed tissues bathed with various concentrations of K^+ (ranging from 10 to 30 mM). The effect of Bay K 8644 was also tested on rubbed depolarised ring preparations preincubated with 30 μM quercetin.

Cell isolation procedure

Smooth muscle cells, freshly isolated from the tail main artery of male rats (350–450 g) by means of collagenase treatment, as previously described [7], exhibited an ellipsoid form (20–30 μm in width, 70–110 μm in length). The cells were continuously superfused with external solution using a peristaltic pump (LKB 2132), at a flow rate of 800 μl/min. Electrophysiological responses were tested at room temperature (22–24 °C) only in those cells that were phase dense. Cell membrane capacitance averaged out at 47.1 ± 2.8 pF ($n = 19$).

Whole-cell patch clamp recording

Conventional whole-cell patch-clamp method [22] 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 internal solution. A low-noise, high-performance Axopatch 200B (Axon Instruments, USA) patch-clamp amplifier, driven by an IBM computer in conjunction with an A/D, D/A board (DigiData 1200 A/B 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 and series resistance, were low-pass filtered at 1 kHz and digitised at 3 kHz prior to being stored on the computer hard disk. Long-lasting nifedipine-blockable inward currents passing through L-type Ca^{2+} channels [$I_{Ca(L)}$], in 5 mM Ca^{2+} -containing external solution, were measured over a range of test potentials (250 ms) from –55 mV to 50 mV from a holding potential (V_h) of –50 mV. Data were collected once the current amplitude had been stabilised (usually 8–10 min after the whole-cell configuration had been obtained). The $I_{Ca(L)}$ did not run down during the following 30 to 40 min under these conditions.

Potassium currents were blocked with 30 mM tetraethylammonium in the external solution and Cs^+ in the internal solution. Values were corrected for leakage using 300 μM Cd^{2+}, which was assumed to completely block $I_{Ca(L)}$.

Solutions and chemicals

Modified Krebs-Henseleit PSS containing (in mM) 124 NaCl, 4 KCl, 1.8 $CaCl_2$, 1.1 $MgCl_2$, 0.4 KH_2PO_4, 25 $NaHCO_3$ and 5.5 glucose, and PSS containing KCl at a concentration of more than 4 mM (prepared by replacing NaCl with equimolar KCl) bubbled with a 95 % O_2 – 5 % CO_2 gas mixture to create a pH of 7.4, were used throughout.

The Ca^{2+}-free external solution contained (in mM) 110 NaCl, 5.6 KCl, 10 HEPES, 20 taurine, 20 glucose, 1.2 $MgCl_2$ and 5 Na-pyruvate (pH 7.4).

The internal solution (pCa 8.4) consisted of (in mM) 100 CsCl, 10 HEPES, 11 EGTA, 2 $MgCl_2$, 1 $CaCl_2$, 5 Na-pyruvate, 5 succinic acid, 5 oxalacetic acid, 3 Na_2ATP and 5 phosphocreatine; pH was adjusted to 7.4 with CsOH.

The osmolarity of the external solution was adjusted to 335 mosmol and that of the internal solution to 310 mosmol by means of an osmometer (Osmostat OM 6020, Menarini Diagnostics, Italy).

The chemicals used included: collagenase (type XI), trypsin inhibitor, bovine serum albumin, tetraethylammonium, Nω-nitro-L-arginine methyl ester (L-NAME), quercetin, rutin, Bay K 8644, and nifedipine (Sigma Chimica, Italy). Quercetin and rutin dissolved directly in dimethyl sulphoxide (DMSO) and nifedipine or Bay K 8644 dissolved in ethanol were diluted at least 1,000 times prior to use.

The resulting concentrations of DMSO and ethanol (below 0.1 %) failed to alter the response of the preparations (data not shown). Final drug concentrations are stated in the text.

The water used in the solutions was first distilled and then passed through a NANOpure II deionisation system (Barnstead-Sybron, Boston, USA), to obtain Type I Reagent Grade water (resistivity 18 MΩ).

Curve fitting and statistics

The acquisition and analysis of data were performed using pClamp 8.1.0.12 software (Axon Instruments, USA) and GraphPad Prism version 3.02 (GraphPad Software, USA). Data are reported as means ± SEM; the number of cells analysed from *n* animals (indicated in parentheses) has also been reported. Statistical analyses and significance as measured by ANOVA (followed by Dunnett's post test) or the Student's *t* test for paired or unpaired samples, were obtained using GraphPad InStat version 3.05 (GraphPad Software, USA) as appropriate. In all comparisons, $P < 0.05$ was considered significant.

