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Electrophysiology, molecular modelling, and functional analysis of the effects of dietary quercetin and flavonoid analogues on $K_{ir}6.1$ channels in rat stomach fundus smooth muscle

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ABSTRACT

Flavonoids, ubiquitously distributed in the plant world, are regularly ingested with diets rich in fruit, vegetables, wine, and tea. During digestion, they are partially absorbed in the stomach. The present work aimed to assess the *in vitro* effects of quercetin and ten structurally related flavonoids on the rat gastric fundus smooth muscle, focussing on ATP-dependent K^+ ($K_{ir}6.1$) channels, which play a central role in the regulation of resting membrane potential, membrane excitability and, consequently, of gastric motility. Whole-cell currents through $K_{ir}6.1$ channels ($I_{Kir6.1}$) were recorded with the patch-clamp technique and the mechanical activity of gastric fundus smooth muscle strips was studied under isometric conditions. Galangin ≈ tamarixetin > quercetin > kaempferol > isorhamnetin ≈ luteolin ≈ fisetin > (±)-taxifolin inhibited pinacidil-evoked, glibenclamide-sensitive $I_{Kir6.1}$ in a concentration-dependent manner. Morin, rutin, and myricetin were ineffective. The steric hindrance of the molecule and the number and position of hydroxyl groups on the B ring played an important role in the activity of the molecule. Molecular docking simulations revealed a possible binding site for flavonoids in the C-terminal domain of the $K_{ir}6.1$ channel subunit SUR2B, in a flexible loop formed by residues 251 to 254 of chains C and D. Galangin and tamarixetin, but not rutin relaxed both high K^+- and carbachol-induced contraction of fundus strips in a concentration-dependent manner. Furthermore, both flavonoids shifted to the right the concentration-relaxation curves to either pinacidil or L-cysteine constructed in strips pre-contracted by high K^+, rutin being ineffective. In conclusion, $I_{Kir6.1}$ inhibition exerted by dietary flavonoids might counterbalance their myorelaxant activity, affect gastric accommodation or, at least, some stages of digestion.

1. Introduction

K^+ channels regulate a wide variety of cell functions including muscle contraction, thus representing a fundamental therapeutic target [1]. In particular, ATP-dependent K^+ ($K_{ir}6.x$) channels represent an important therapeutic target: both activators and inhibitors of the channel, in fact, are in clinical use. A study by Li et al. [2], using synthetic molecules as activators, and sulfonylureas (glibenclamide) as inhibitors suggests that the smooth muscle $K_{ir}6.1$ channel plays an important role in modulating gastrointestinal motility in dogs, particularly gastric tone and accommodation. Furthermore, $K_{ir}6.x$ channels expressed in murine colonic smooth muscle cells regulate resting membrane potential, excitability, and contractility [3].

Flavonoids are secondary metabolites widely distributed in the plant kingdom. Abundantly present, either as glycosides or aglycones, in fruit, vegetables, wine, and tea, flavonoids are ingested daily with the diet. The gastrointestinal tract, physically and chemically modifying food to make it available for absorption, distribution, metabolism, and excretion, plays a central role in shaping the beneficial health effects of this class of nutraceuticals [4]. Their bioavailability in the gastrointestinal tract can be influenced by structure, interactions with food matrices, the presence of gastric hydrolytic enzymes and bacterial flora [5]. Flavonoids can exert their biological actions systemically and/or locally targeting, for example, different gastrointestinal tract cell types. More importantly, they can modulate gut microbiota composition, thus controlling the pathogenesis of metabolic and cardiovascular diseases [6].

Abbreviations: DMSO, dimethyl sulfoxide.

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The digestion process depends on controlled movements of smooth muscle, the so-called peristalsis, which occurs in the stomach and small intestine. Several studies suggest that flavonoids can relax gastrointestinal smooth muscle: for example, apigenin and genistein are effective on mouse gastric tone [7]. This activity is independent of their antioxidant capacity; rather, it seems to be mediated by direct interaction with specific proteins and/or signal pathways, whose activation/inhibition causes spasmolysis [8].

Among the several proteins targeted by flavonoids, ion channels have recently attracted the attention of many laboratories. Recent comprehensive reviews demonstrated that this class of natural compounds specifically interacts with Ca_V [9] and K^+ channels [10,11], including hERG channels [12].

To our knowledge, the first work analysing the effect of a flavonoid, namely genistein, on smooth muscle $K_{ir}6.x$ channels dates to 1997. In smooth muscle cells of the rabbit portal vein, genistein reversibly inhibits the ATP-sensitive, pinacidil-induced K^+ current with a mechanism likely involving tyrosine phosphorylation [13]. Later, (-)-epigallocatechin gallate [along with (-)-epicatechin gallate], but not (-)-epigallocatechin and (-)-epicatechin, was shown to block native (rat pancreas islets of Langerhans) and expressed (*Xenopus* oocytes) $K_{ir}6.2$ channels [14]. The flavonoid exerts its blockade by acting from the intracellular side without interacting with the cytoplasmic regions of the channel, the gallate-ester moiety being critical for channel inhibition, which was also observed in $K_{ir}6.2$/SUR2A and $K_{ir}6.2$/SUR2B channels. On the other hand, luteolin relaxes rat coronary arteries, at least in part, through the stimulation of K_{ir} channels [15]. In a rat model of lipopolysaccharides-mediated sepsis, upregulated $K_{ir}6.x$ channel currents were normalised by dihydromyricetin, *in vivo* as well as *in vitro*, likely through the inhibition of oxidative stress, thus restoring vascular responsiveness of the thoracic aorta [16]. Quercetin, and to a lesser extent 5-hydroxyflavone, but not rutin, inhibit $K_{ir}6.1$ channel currents ($I_{Kir6.1}$) in vascular myocytes. The *in-silico* analysis suggested that the network of hydrogen bonds formed by these molecules at the binding site is crucial for their activity, supporting the evidence that the greater the number of hydroxyl groups in the structure the greater the inhibitory effect on the current [17].

Worthy of note, when quercetin, isoquercetin, and rutin aglycones are administered to rats by gavage, they are partially absorbed in the stomach, as opposed to the corresponding glycosides [18]. Taken together, these three pieces of evidence ($K_{ir}6x$ channels regulate gastric tone, flavonoids modulate $K_{ir}6.x$ channels, and flavonoids are adsorbed in the stomach) prompted us to investigate the effect of quercetin and ten structurally related flavonoids (Fig. 1) on the rat gastric fundus smooth muscle *in vitro*: single myocyte $I_{Kir6.1}$ and the mechanical activity of muscle strips were recorded. Electrophysiology data were corroborated by a molecular docking simulation to identify possible residue(s) involved in flavonoid binding. Results demonstrate that these dietary components indeed modulate $K_{ir}6.1$ channels in single myocytes as well as in isolated strips. This might have functional consequences in some stages of digestion.

2. Materials and methods

2.1. Cell isolation procedure from rat gastric fundus

All the study procedures were in strict accordance with the European Union Guidelines for the Care and the Use of Laboratory Animals (European Union Directive 2010/63/EU) and approved by the Animal Care and Ethics Committee of the University of Siena and the Italian Department of Health (7DF19.N.TBT). Male Wistar rats, weighing 250–350 g (Charles River Italia, Calco, Italy) were anaesthetized with an isoflurane (4 %) and O_2 gas mixture using Fluovac (Harvard Apparatus, Holliston, Massachusetts, USA), decapitated, and exsanguinated. The stomach was removed, opened along the longitudinal axis of the greater curvature and washed in cold Ca^{2+}-free physiological salt solution (Ca^{2+}-free PSS) containing (mM): NaCl 118, KCl 4.7, KH_2PO_4 1.2, $MgCl_2$ 1.2, $NaHCO_3$ 25, and glucose 11.5, bubbled with a 95 % O_2 − 5 % CO_2 gas mixture to create a pH of 7.4. Smooth muscle strips (1–2 mm in width; 1.5–2 cm in length), dissected from the circular layer of the anterior fundus wall, were digested for 40–45 min at 37 °C in 2 ml of nitrate-rich digestion solution (in mM: NaCl 55, $NaNO_3$ 65, KCl 5, Na-

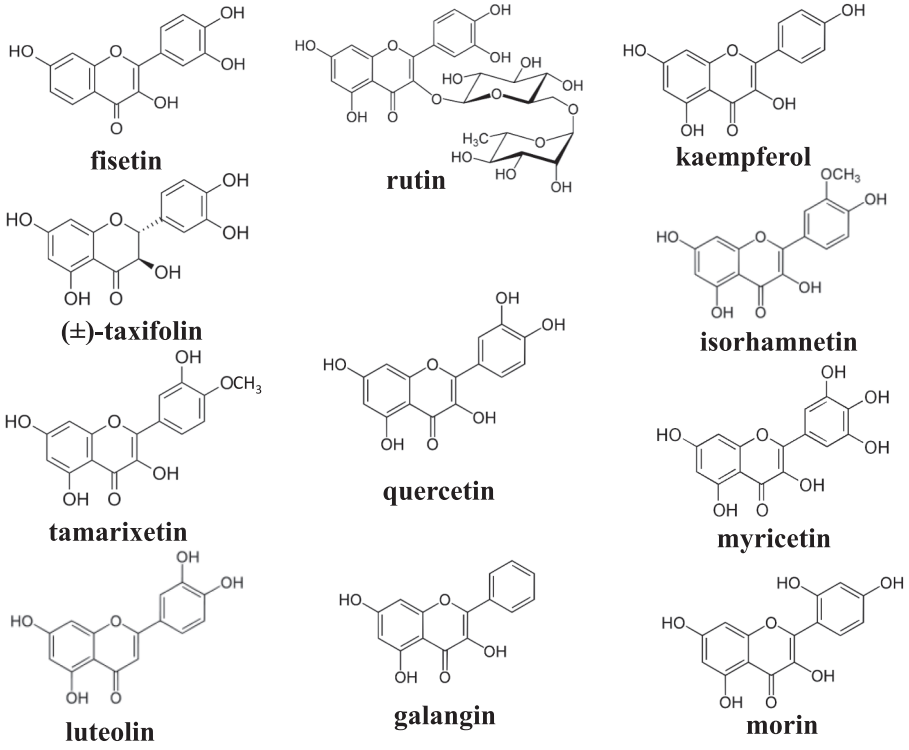

Fig. 1. Molecular structures of the flavonoids analysed in the present study.