The current-voltage relationships were calculated on the basis of the peak values (leakage corrected) from the original currents.

The pharmacological response to each substance was described in terms of pEC_{50} or pIC_{50} (the negative logarithm to base 10 of the EC_{50} or IC_{50}, respectively).

Results

Effects of flavonoids on aorta rings

The cumulative addition of rutin relaxed intact rat aorta rings precontracted with phenylephrine with pIC_{50} of 5.65 ± 0.31 ($n = 3$–4) (Fig. 1a). This effect depended on the intactness of the endothelium, since it was no longer evident in the rubbed rings, or suppressed by preincubation of tissues with 100 μM L-NAME. The addition of rutin failed to produce any effect up to the maximum concentration tested (100 μM) in rings depolarised with 60 mM K^+.

The effects of quercetin on aorta rings were different from those produced by rutin. In fact, the quercetin-induced relaxation of intact rings precontracted with phenylephrine exhibited two components characterised by 6.23 ± 0.38 and 4.66 ± 0.09 pIC_{50} ($P < 0.001$, Student's *t* test for unpaired samples; $n = 10$), respectively (Fig. 1b). The removal of the endothelium ($pIC_{50} = 4.66 \pm 0.07$, $n = 5$–6) as well as preincubation with 100 μM L-NAME ($pIC_{50} = 4.62 \pm 0.07$, $n = 3$–4) abolished the first component, leaving the second unaltered. Furthermore, quercetin was observed to relax rings depolarised with 60 mM K^+ displaying a pIC_{50} of 4.59 ± 0.03 ($n = 7$).

Effects of flavonoids on $I_{Ca(L)}$

As shown in Fig. 2a, 100 μM rutin did not affect $I_{Ca(L)}$. On the contrary, 30 μM quercetin when applied to single smooth muscle cells, caused a significant and sustained increase of $I_{Ca(L)}$ (Fig. 2b). Quercetin increased $I_{Ca(L)}$ in a concentration-dependent manner (Fig. 2c) with a pEC_{50} of 5.09 ± 0.05 (3–7 cells, $n = 4$). The maximum stimula-

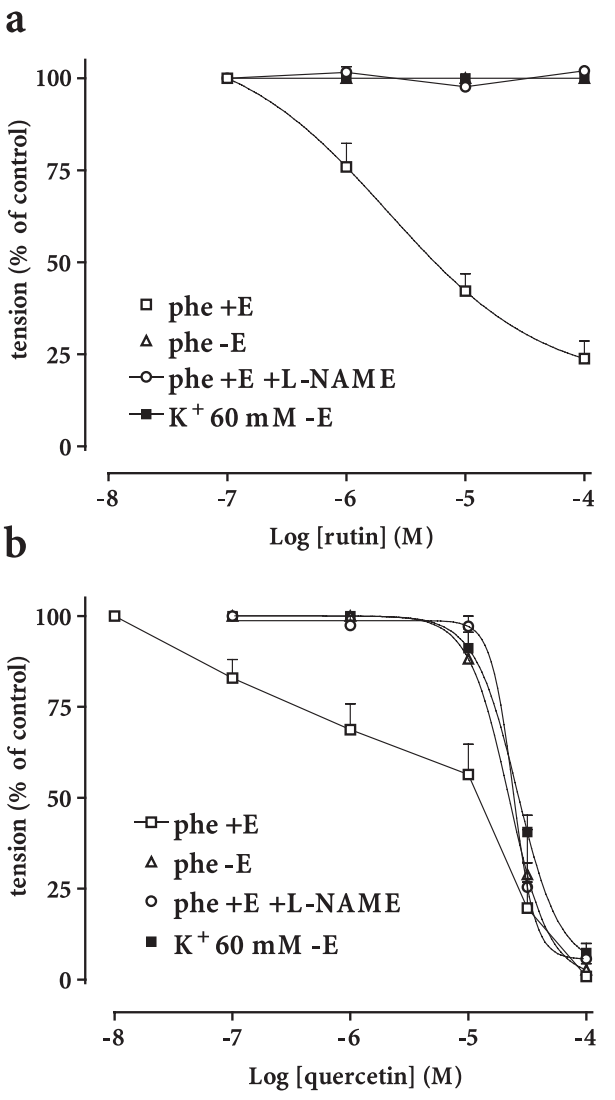

Fig. 1 Effects of quercetin and rutin on rat aorta rings. Concentration-relaxation curves for rutin (**a**) and quercetin (**b**) either in the absence (–E) or presence of functional endothelium (+E), and with functional endothelium preincubated for 15 min with 100 μM L-NAME. Rings were precontracted either with 0.3 μM phenylephrine or with 60 mM K^+ . Ordinate scale, relaxation is reported as a percentage of the initial tension induced by phenylephrine or K^+ (control). Data points are means ± SEM (n = 3–10). pIC_{50} values of the two components of quercetin-induced relaxation were calculated normalising the endothelium-dependent and -independent components to 0–100 % of the effect