pyruvate 5, glucose 10, taurine 10, HEPES 10, and $MgCl_2$ 1.2; pH 7.4) containing 2 mg collagenase (type I), 2.5 mg bovine serum albumin, and 3 mg soybean trypsin inhibitor, bubbled with an O_2 (95 %) and CO_2 (5 %) gas mixture [19]. Thereafter, cells were mechanically dispersed with a plastic pipette in a modified Kraft-Bruhe solution [containing 1 mg bovine serum albumin and (in mM): NaCl 105, KH_2PO_4 7, KCl 5, glucose 5, taurine 10, HEPES 10, $MgCl_2$ 1.6, Na-pyruvate 2.5, creatine 1.7, oxalacetate 2, Na_2ATP 1.5, and EGTA 0.1; pH adjusted to 7.25 with NaOH], and used for experiments within 10 h of isolation. During this time, cells were stored at 4 °C in a Kraft-Bruhe solution containing bovine serum albumin.

2.2. Whole-cell $I_{Kir6.1}$ recordings in gastric fundus myocytes

The conventional whole-cell patch-clamp method was applied to perform voltage-clamp measurements in smooth muscle cells. Cells were continuously superfused with recording solution [containing (in mM): NaCl 25, KCl 140, HEPES 10, glucose 10, $MgCl_2$ 1, $CaCl_2$ 0.1, and tetraethylammonium 1; pH was adjusted to 7.4 with NaOH] using a peristaltic pump (LKB 2132, Bromma, Sweden), at a flow rate of 400 μl min^{-1}. Recording electrodes were pulled from borosilicate glass capillaries (WPI, Berlin, Germany) and fire-polished to obtain a pipette resistance of 2–4 MΩ when filled with internal solution [containing (in mM): KCl 140, HEPES 10, EGTA 10, $MgCl_2$ 1, glucose 5, Na_2ATP 0.1, KADP 1, and Na_2GTP 0.1; pH was adjusted to 7.3 with KOH]. The osmolarity of the recording solution (320 mosmol) and that of the internal solution (290 mosmol) were measured with an osmometer (Osmostat OM 6020, Menarini Diagnostics, Florence, Italy).

To minimize K_V currents, $I_{Kir6.1}$ were recorded at a steady membrane potential (V_h) of −50 mV using a continuous gap-free acquisition protocol. Currents, activated by the $K_{ir}6.1$ channel opener pinacidil (10 μM), did not run down during the following 10 min under these conditions [19]. Care was taken to complete each experiment within this period. As few cells did not respond to pinacidil stimulation, flavonoids were always added after pinacidil had induced a stable current amplitude.

In a second series of experiments, the effect of galangin was assessed on outward K^+ current elicited with depolarising steps from a V_h of −80 mV to 80 mV (50 ms length) every 10 s. The recording solution contained (in mM): NaCl 140, KCl 5, HEPES 10, glucose 10, $MgCl_2$ 1.2, $CaCl_2$ 0.1, and tetraethylammonium 1; pH was adjusted to 7.4 with NaOH. The internal solution contained (in mM): KCl 140, HEPES 10, EGTA 10, $MgCl_2$ 1, glucose 5, Na_2ATP 0.1, KADP 1, and Na_2GTP 0.1; pH was adjusted to 7.3 with KOH.

An Axopatch 200B patch-clamp amplifier (Molecular Devices Corporation, Sunnyvale, CA, USA) in conjunction with an ADC/DAC interface (DigiData 1200 A/B series, Molecular Devices Corporation) was used to generate and apply voltage pulses to the clamped cells and record the corresponding membrane currents. At the beginning of each experiment, the junction potential between the pipette and bath solution was electronically adjusted to zero. Current signals, after compensation for whole-cell capacitance and series resistance (between 70 % and 75 %), were low-pass filtered at 1 kHz and digitized at 3 kHz before being stored on the computer's hard disk. Electrophysiology responses were tested at room temperature (20–22 °C).

Analysis of data was accomplished by using pClamp 9.2.1.8 software (Molecular Devices Corporation). Current values were corrected for leakage using 10 μM glibenclamide, which completely blocked $I_{Kir6.1}$ [17].

2.3. Molecular docking simulations

The atomic structure of the $K_{ir}6.1$ channel was defined using the PDB entry 7MIT [20]. Docking calculations were performed using HADDOCK webserver version 2.4 [21,22] for all the flavonoids considered in the experimental analyses plus rosiglitazone. Rosiglitazone, in fact, is a known blocker of $K_{ir}6.1$ channels [23], and it was included in the calculations as a positive control. The chemical structures of drugs were retrieved from the PubChem database [24] and parameterized using UCSF Chimera version 1.17.3 [25] with Amber14SB forcefield [26]. The binding site of flavonoids and rosiglitazone was estimated using an iterative procedure. Firstly, docking calculations were performed considering as active residues in HADDOCK the residues previously identified as part of the binding sites for class III antiarrhythmic drugs by Chen et al. [27], namely Phe61(A), Lue64(A), Phe76(A), Ser79(A), Phe80(A), Ser83(A), Ile168(A), Ile169(A), Met173(A), Cys176(A), Trp69(D), Leu73(D), with the letter in parenthesis identifying the protein chain. In these calculations, drugs were considered as part of the active region, meaning that they were forced to bind at least one, but not necessarily all the active resides of the protein, while still being free to further explore other possible binding sites. The best poses obtained for all the molecules at these steps were exported in PDB format and the Protein-Ligand Interaction Profiler (PLIP) tool was used to assess the role of specific residues [28]. Then, docking calculations were repeated considering active protein residues those identified by the PLIP analyses. Default settings for small-molecule docking were used both for data production and clustering, as recommended by the developers of HADDOCK [29].

2.4. Rat gastric fundus functional assay

Gastric fundus strips obtained as described above were transferred into 25 ml organ bath chambers filled with Ca^{2+}-free PSS, bubbled with O_2 (95 %) and CO_2 (5 %) gas mixture, and maintained at 37 °C. Strips, connected to isometric transducers (FORT25, WPI, Berlin, Germany) were stretched to a tension of 1 g. After equilibration for 15 min, 2.5 mM Ca^{2+} was added and the strips were allowed to develop stable, spontaneous tone over a 30-min period. Afterwards, they were challenged with consecutive stimulations by 60 mM KCl interspersed by three 15-min washing periods, until a stable response was obtained (usually two challenges). After 45 min of washing in PSS, the experimental protocols detailed below were performed. At the end of each experimental session, 100 μM sodium nitroprusside was added to test the functional integrity of smooth muscle.

Data were collected and analysed by using a digital PowerLab acquisition system (PowerLab 8/30; ADInstruments, Castle Hill, Australia) driven by LabChart 7.3.7 Pro (PowerLab; ADInstruments). Signals were low-pass filtered at 30 Hz and digitized at 100 Hz before being stored on the computer's hard disk.

2.5. Flavonoid concentration–response curves in strips stimulated by either high KCl or carbachol

A flavonoid concentration–response curve was constructed in strips pre-contracted by either 25 mM KCl or 1 μM carbachol. On the plateau of the contraction, each flavonoid was added at cumulative concentrations (0.1–100 μM) in half-log increments. The myorelaxant effect was calculated as a percentage of the response obtained with either 25 mM KCl or carbachol.

2.6. Pinacidil concentration–response curve

A concentration–response curve was constructed for pinacidil, a well-known activator of $K_{ir}6.1$ channels [30]. Stomach fundus smooth muscle strips were stimulated by either 25 mM KCl or 1 μM carbachol. On the plateau of contraction, pinacidil was added at cumulative concentrations (0.1–100 μM) in half-log increments. In different preparations, a pinacidil concentration–response curve was performed in the presence of the selective blocker of $K_{ir}6.1$ channels glibenclamide (1–10 μM) [30], added on the plateau of the contraction induced by 25 mM KCl or carbachol. The muscle relaxant effect of pinacidil was calculated as a percentage of the response obtained with KCl or carbachol.

2.7. Effect of various flavonoids on the pinacidil concentration–response curve

Different strips from those used to construct the concentration–response curve to pinacidil under control conditions were pre-incubated with each flavonoid (10 μM) for 20 min and then stimulated by 25 mM KCl. On the plateau of the contraction, pinacidil was added at cumulative concentrations (0.1–100 μM) in half-log increments. The relaxant effect of pinacidil was calculated as a percentage of the response obtained with 25 mM KCl.

2.8. Effect of various flavonoids on the L-cysteine concentration–response curve

An L-cysteine concentration–response curve was constructed in strips pre-contracted by 25 mM KCl. L-cysteine was added at cumulative concentrations (0.03–30 mM) in half-log increments. Preliminary experiments proved that two consecutive L-cysteine concentration–response curves could be carried out in the same strip giving rise to similar results (first curve: 25 mM KCl-induced contraction 2.4 ± 0.3 g, n = 5; L-cysteine estimated pIC_{50} value 1.76 ± 0.09; second curve: 2.5 ± 0.3 g, n = 5; P = 0.282; pIC_{50} value 1.68 ± 0.14 mM; P = 0.5207, Student's *t* test for paired samples). Therefore, in this setting the first curve was used as control and the second one to analyze the effect of various agents. Between the two curves, preparations were washed with PSS until the spontaneous tone was restored.