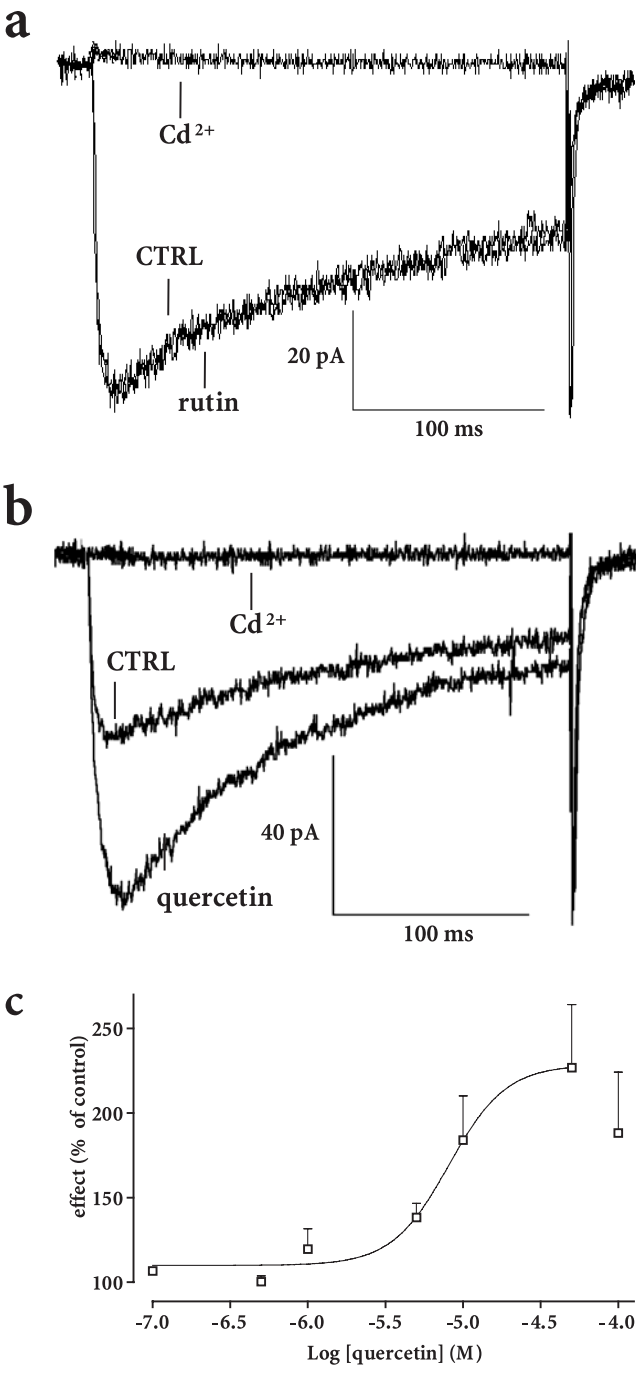

Fig. 2 Effects of rutin and quercetin on $I_{Ca(L)}$. **a, b** Original recordings of conventional whole-cell $I_{Ca(L)}$ in rat tail artery myocytes elicited with 250 ms depolarising voltage-clamp pulses from a V_h of –50 mV to test potentials of +5 (**a**) or +10 mV (**b**) measured in the absence (CTRL) or presence of 100 μM rutin or 30 μM quercetin, respectively. $I_{Ca(L)}$ suppression by 300 μM Cd^{2+} is also shown. **c** Concentration-dependent effect of quercetin on $I_{Ca(L)}$. On the ordinate scale, response is reported as a percentage of control. The curve shows the best fit of the points (pEC_{50} = 5.09 ± 0.05), excluding that at concentration higher than 50 μM. Data points are means ± SEM of 3 to 7 cells (n = 4)

tion was observed at 50 μM quercetin with higher concentrations proving less effective.