The L-cysteine concentration–response curve was performed also in strips pre-incubated with either 1 μM glibenclamide (for 20 min) or the irreversible inhibitor of cystathionine-γ-lyase DL-propargylglycine (10 mM for 30 min) [31]. A similar protocol was followed to assess the effect of flavonoids (10 μM final concentration, 20 min pre-incubation) on L-cysteine myorelaxant activity.

2.9. Statistical analysis

Individual values, contributing to the calculation of the group mean ± SEM values, derived from independent cells or strips that, sometimes, were isolated from the same animal. To ensure that mean values are representative of the population, however, cells or strips included in the same group were isolated from at least three different animals. Data were normally distributed and variances were not significantly different.

Statistical analysis and significance, as measured by either one-way or repeated measures ANOVA (followed by Dunnett or Bonferroni post hoc test) or Student's *t* test for paired or unpaired samples (two-tailed), were obtained using GraphPad Prism version 5.04 (GraphPad Software Inc., San Diego, CA, USA). Post hoc tests were performed only when ANOVA found a significant value of F and no variance inhomogeneity. In all comparisons, $P < 0.05$ was considered significant.

2.10. Materials

Collagenase (type IA), soybean trypsin inhibitor, bovine serum albumin, tetraethylammonium chloride, carbachol, isorhamnetin, (±)-taxifolin, quercetin, morin, myricetin, and rutin were purchased from Sigma Chimica (Milan, Italy). Fisetin and luteolin were from Abcam (Milan, Italy). L-cysteine and DL-propargylglycine were supplied by Merck KGaA (Darmstadt, Germany). Galangin, tamarixetin, and kaempferol were supplied by Extrasynthese (Genay Cedex, France). Pinacidil and glibenclamide were a kind gift from Prof. Bova (Department of Pharmaceutical and Pharmacological Sciences, University of Padua, Italy). Sodium nitroprusside was supplied by Riedel-De Haen AG (Seelze-Hannover, Germany). Flavonoids, pinacidil, and glibenclamide, dissolved directly in dimethyl sulfoxide (DMSO), were diluted at least 1000 times before use. All solutions were stored at −20 °C and protected from light by wrapping containers with aluminium foil. The resulting concentrations of DMSO (below 0.1 %, v/v) did not influence tissue or cell responses. L-cysteine, DL-propargylglycine and sodium nitroprusside were solubilized in deionized water. L-cysteine and DL-propargylglycine were prepared immediately before use.

The final concentrations of the drugs used are indicated in the text. All other substances were analytical grade and were used without further purification.

3. Results

3.1. Flavonoids inhibited $I_{Kir6.1}$ in gastric fundus myocytes

The effect of quercetin and ten structurally related flavonoids was assessed on $I_{Kir6.1}$ recorded in myocytes freshly isolated from the rat gastric fundus. To limit the activation of K_V and $K_{Ca}1.1$ channels, $I_{Kir6.1}$ was evoked at a V_h of −50 mV in the presence of 0.1 mM ATP and 1 mM ADP in the pipette solution, and 1 mM tetraethylammonium in the recording solution. As shown in Fig. 2A, the $K_{ir}6.1$ channel opener pinacidil (10 μM) activated an inward current that reached a plateau after about two minutes. The addition of quercetin (10 μM and 50 μM) reduced the amplitude of the current in a concentration-dependent manner. At the maximum concentration tested, inhibition was statistically significant (Fig. 2B). The $K_{ir}6.1$ channel antagonist glibenclamide (10 μM) blocked the current amplitude. Among the flavonoids assessed, galangin was the most potent agent with an estimated pIC_{50} value of 5.11 (Fig. 2C) and an efficacy of 99.5 %. Also tamarixetin was very effective showing a 95 % inhibition of the current amplitude (Fig. 2D). Maximal inhibition decreased to 78 % with quercetin, 62 % with kaempferol (Fig. 2E) and about 35 % with fisetin (Fig. 2F), luteolin (Fig. 2G), and isorhamnetin (Fig. 2H). (±)-Taxifolin (Fig. 2I), morin (Fig. 2J), and rutin (Fig. 2K) were almost ineffective, whereas myricetin (Fig. 2L) caused a modest stimulation of the current density.

A second series of experiments was performed to investigate whether $I_{Kir6.1}$ inhibition by flavonoids was dependent on the current direction. The most effective flavonoid, namely galangin (50 μM), inhibited not only the 10 μM pinacidil-elicited component but also other components of the outward current (Fig. 3). Replacement of galangin with glibenclamide, still in the presence of pinacidil, partially reverted current amplitude toward control values.

3.2. Flavonoids docked to the $K_{ir}6.1$ channel

An *in silico* analysis was performed to identify the putative $K_{ir}6.1$ channel binding site(s) of flavonoids and provide a rationale for their different activity. Flavonoids did not occupy any of the three binding sites previously identified by Chen et al. [27] and used by HADDOCK to select active residues in the first round of docking calculations. Instead, PLIP calculations identified an alternative binding site in the C-terminal domain of the $K_{ir}6.1$ channel subunit SUR2B (Fig. 4A-C), which was confirmed by HADDOCK in a second round of docking calculations. This binding site includes a flexible loop, formed by the amino acids Asn251-Ile254, which is located in the proximity of the intracellular entrance of the pore cavity (Fig. 4). Rosiglitazone bound at this site with its polar head deeply inserted into the binding pocket, and its lipophilic tail extending outwards (Fig. 4B-C). This pose is stabilized by van der Waals interactions between pyridine and benzene moieties of the drug and the hydrophobic residues in the protein loop, and hydrogen bonds between protein residues and the thiazolidinedione ring of the drug (Table 1). The binding score estimated by HADDOCK only partially agreed with experimental data. The potent and medium flavonoid blockers (galangin, tamarixetin, kaempferol, fisetin, luteolin, and isorhamnetin) had scores ranging from −3.7 to −7.0 (Table 1), as compared to −12.5 for rosiglitazone. The ineffective blockers morin, rutin, and myricetin had score values of −1.9, 0.5 (the only positive value), and −2.8, respectively, in agreement with their experimental behaviour. Instead, the score of (±)-taxifolin (-4.1) suggested a strong binding, in disagreement with the lack of blocking observed experimentally. Binding scores are

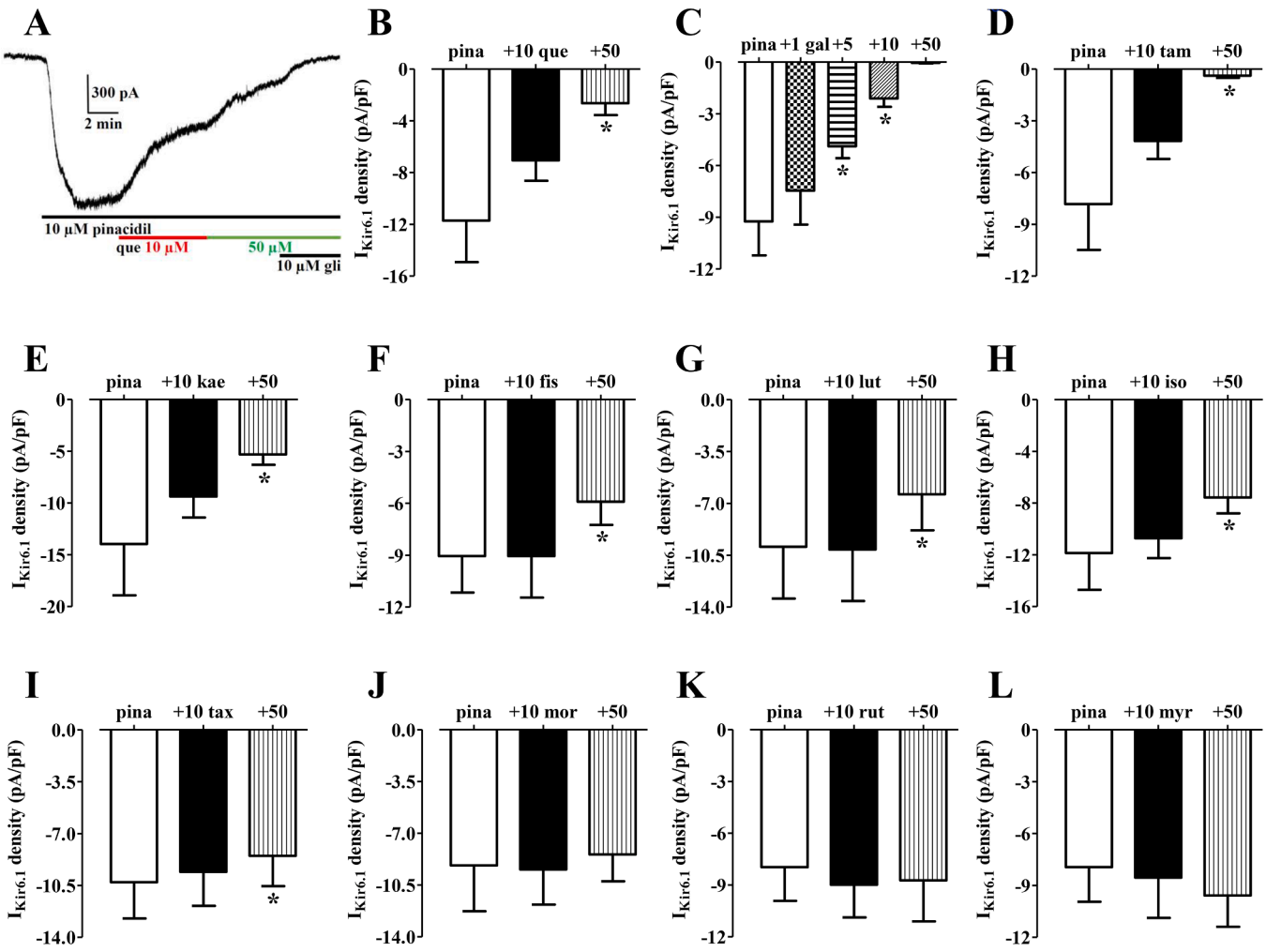

Fig. 2. Effects of flavonoids on $I_{Kir6.1}$ recorded in rat gastric fundus myocytes. (A) Representative whole-cell recording of the inward current elicited by 10 μM pinacidil at a V_h of −50 mV. The effect of quercetin (que; 10 μM and 50 μM) as well as of 10 μM glibenclamide (gli) is shown. The trace is representative of seven similar experiments. (B-L) Effects of μM concentrations of (B) quercetin (que), (C) galangin (gal), (D) tamarixetin (tam), (E) kaempferol (kae), (F) fisetin (fis), (G) luteolin (lut), (H) isorhamnetin (iso), (I) (±)-taxifolin (tax), (J) morin (mor), (K), rutin (rut), and (L) myricetin (myr) on $I_{Kir6.1}$ elicited by pinacidil (pina). The amplitude of the current normalized to the cell surface (current density) in pA/pF is shown on the ordinate axis. The columns represent the mean ± SEM (n = 5–8). *P < 0.05 vs pinacidil, one-way ANOVA and Dunnett post-test.