Effect of K^+ on aorta ring response to quercetin and Bay K 8644

The effects of quercetin on aorta rings were compared to those produced by Bay K 8644, a well-characterised L-type Ca^{2+} channel activator [23]. Application of either

quercetin (at concentrations of 1 to 100 µM) or Bay K 8644 (1, 10 and 100 nM) to rat aorta rings failed to induce mechanical responses. When the preparations were incubated in PSS containing levels of K^+ above that of PSS (10, 20 or 30 mM, respectively), contractions to Bay K 8644, which depended on the concentration of K^+, were obtained (Fig.3a–c). On the contrary, quercetin did not cause any mechanical response, but rather tended to relax the preparations (data not shown).

Pharmacological interaction between Bay K 8644 and quercetin

In order to test for pharmacological interactions between Bay K 8644 and quercetin, the effects of Bay K 8644 were studied in either aorta rings or tail artery myocytes pretreated with quercetin.

The application of Bay K 8644 (1–100 nM) to aorta preparations preincubated with 30 µM quercetin as well as bathed with 10, 20 or 30 mM K^+ brought about mechanical responses that were not significantly different from those obtained in the absence of quercetin (Fig. 3a–c).

Quercetin (30 µM) caused a significant increase of $I_{Ca(L)}$ in rat tail artery myocytes (Fig. 4). The subsequent addition of Bay K 8644 (10 and 100 nM) on the plateau of the quercetin effect induced a significant additional increase of the current.

Discussion

Red wine components (e. g. quercetin) have become a matter of particular health concern and scientific investigation ever since the French paradox was first presented [24]. Previous studies have investigated the effects of quercetin on vascular functions. Duarte et al. [9] demonstrated that quercetin produced a vasodilator effect in isolated rat aorta that had been attributed to the inhibition of protein kinase C. Protein kinase C inhibition by quercetin is furthermore independent of Ca^{2+} [25]. The data on aorta ring preparations presented here are in agreement with those presented by Duarte et al. [9]. We have also demonstrated that, at low concentrations, quercetin is capable of eliciting an endothelium-dependent relaxation, in agreement with what already observed by other authors [18, 19]. This could well reflect the activation of Ca^{2+} channels (i. e. Ca^{2+} influx) in the endothelial cells [26], which, in turn, activates NO synthase. Therefore, it is conceivable that the biphasic effect observed on intact aorta rings results hierarchically from quercetin effect on the endothelium. At low concentrations (< 10 µM) the endothelial target predominates, while at higher concentrations (≥ 10 µM) its effect on smooth muscle prevails. Therefore this effect of