particularly hard to estimate [32] and, consequently, the partial agreement with experimental data is not entirely surprising.

To further assess the characteristics of the estimated binding site, the best binding poses of all the flavonoids were evaluated. Flavonoids were grouped according to the activity recorded in the electrophysiology experiments as follows: ineffective or weak inhibitors (morin, rutin, (±)-taxifolin, and myricetin) (Fig. 5A), medium inhibitors (luteolin, quercetin, fisetin, kaempferol, and isorhamnetin) (Fig. 5B), potent inhibitors (galangin and tamarixetin) (Fig. 5C). This analysis revealed marked differences among the three groups of molecules. Potent inhibitors established hydrogen bonds with residues Asn-252(C) and Ile-254(D). This characteristic is shared with the best binding pose observed for rosiglitazone and described above, but not with the binding poses observed for medium and weak blockers (compare Figs. 4 and 5C with Fig. 5A, B). In particular, galangin and tamarixetin bound deeply to the binding pocket, orienting their hydroxyl groups facing the loop of both adjacent chains C and D, while myricetin, morin, and (±)-taxifolin did not establish strong hydrophobic interactions with the loop (Table 1 and Fig. 5A–C). This interaction profile suggested that the inhibitory activity might be site-specific because the inhibitory strength is maximized only if the flavonoid interactions with the channel involve both the C and D chains. The remaining flavonoids, devoid of blocking activity, bound to the opposite site of the loop residues concerning potent/medium flavonoid blockers and rosiglitazone (compare Fig. 5A with Figs. 4 and 5B, C).

3.3. Flavonoids relaxed high KCl- and carbachol-induced contraction of fundus strips

In the first series of experiments, the effects of the neurotransmitter acetylcholine, the muscarinic agonist carbachol, and high KCl concentrations were assessed on the mechanical function of the gastric fundus strips to select the best stimulating agent(s) for the analysis of the flavonoid activity. These agents caused a concentration-dependent contraction with pEC_{50} values of 5.58 ± 0.17 (n = 8), 6.35 ± 0.16 (n = 6), and 1.60 ± 0.06 (n = 6), respectively. Similar maximal effects were elicited by 100 μM acetylcholine and 80 mM KCl (106.8 ± 10.4 % and 114.9 ± 5.2 % of the contraction induced by 60 mM KCl, respectively), while that of 100 μM carbachol was 164.2 ± 16.2 %. Based on these results and due to the metabolic instability of acetylcholine, carbachol and high KCl were used in the following experiments to stimulate fundus preparations.

In a second series of experiments, the effects of the flavonoids selected based on the inhibitory activity recorded in the

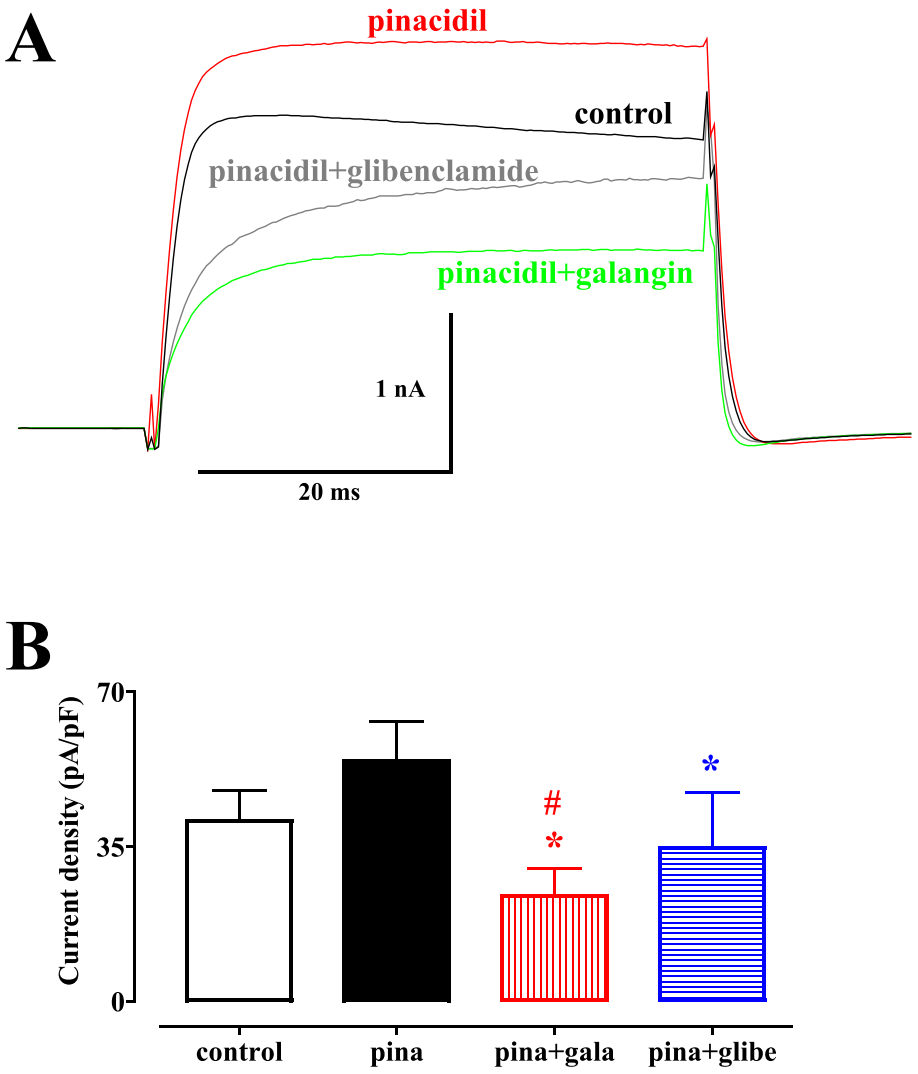

Fig. 3. Effects of galangin on outward K^+ current recorded in rat gastric fundus myocytes. (A) Representative whole-cell recordings of the outward current elicited by depolarizing steps to 80 mV from a V_h of −80 mV recorded under control conditions, in the presence of 10 μM pinacidil, pinacidil plus 50 μM galangin, and after the replacement of galangin with 10 μM glibenclamide. Traces are the average of seven cells. (B) Effects of pinacidil, galangin, and glibenclamide on outward K^+ current. The amplitude of the current normalized to the cell surface (current density) in pA/pF is shown on the ordinate axis. The columns represent the mean ± SEM (n = 7). $^{\#}P < 0.05$ vs control, $^{*}P < 0.05$ vs pinacidil, repeated measures ANOVA and Bonferroni post-test.

electrophysiology experiments (i.e., galangin, tamarixetin, quercetin, kaempferol, fisetin, luteolin, and rutin as a negative control), were investigated on fundus smooth muscle strips either depolarized by high KCl or stimulated by carbachol, to quantify their myorelaxant activity. Stimulation by 25 mM KCl caused a sustained tonic contraction of the preparations. The addition of cumulative concentrations of each flavonoid, except rutin (data not shown), caused a concentration-dependent relaxation (Fig. 6) with pIC_{50} values of 4.53 ± 0.07 (galangin; n = 8), 4.53 ± 0.21 (tamarixetin; n = 7), 4.21 ± 0.13 (quercetin; n = 6, estimated), 4.76 ± 0.11 (kaempferol; n = 6), 4.24 ± 0.25 (fisetin; n = 7), and 4.34 ± 0.18 (luteolin; n = 5, estimated). Only galangin, in addition to completely reverting the 25 mM KCl-induced tone, also caused a marked relaxation of the spontaneous tone (Fig. 6A).

The same experiment was then repeated in preparations stimulated by 1 μM carbachol (Fig. 6). The addition of galangin caused a concentration-dependent relaxation with a pIC_{50} value of 4.42 ± 0.15 (n = 8), which was not significantly different from that recorded on 25 mM KCl-induced contraction (P = 0.5213), though the curve was shifted to the right; galangin efficacy, however, was significantly reduced (P = 0.0289; Fig. 6A). On the contrary, quercetin was more potent and effective in preparations stimulated by carbachol (pIC_{50} value of 4.71 ± 0.17; n = 6; P = 0.0432) than by KCl. The myorelaxant activity of the remaining flavonoids was similar to that observed in KCl-stimulated strips (pIC_{50} values: 4.69 ± 0.12 tamarixetin, n = 6, P = 0.5404; 4.44 ± 0.20 kaempferol, n = 8, P = 0.1865; 4.20 ± 0.28 fisetin, n = 6, P = 0.9274), except that of luteolin, which was markedly reduced, and that of rutin that, at 100 μM caused a maximal relaxation of 29.5 ± 11.1 % (n = 7).

Based on the electrophysiology and functional data, the 10 μM concentration was considered appropriate to analyse the effects of flavonoids on fundus smooth muscle strip $K_{ir}6.1$ channels.

3.4. *Flavonoids antagonized pinacidil-induced myorelaxation of fundus strips*

This series of experiments was performed to analyse the effects of flavonoids on the myorelaxation induced by the $K_{ir}6.1$ channel activator pinacidil in fundus smooth muscle strips.

In a preliminary experiment, a concentration–response curve to pinacidil was constructed in preparations either depolarized by high KCl

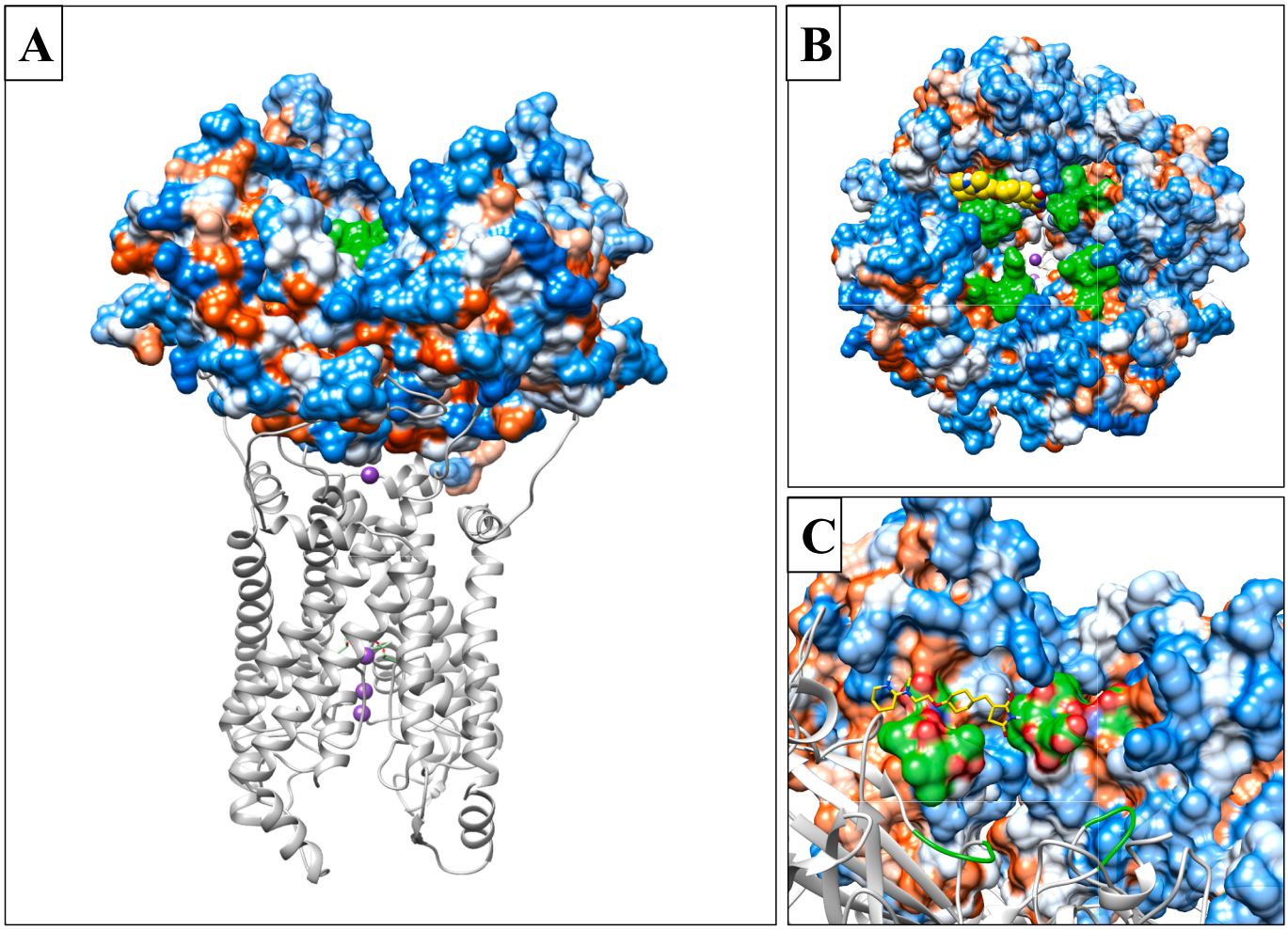

Fig. 4. ***In silico*** **depiction of the most representative pose of rosiglitazone.** (A) Side view and (B) top view of the $K_{ir}6.1$ channel subunit SUR2B, and (C) enlarged view of the binding pocket in cartoon and surface representation (Chimera default hydropathicity colouration). The binding sites of both chains C and D are coloured green. Rosiglitazone is coloured yellow with (B) dots representation or (C) liquorice sticks representation.

Table 1
Characteristic of the binding site for flavonoids and rosiglitazone in the $K_{ir}6.1$ channel as estimated by docking calculations.

	HADDOCK score	Van der Waals energy (kcal/mol)	Electrostatic energy (kcal/mol)	Hydrophobic interactions	Hydrogen bonds
Myricetin	−2,8	−21,1	−20,9	226(B), 254(C), 257(C)	226(B), 228(B), 247(B), 248(B), 257 (C)
Rutin	0,5	−31,3	−17,4	274(B), 253(C)*	/
Morin	−1,9	−26,9	−19,3	231(B), 235(B), 244(B), 246 (B)	231(B), 233(B), 244(B), 246(B)
(±)-Taxifolin	−4,1	−24,8	−52,0	226(B), 247(B), 254(C)	224(B), 227(B), 228(B)*, 256(C), 257 (C)
Isorhamnetin	−4,6	−25,9	−43,4	251(C), 253(C)	246(B), 249(C), 251(C), 252(C)
Fisetin	−3,7	−22,7	−39,9	255(C)	246(B), 253(C)
Luteolin	−7,0	−25,0	−58,8	251(C)	246(B)*, 250(C), 251(C)
Kaempferol	−4,7	−27,7	−30,5	253(C), 255(C)	246(B), 250(C), 252(C)
Quercetin	−5,5	−24,5	−57,9	253(C)	244(B), 246(B), 247(B), 203(C)
Tamarixetin	−3,9	−24,2	−20,9	254(D)	252(C)*, 274(C), 253(D)
Galangin	−4,00	−24,7	−9,5	255(C)	252(C), 255(C)*, 256(C), 254(D)
Rosiglitazone	−12,5	−25,5	−89,3	253(C)	203(C), 251(C), 252(C), 254(D)

HADDOCK scores, van der Waals, and electrostatic intermolecular energies of the best cluster of each complex. The residues that can form either hydrophobic or hydrogen bonds with each ligand are also shown (residues involved in more than one contact are marked with an asterisk).

or stimulated by carbachol, in the absence and presence of the $K_{ir}6.1$ antagonist glibenclamide. Stimulation by 25 mM KCl resulted in a sustained tonic contraction of the preparations (Fig. 7A). The addition of cumulative concentrations of pinacidil, besides reverting the active tone induced by KCl, also markedly relaxed the spontaneous tone in a concentration-dependent manner with a pIC_{50} value of 5.85 ± 0.08 (n = 8) and a maximal effect equal to −48.6 ± 9.8 % of the contraction induced by KCl (Fig. 7C). The presence of 1 μM glibenclamide caused a significant rightward shift of the concentration–response curve to pinacidil (pIC_{50} of 5.14 ± 0.14, n = 8; P = 0.0006, Student's *t* test for

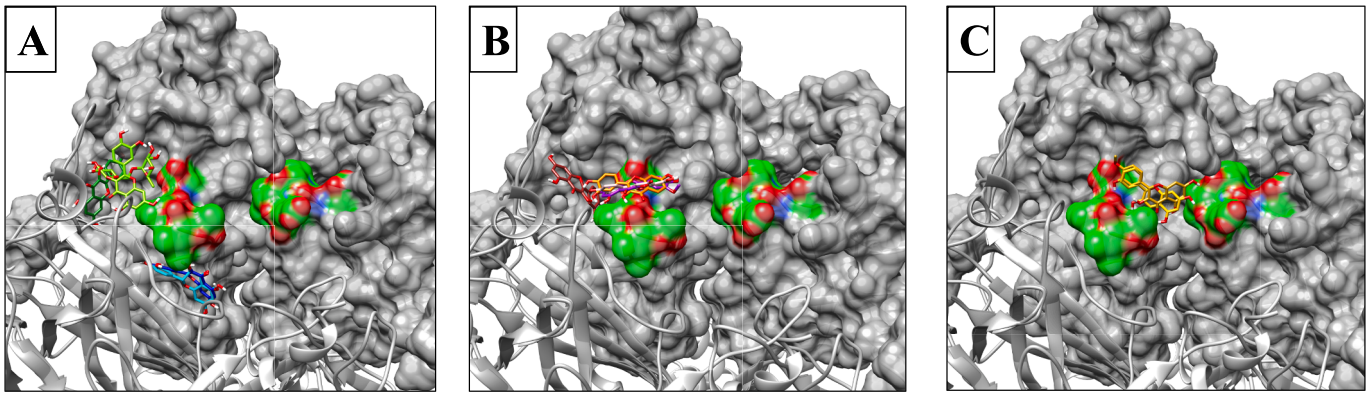

Fig. 5. The most representative poses of flavonoids as a result of molecular docking analysis. In each panel, the binding pocket is displayed with a cartoon (chains A and B) and surface representation (chains C and D). The binding site is coloured in green with heteroatom functional groups displayed. Flavonoids, represented in liquorice sticks, are grouped according to their inhibitory activity: (A) no effect or weak inhibitors [morin (forest green), rutin (chartreuse), (±)-taxifolin (cyan), and myricetin (blue)]; (B) medium inhibitors [luteolin (pink), quercetin (brown), fisetin (orange), kaempferol (purple), and isorhamnetin (magenta)]; (C) potent inhibitors [galangin (gold) and tamarixetin (goldenrod)].

unpaired samples) without affecting the maximal response (-41.2 ± 7.8 %; P = 0.5672) (Fig. 7B,C).