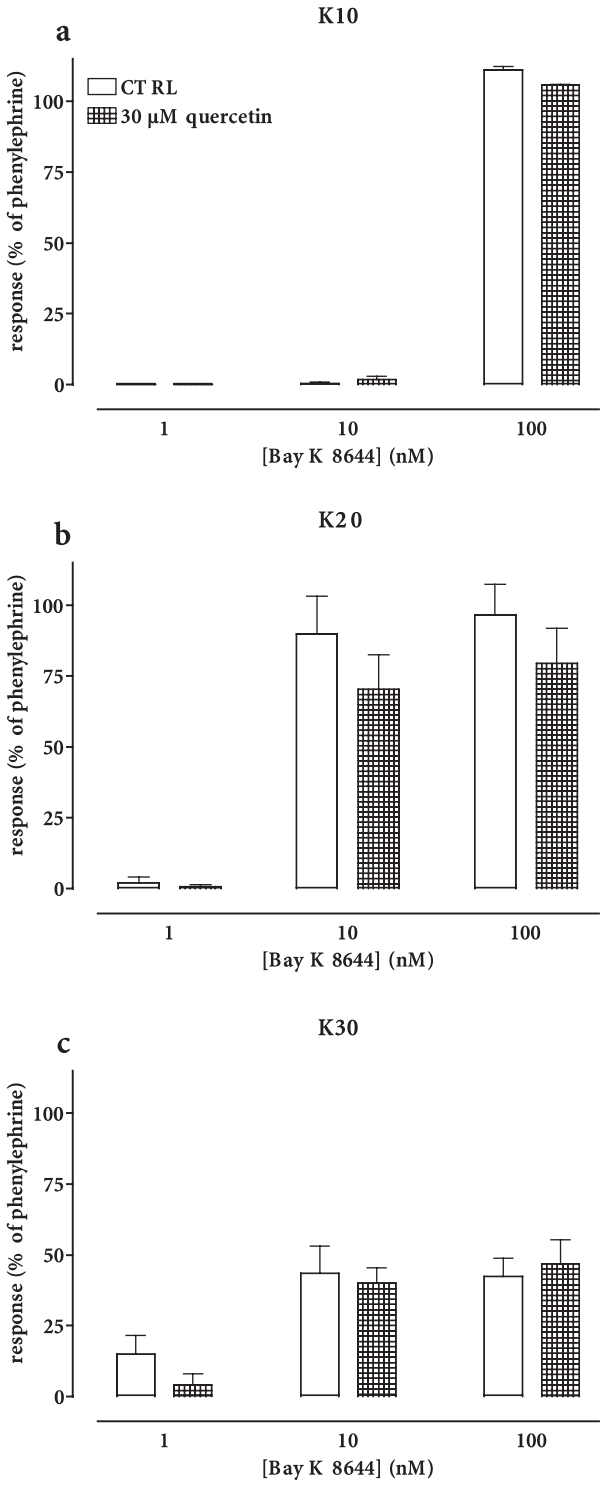

Fig. 3 Effect of quercetin on aorta ring contractile responses to Bay K 8644 in the presence of various K^+ concentrations (mM): **a** 10; **b** 20; **c** 30. Concentration-dependent responses to Bay K 8644 were determined in K^+ depolarised rubbed aorta rings either in the absence (CTRL) or in the presence of 30 µM quercetin. Responses (%) were calculated with respect to 0.3 µM phenylephrine. The columns represent means ± SEM of 3 to 5 preparations (n = 3–5)

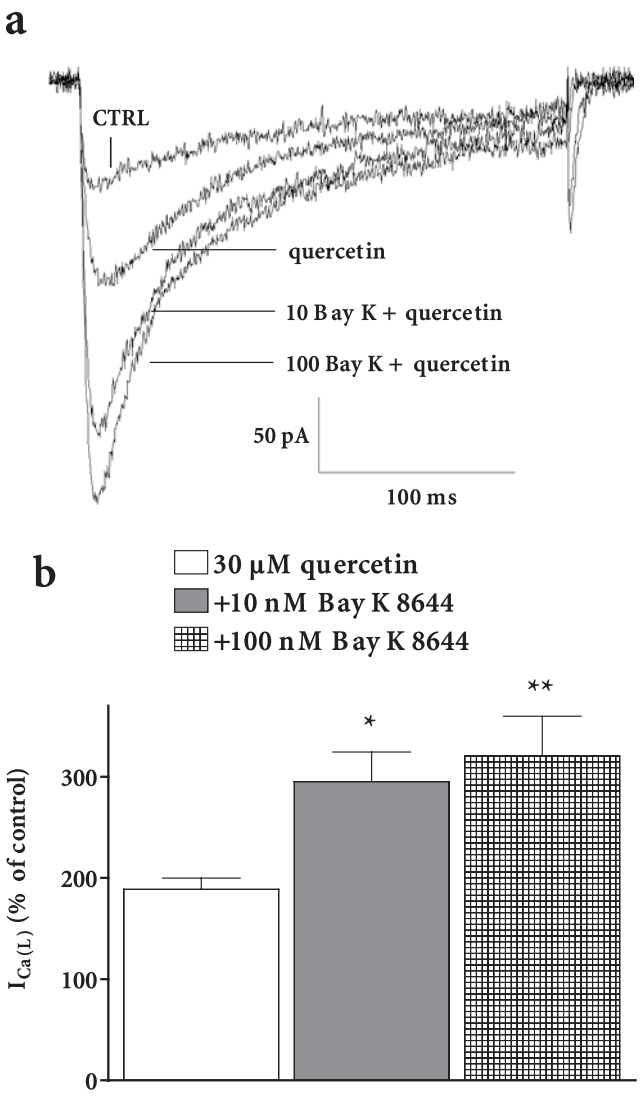

Fig. 4 The effect of Bay K 8644 on $I_{Ca(L)}$ in rat tail artery myocytes pretreated with quercetin. **a** Original recordings of conventional whole-cell $I_{Ca(L)}$ in rat tail artery myocytes elicited with 250 ms depolarising voltage-clamp pulses from a V_h of –50 mV to test potentials of +10 mV measured in the absence (CTRL) or presence of 30 µM quercetin, quercetin plus 10 or 100 nM BayK 8644, respectively. Bay K 8644 was applied to cells where the response to quercetin had reached a steady value. **b** Responses (%) were calculated with respect to the control (i. e. before the addition of quercetin). The columns represent means ± SEM of 6 to 8 cells ($n = 5$). * $P < 0.05$, ** $P < 0.01$, Dunnett's post test

quercetin, together with that of rutin, should be taken into account when considering the effects of flavonoids on the vascular system since sub-µM or µM concentrations of these substances (either as aglycon or as glucosilate/glycosilate metabolite) are expected in plasma of humans [27–29]. Furthermore, the rate of elimination of quercetin is relatively low and, therefore, repeated consumption of quercetin-containing foods might cause accumulation of quercetin in blood [30] at levels very similar to those found to be active in the present study.