A similar protocol was then repeated in preparations stimulated by 1 μM carbachol. Under these experimental conditions, three concentrations of glibenclamide were assessed on the myorelaxant activity of pinacidil. The pIC_{50} values were: 5.32 ± 0.13 (DMSO; n = 7; P = 0.0034 vs 25 mM KCl, Student's *t* test for unpaired samples), 4.87 ± 0.16 (1 μM glibenclamide; P > 0.05 vs DMSO, Dunnett post hoc test), 4.94 ± 0.17 (3 μM glibenclamide; P > 0.05), and 4.44 ± 0.09 (10 μM glibenclamide; n = 7; P < 0.05). Maximal effects were not significantly different among the various treatments (-41.5 ± 12.4 %, −54.6 ± 21.2 %, −14.8 ± 5.4 %, and −8.1 ± 9.7 % of the contraction induced by carbachol; P = 0.0705).

Glibenclamide efficacy was higher in strips stimulated by KCl than carbachol, so the former protocol was preferred to assess flavonoid activity. In preparations pre-incubated with 10 μM galangin, the contraction induced by 25 mM KCl (1.8 ± 0.2 g, n = 7) was similar to that obtained with the solvent alone (1.8 ± 0.4 g, n = 7; P = 0.979, Student's *t* test for unpaired samples). However, the concentration–response curve to pinacidil was significantly shifted to the right (pIC_{50} 5.76 ± 0.14 DMSO, and 5.29 ± 0.11 galangin, n = 7; P = 0.0223, Student's *t* test for unpaired samples) (Fig. 7D). Conversely, the efficacy values were not significantly different (-65.8 ± 20.7 % DMSO, –33.7 ± 8.5 % galangin; P = 0.1766).

The same experiment was replicated in preparations pre-incubated with either 10 μM tamarixetin or 10 μM kaempferol. Both flavonoids did not affect 25 mM KCl-induced tone (1.5 ± 0.3 g, n = 7, P = 0.540 vs DMSO, and 1.6 ± 0.3 g, n = 7; P = 0.791) but, similarly to galangin, antagonized the myorelaxant activity of pinacidil (pIC_{50} of 5.17 ± 0.16 tamarixetin, n = 7, P = 0.0172 vs. DMSO, Fig. 7E; pIC_{50} of 5.41 ± 0.09 kaempferol, n = 7, P = 0.0562 vs. DMSO, Fig. 7F). The efficacy, however, was similar to that recorded in the presence of DMSO (-45.3 ± 10.3 %, n = 7, P = 0.3924, and −47.4 ± 17.6 %, n = 7, P = 0.5093, respectively). Among the remaining flavonoids, quercetin, luteolin, and rutin did not affect 25 mM KCl-induced tone, the pIC_{50} value or maximal effect for pinacidil, whereas fisetin significantly reduced both maximal effect and KCl-induced tone (data not shown).

3.5. Flavonoids antagonized L-cysteine-induced myorelaxation of fundus strips

This series of experiments was performed to analyse the effects of flavonoids on the myorelaxation induced by L-cysteine, the metabolic precursor of the $K_{ir}6.1$ channel stimulator H_2S.

In a preliminary experiment, fundus strips challenged by 25 mM KCl did not show variations in the response to two consecutive concentration–response curves to L-cysteine interspersed by several washing to restore spontaneous tone (pIC_{50} of 1.96 ± 0.10 first curve and 1.81 ± 0.05 second curve, n = 5, P = 0.0889, Student's *t* test for paired samples; efficacy 74.1 ± 6.3 % and 77.2 ± 8.6 %, respectively, P = 0.3921). However, preincubation of the preparations with either the $K_{ir}6.1$ channel blocker glibenclamide (1 μM; [30]) or the selective and irreversible inhibitor of cystathionine-γ-lyase DL-propargylglycine (10 mM; [31]) caused a significant rightward shift of the concentration–response curve to L-cysteine (Fig. 8A,B) (pIC_{50} values of 1.95 ± 0.12 DMSO, >1.5 glibenclamide, n = 5; 2.23 ± 0.08 control, 1.94 ± 0.09 DL-propargylglycine, n = 7; P = 0.0135). Furthermore, glibenclamide significantly reduced the efficacy of L-cysteine as well.

In strips pre-incubated for 20 min with 10 μM galangin the concentration–response curve to L-cysteine was significantly shifted to the right (pIC_{50} of 2.08 ± 0.17 DMSO, 1.81 ± 0.09 galangin, n = 5; P = 0.0074, Student's *t* test for paired samples) along with a significant reduction of the efficacy (Fig. 8C). Similar results were obtained with tamarixetin (pIC_{50} of 2.12 ± 0.09 DMSO, 1.80 ± 0.13 tamarixetin, n = 5; P = 0.0019; Fig. 8D). Pre-incubation of tissues with 10 μM kaempferol, quercetin, fisetin, luteolin, and rutin did not change either the potency or efficacy of L-cysteine (data not shown).

4. Discussion

The main findings of the present work are as follows: some flavonoids, structurally related to quercetin and abundant in our diet, inhibit $I_{Kir6.1}$ in rat fundus myocytes and this activity counteracts the myorelaxant effect exerted by several agents and mediated, at least in part, by $K_{ir}6.1$ channel activation, in gastric fundus smooth muscle tissue.

Recently, quercetin-like flavonoids have been described as blockers of vascular smooth muscle $K_{ir}6.1$ channels [17], inhibition of the current being directly related to the number of free hydroxyl groups existing on the molecule and markedly hampered in glycosides by the presence of sugar molecule(s), which represent a steric hindrance limiting the access to the binding site on the channel. The present findings demonstrate that flavonoids structurally related to quercetin are capable of inhibiting the same channel present on gastric fundus smooth muscle. These results, along with those obtained by Kittl et al. [33] in rat INS-1 β-cells, where quercetin stimulates the release of insulin by inhibiting $K_{ir}6.1$ channels, demonstrate that the inhibitory activity, at least of quercetin, is not tissue-specific.

Some clear considerations emerged from the structure–activity relationship analysis that revealed a few requirements necessary for the $K_{ir}6.1$ channel blockade. Ring B substitution: the inhibitory effect was

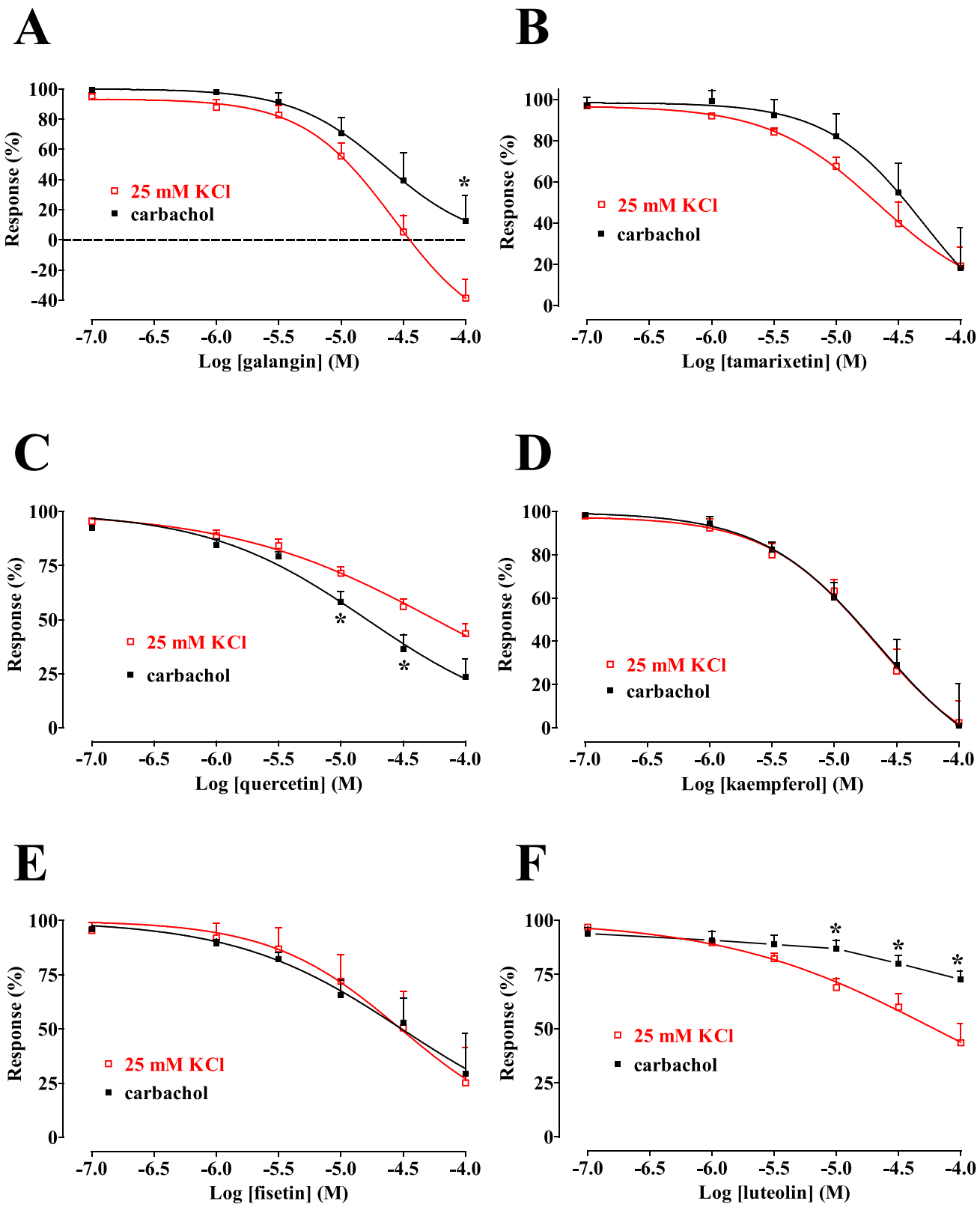

Fig. 6. Effects of flavonoids on rat gastric fundus smooth muscle strips pre-contracted by high KCl or carbachol. The addition of cumulative concentrations of (A) galangin, (B) tamarixetin, (C) quercetin, (D) kaempferol, (E) fisetin, or (F) luteolin caused concentration-dependent relaxation of the preparations pre-contracted by either 25 mM KCl or 1 μM carbachol. On the ordinate axis, the response is reported as a percentage of the contraction evoked by KCl or carbachol. The dotted line represents the spontaneous tone of the preparation. Data represent the mean ± SEM (n = 6–8). *P < 0.05 vs. 25 mM KCl, Student's *t* test for unpaired samples.

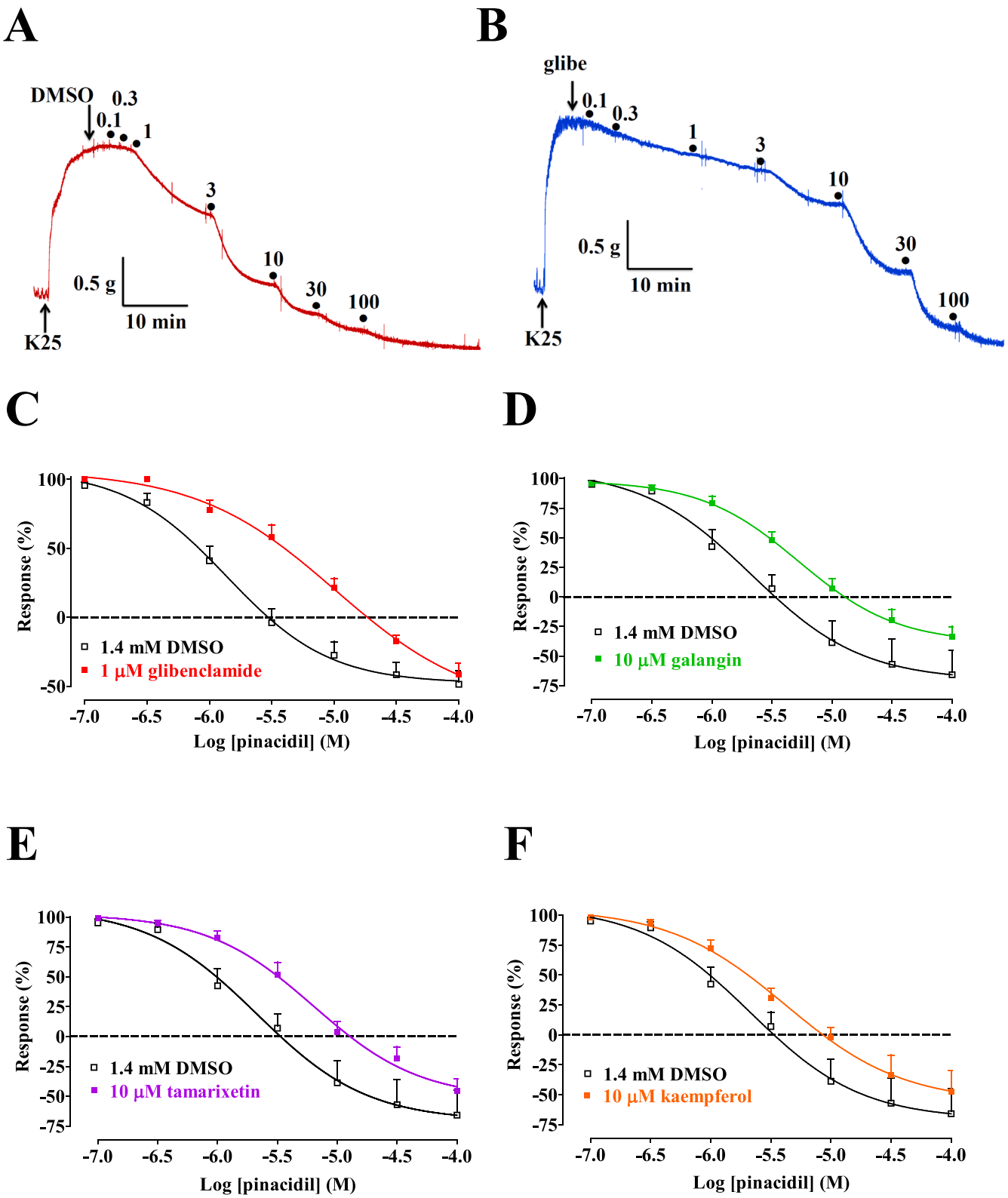

Fig. 7. Effects of flavonoids on pinacidil-induced relaxation of rat gastric fundus smooth muscle strips pre-contracted by high KCl. The addition of cumulative concentrations (μM) of pinacidil in the presence of either (A) 1.4 mM DMSO or (B) 1 μM glibenclamide (glibe) caused the relaxation of preparations stimulated by 25 mM KCl (K25). Traces are representative of 7 similar experiments. (C-F) Concentration-response curves to pinacidil constructed in the presence of vehicle (DMSO), (C) 1 μM glibenclamide, (D) 10 μM galangin, (E) 10 μM tamarixetin, or (F) 10 μM kaempferol. On the ordinate axis, the response is reported as a percentage of the contraction evoked by KCl. The dotted line represents the spontaneous tone of the preparation. Data represent the mean ± SEM (n = 7–8).

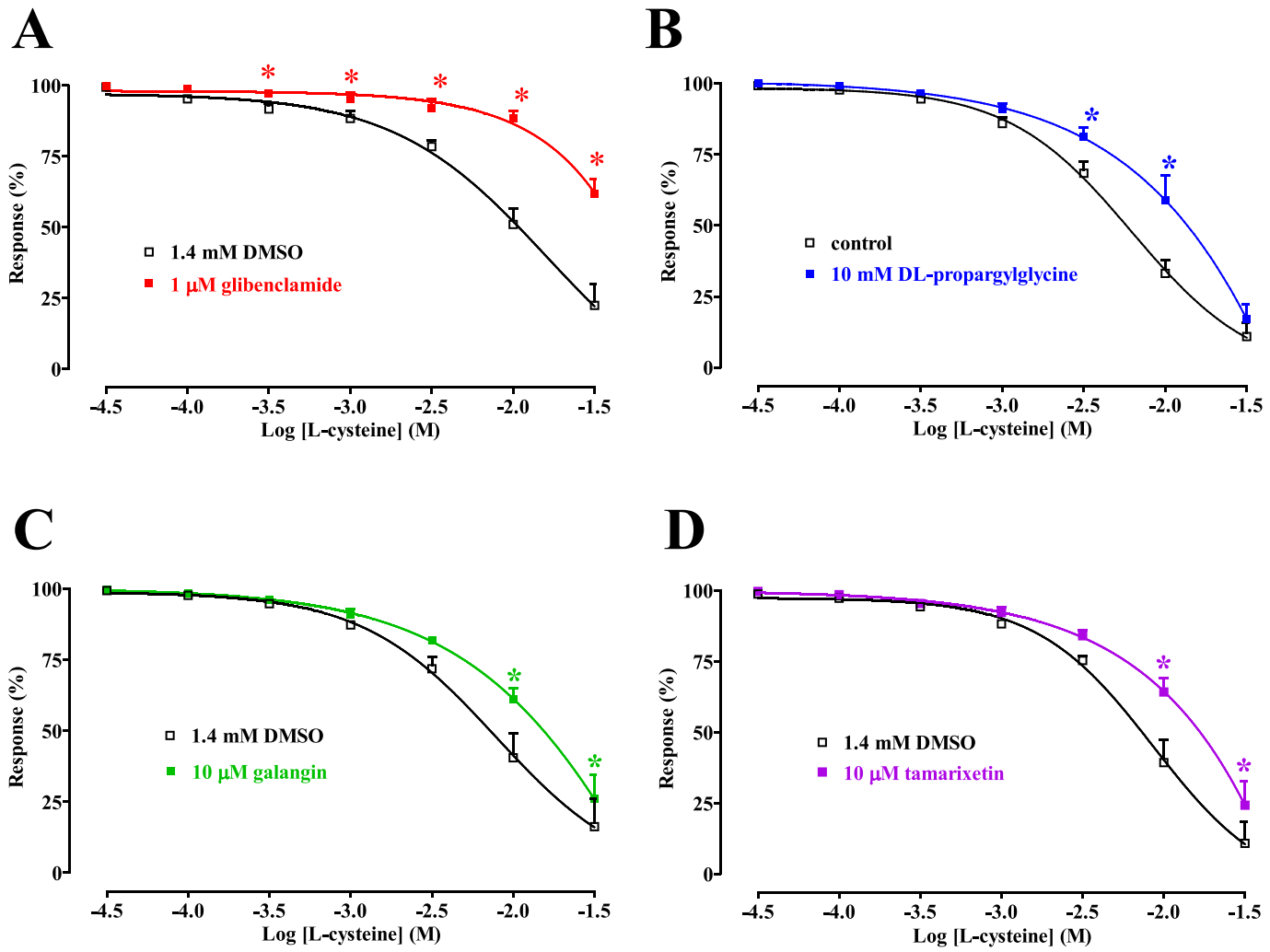

Fig. 8. Effects of flavonoids on L-cysteine-induced relaxation of rat gastric fundus smooth muscle strips pre-contracted by high KCl. Concentration-response curves to L-cysteine constructed in preparations pre-incubated for 20 min with 1.4 mM DMSO or water, (A) 1 μM glibenclamide, (B) 10 mM DL-propargyl glycine (DL-PAG), (C) 10 μM galangin, or (D) 10 μM tamarixetin, and contracted by 25 mM KCl. On the ordinate axis, the response is reported as a percentage of the contraction evoked by 25 mM KCl. Data represent the mean ± SEM (n = 5–7). *P < 0.05 vs. control or DMSO, Student's *t* test for paired samples.