Quercetin, unlike rutin, was shown in our study to antagonise K^+-induced contraction in a concentration-dependent manner. K^+-induced contraction is the result of an increased Ca^{2+} influx through voltage-dependent Ca^{2+} channels and is specifically inhibited by Ca^{2+}-antagonists. Therefore, the inhibition of K^+-induced contraction by quercetin might be interpreted as a consequence of the blockade of voltage-dependent Ca^{2+} channels, as previously hypothesised by others [8]. Surprisingly enough, however, the electrophysiological data presented here showed a clear, potent activation of $I_{Ca(L)}$ by quercetin (see also [21]). In a recent paper, Summanen et al. [31] demonstrated that quercetin increases I_{Ca} in clonal rat pituitary GH_4C_1 cells, possibly via a cAMP-induced activation of protein kinase A. However, in vascular smooth muscle, both cAMP and its related kinase have been shown to inhibit L-type Ca^{2+} channels [32]. Therefore, the quercetin-induced activation of $I_{Ca(L)}$ observed here should not be attributed to changes in the intracellular levels of this cyclic nucleotide.

Ca^{2+} channel activators, e. g. Bay K 8644 and FPL 64176, evoke contractile tonic responses, although only in vascular smooth muscle preparations depolarised with low concentrations of K^+ [33]. This feature is characteristic of such substances and may be explained by the fact that channel activation is voltage-dependent, therefore Ca^{2+} channels have to be activated in order to respond to Ca^{2+}-agonist drugs. In fact, in this paper, Bay K 8644 elicited a contractile response under the above-mentioned experimental conditions. On the contrary, quercetin failed to evoke a contractile response but rather relaxed depolarised rings. In other words, L-type Ca^{2+} channel activation by quercetin determined an influx of Ca^{2+} from the extracellular space, which was not sufficient to overcome the myorelaxing mechanism subsequent to protein kinase C inhibition. This hypothesis was supported by the observation that the further activation of channels by Bay K 8644 (i. e. larger influx of Ca^{2+} from the extracellular space), despite the presence of 30 µM quercetin, was capable to antagonise the myorelaxing effect of the flavonoid, as previously observed in rabbit ear arteries [34]. As such, our observations might explain the discrepancy between electrophysiological and mechanical data and suggest that the myorelaxing effect of quercetin in tissue preparations originates from its interaction with protein kinase C (beyond the Ca^{2+} channel) which hierarchically prevails over the increase in the Ca^{2+} influx to be expected from $I_{Ca(L)}$ stimulation. Under conditions where Ca^{2+} channel activation was maximal, however, as in the presence of Bay K 8644, Ca^{2+} influx from the extracellular space prevailed over protein kinase C inhibition thus causing smooth muscle contraction, despite the presence of quercetin. Experiments performed with intact tissues, therefore, seem to indicate the existence of a threshold in Ca^{2+} influx over which protein kinase C inhibition be-

comes secondary and muscle contraction may occur. At present, however, we can not rule out that Bay K 8644 also contracts vascular tissues preincubated with quercetin by directly stimulating protein kinase C activity; the latter effect, in fact, has been observed in isolated lung epithelial type II cells [35].

Rutin, on the contrary, had only one effect in common with quercetin, lying in its ability to evoke an endothelium-dependent relaxation of aorta rings. Moreover, rutin was ineffective on K^+-induced contraction as well as on $I_{Ca(L)}$. The lower liposolubility of rutin, perhaps originating from its glycoside polar group, might account for its ineffectiveness towards $I_{Ca(L)}$.

In conclusion, quercetin is a natural flavonoid exerting pleyotipic effects on vascular smooth muscle. The comparison between the electrophysiological and mechanical properties of quercetin alone, as well as quercetin and Bay K 8644 presented here, indicates that quercetin-induced vasorelaxant mechanisms are more relevant than the increase in Ca^{2+} influx to be expected from L-type Ca^{2+} channel activation in the hierarchy of functional competences.

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