marked in the absence of hydroxyl groups (galangin), but was reduced by the presence of two hydroxyl groups in ortho (in 3′ and 4′ as in quercetin), to a lesser extent if that in the 4′ position (tamarixetin) or the 3′ position (isorhamnetin) was replaced by a methoxy group, and to a higher extent when only one hydroxyl group was present (kaempferol), disappearing when two hydroxyl groups were in meta (in 2′ and 4′ positions as in morin) or three hydroxyl groups were present (myricetin). The inhibitory effect markedly decreased following the loss of the hydroxyl group in 3 (luteolin) or 5 position (fisetin) and disappeared when the C3-C4 double bond was absent. Finally, the presence of two glycosidic groups linked to the 3′ hydroxyl group in ring C caused the disappearance of the inhibition, likely due to the increased steric hindrance of the molecule, which precluded its access to the binding site in the channel protein, as previously observed *in vitro* and analysed *in silico* on vascular $K_{ir}6.1$ channels [17].

Though the precise mechanism through which flavonoids inhibit $K_{ir}6.1$ channels was not investigated, it is possible to speculate that their antioxidant activity is not involved in this effect. Ion channels are regulated by multiple redox mechanisms [34] and by the concentration of free radicals in the vicinity of the channel protein. Quercetin, which inhibits $I_{Kir6.1}$, is an excellent antioxidant and radical scavenger like rutin, myricetin, and morin [35] that, however, are inactive on $K_{ir}6.1$ channels. This evidence is consistent with the hypothesis that flavonoids cannot modulate $I_{Kir6.1}$ by their antioxidant activity.

Some drugs or ions are known to inhibit current flow through ion channels depending on its direction (e.g., [36,37]). The results obtained during the recordings of the more physiologically relevant outward K^+ current demonstrated that at least galangin-induced inhibition is not related to the direction of the current. Furthermore, this experiment suggested that the flavonoid likely affected K^+ channels other than $K_{ir}6.1$, because its inhibitory effect went beyond the outward component elicited by the selective $K_{ir}6.1$ channel activator pinacidil. Future experiments will help clarify this hypothesis.

Docking calculations identified a binding site that might explain the different blocking capacities observed experimentally for flavonoids. Potent blockers showed strong interactions with the loops formed by residues Asn251-Ile254, as also observed for the known $K_{ir}6.1$ channel blocker rosiglitazone. These interactions diminished in weak blockers and were almost abolished in non-blockers. Taken together, these observations suggest that the inhibitory activity of flavonoids depends on the pose they assume around the loop of this binding site. As access to this binding site is allowed only by directly entering the channel cavity from the cytoplasmic side, it is assumed that flavonoids cross the cell membrane to block the channel. The binding of flavonoids to the intracellular loops of the channel might stabilize the structure of its C-terminal domain, thus inhibiting the dimerization of the paired

nucleotide-binding domains, which is required to trigger the activation of the channel. It should be noted that the proposed binding site for potent flavonoid blockers and rosiglitazone differs from the one previously proposed by Chen et al. [27]. This apparent inconsistency could easily be explained by the different models used for the $K_{ir}6.1$ channel, a homology one by Chen et al. [27] and one based on cryo-EM data in the present study.

As $K_{ir}6.1$ channels play an important role in the modulation of gastric tone and accommodation [2], it is conceivable that flavonoids may impact gastric smooth muscle mechanical function. To verify this hypothesis, the effects of quercetin and its structural analogues were assessed on the whole tissue. The two stimulating agents used, namely carbachol and high KCl, characterised by different mechanisms of action, gave rise to stable contractions with pEC_{50} values similar to that described in the literature [38,39]. The six flavonoids selected based on the electrophysiology analysis showed myorelaxant activity in both experimental settings, galangin and luteolin being more effective in depolarised tissue whereas quercetin in carbachol-stimulated ones. These differences may be ascribed to the $Ca_V1.2$ channel blocking and stimulating activities of galangin and quercetin, respectively [40], and to the capacity of luteolin to potentiate gastric cholinergic contractions [41]. Based on the electrophysiology and functional data, the 10 μM concentration was considered a reasonable compromise to investigate the effects of flavonoids on fundus smooth muscle strip $K_{ir}6.1$ channels.

The gastric fundus is a complex system where, in addition to $K_{ir}6.1$ channels, other channels and cellular signalling pathways contribute to the shaping of the smooth muscle tone. Stimulation operated by 25 mM KCl evoked an active tone sufficiently high to allow the study of myorelaxant agents; moreover, depolarization induced by 25 mM KCl is not particularly marked (see [42]) and does not represent a hurdle to the analysis of potassium channel stimulators [43]. In fact, both L-cysteine, a metabolic precursor of H_2S that regulates smooth muscle tone by activating $K_{ir}6.1$ channels [44,45], and the $K_{ir}6.1$ channel activator pinacidil relaxed the high KCl-induced contraction, the latter reverting also the spontaneous tone of the preparations. Furthermore, the specific $K_{ir}6.1$ channel blocker glibenclamide shifted the concentration–response curve to both L-cysteine and pinacidil to the right, displaying a competitive antagonism. Finally, DL-propargylglycine antagonised L-cysteine-induced myorelaxation, a piece of evidence consistent with the conversion of the amino acid into H_2S by cystathionine-γ-lyase. In fact, DL-propargylglycine is a selective and irreversible inhibitor of this enzyme [46], which is more expressed than cystathionine-β-synthase in the gastrointestinal system [47], thus representing the predominant pathway for the production of H_2S in the stomach and intestines of rodents [48]. Taken together, this evidence is consistent with the hypothesis that the myorelaxant action of L-cysteine is specifically due to its conversion to H_2S and the subsequent stimulation of $K_{ir}6.1$ channels. Therefore: 1) $K_{ir}6.1$ channels underpin, at least in part, the spasmolytic effect of both pinacidil and L-cysteine; 2) $K_{ir}6.1$ channels can be activated even in conditions of medium depolarization of the cell membrane, thus contributing to its repolarization, with consequent closure of $Ca_V1.2$ channels and spasmolysis; 3) $K_{ir}6.1$ channels play a significant role in the development of tissue active as well as passive tone. Taken together, these observations demonstrated that the protocol was functional in the study of molecules capable of blocking $K_{ir}6.1$ channels. On the contrary, stimulation produced by carbachol significantly reduced the potency of pinacidil and higher concentrations of glibenclamide were required to detect a significant competitive antagonism, likely due to the blockade of $K_{ir}6.1$ channels consequent to the activation of protein kinase C by the cholinergic agonist [49,50].

When gastric fundus strips were pre-incubated with galangin, tamarixetin, or kaempferol, the concentration–response curve to pinacidil was significantly shifted to the right, similarly to what was observed with glibenclamide. Likewise, galangin and tamarixetin antagonised the vasorelaxant effect of L-cysteine, further supporting the hypothesis that the blockade of $K_{ir}6.1$ channels affects gastric tissue function *in vitro*. The less effective $K_{ir}6.1$ channel blockers fisetin, luteolin, isorhamnetin, and (±)-taxifolin did not modify the response of the preparations to both relaxant agents, confirming the structure–activity relationship previously hypothesized in the electrophysiology study.

$K_{ir}6.x$ channels provide the link between cellular metabolism and electrical activity. Inhibitors of these channels, which are used for the treatment of type 2 diabetes, have recently gained much new interest for their potential usefulness in treating other disease conditions, such as gastrointestinal disorders. In fact, mutations-driven gain-of-function of $K_{ir}6.1$ channels reduces gut contractility and intestinal transit [51]. Therefore, the results obtained in the present study suggest that some dietary, quercetin-like flavonoids might help to normalize gastrointestinal transit in diseases such as Cantú syndrome.

In conclusion, a series of flavonoids structurally related to quercetin are effective spasmolytic agents of gastric fundus smooth muscle stimulated by either electro-mechanical or pharmaco-mechanical coupling. More importantly, this work demonstrates for the first time that they are effective $I_{Kir6.1}$ inhibitors capable of antagonising muscle relaxation induced by the endogenous gaseous transmitter H_2S. As these flavonoids, daily ingested with the diet, can reach high concentrations in the stomach [4], where they can also be absorbed [18], it is conceivable to hypothesize that, once into contact with smooth muscle cells located in the gastric fundus wall, they might affect the tone and motility of this organ during the phases of digestion. Last, but not least, these $K_{ir}6.1$ channel inhibitors might be exploited as tools to unveil the still largely undeveloped pharmacology of some members of this family of channels or as models for the design and synthesis of novel $K_{ir}6.1$ channel modulators.

CRediT authorship contribution statement

Francesco Pettini: Investigation, Validation, Visualization, Writing – original draft. **Ottavia Spiga:** Conceptualization, Supervision, Writing – review & editing. **Simone Furini:** Supervision, Writing – review & editing. **Fabio Fusi:** Conceptualization, Investigation, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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