From in silico to in vitro: a trip to reveal flavonoid binding on the *Rattus norvegicus* Kir6.1 ATP-sensitive inward rectifier potassium channel

Alfonso Trezza¹, Vittoria Cicaloni¹,², Piera Porciatti¹, Andrea Langella¹, Fabio Fusi³, Simona Saponara³ and Ottavia Spiga¹

¹ Department of Biotechnology, Chemistry and Pharmacy, University of Siena, Siena, Italy
² Toscana Life Sciences Foundation, Siena, Italy
³ Department of Life Sciences, University of Siena, Siena, Italy

ABSTRACT

**Background:** ATP-sensitive inward rectifier potassium channels (Kir), are a potassium channel family involved in many physiological processes. K<sub>ATP</sub> dysfunctions are observed in several diseases such as hypoglycaemia, hyperinsulinemia, Prinzmetal angina–like symptoms, cardiovascular diseases.

**Methods:** A broader view of the K<sub>ATP</sub> mechanism is needed in order to operate on their regulation, and in this work we clarify the structure of the *Rattus norvegicus* ATP-sensitive inward rectifier potassium channel 8 (Kir6.1), which has been obtained through a homology modelling procedure. Due to the medical use of flavonoids, a considerable increase in studies on their influence on human health has recently been observed, therefore our aim is to study, through computational methods, the three-dimensional (3D) conformation together with mechanism of action of Kir6.1 with three flavonoids.

**Results:** Computational analysis by performing molecular dynamics (MD) and docking simulation on rat 3D modelled structure have been completed, in its closed and open conformation state and in complex with Quercetin, 5-Hydroxyflavone and Rutin flavonoids. Our study showed that only Quercetin and 5-Hydroxyflavone were responsible for a significant down-regulation of the Kir6.1 activity, stabilising it in a closed conformation. This hypothesis was supported by in vitro experiments demonstrating that Quercetin and 5-Hydroxyflavone were capable to inhibit K<sub>ATP</sub> currents of rat tail main artery myocytes recorded by the patch-clamp technique.

**Conclusion:** Combined methodological approaches, such as molecular modelling, docking and MD simulations of Kir6.1 channel, used to elucidate flavonoids intrinsic mechanism of action, are introduced, revealing a new potential druggable protein site.

Subjects Biochemistry, Bioinformatics, Computational Biology

Keywords Potassium channel, ATP-sensitive inward rectifier potassium channel, Binding site, Homology modeling, Patch clamp, Molecular dynamics, Molecular docking, Kir6.1, Flavonoid

BACKGROUND

Potassium channels are the most various ion channel family group (*Perney & Kaczmarek, 1991; Luneau et al., 1991*). Each category of K<sup>+</sup> channel is activated by several signals and
environments depending on their nature of regulation: some open in reaction to
depolarisation of the plasma membrane; others in reaction to hyperpolarisation or a
growth in intracellular calcium concentration; some can be regulated after the binding
of a transmitter, others are regulated by GTP-binding proteins or other messengers
(Schwarz et al., 1988). Inwardly rectifying potassium channels (Kir) are the main
important group of two TMD potassium channels. The Kir superfamily comprises 16
members in seven different subfamilies, from the Kir1 to the Kir7 (Doupnik, Davidson &
Lester, 1995; Lu, 2004) and their function is influenced by their gating features, which is
attended by conformational transitions. Four Kir subunits are assembled into a tetrameric
channel complex which is composed by hetero or homomeric subunits (Glaaser &
Slesinger, 2015). Inwardly rectifying potassium channels is a ubiquitous potassium
channel family ordered in two transmembrane domains (TMDs) which regulate several
physiological processes including cellular excitability, heart rate, vascular tone, renal salt
flow and insulin release (Minor et al., 1999). Physiological activity and role of Kir channels
depend on regulation of the pore opening, ion flux and channel position (Meng et al.,
2016). Our study was focused on Rattus norvegicus ATP-sensitive inward rectifier
potassium channel 8 (Kir6.1) belonging to the K_{ATP} subfamily (Stephan et al., 2006). It
shows four subunits with two TMDs that are linked with a P-domain (P loop), the latter
representing the ion-selective domain (selectivity filter). All K^+ channels have a core of
alpha subunits, each comprising one or two copies of conserved pore loop domain
(P-domain), which contains the sequence (T/SxxTxGxG) (Miller, 2000). A second ‘pore’
known as G loop, is localised in the cytoplasmic domain (CTD), where the pore is a
typical architecture of Kir channels (Li et al., 2014). Thus, three gates are distributed along
the permeation way: selective filter gate, the bundle-crossing gate in transmembrane pore
and G loop gate, in the cytoplasmic one. The TMD and CTD are linked by about 20 amino
acids and four supporting proteins, the sulfonylurea receptors, which envelop the four
subunits of K_{ATP} forming a hetero-octameric complex (Sepúlveda et al., 2015). The Kir
channel open-closed mechanism depends on different conformational changes which
regulate its state (Li et al., 2016; Lü et al., 2016). Small molecules regulate functions of Kir
channels for instance: H^+, Mg^{2+}, Na^+; polyanines, phosphorylation and membrane-bound phospholipids and proteins (Xie et al., 2007; Hibino et al., 2010; Li et al., 2016; Fowler et al., 2014). K_{ATP} channels act as an endogenous homeostatic transducer in
response to an altered demand (Carrasco et al., 2001; Zingman et al., 2002; Miki et al.,
2002; Gumina et al., 2003). In the heart, they protect against ischemia metabolic insult
and contribute, as molecular mediator, to the adaptive response to stress. They regulate
vascular tone, metabolic resource delivery (Lawson & Dunne, 2001; Cole & Clément-
Chomienne, 2003) and are crucial in blood–glucose level setting by regulating pancreatic
β-cells insulin secretion and skeletal muscle insulin-dependent glucose uptake
(Aguilar-Bryan, Bryan & Nakazaki, 2001; Minami et al., 2004). Similarly, in the brain,
K_{ATP} channel stimulation has a protective role against metabolic challenge (Yamada et al.,
2001). Therefore, K_{ATP} channels, combined with cellular and systemic metabolism, act at
various levels to guarantee metabolic health under stress challenge (Zingman et al., 2002).
Potassium channel openers play a role in matching membrane electrical excitability
with variations in energetic state, and also in preserving metabolic expenditure (Yamada, 2010), making these molecules cytoprotective agents under varied conditions (Coghlan, Carroll & Gopalakrishnan, 2001; Jahangir, Terzic & Shen, 2001; Campbell, Sansom & Ashcroft, 2003; Mannhold, 2004). Thus, potassium channel openers could have important benefit as myocardial protectors, antihypertensive vasodilators, bronchodilators, bladder relaxants, islet cell protectors and antiepileptics (Yokoshiki et al., 1998; Tinker, Aziz & Thomas, 2014; Villa & Combi, 2016). Li et al. (2017) and Martin et al. (2017) discovered the first structure of a Pancreatic ATP-sensitive Potassium Channel in its closed state (Kir6.2), elucidating many of its structural and regulating aspects (Martin et al., 2017; Li et al., 2017).

Several studies showed that Kir channel have the capacity to interact with different types of molecules (Mackie et al., 1995; Zhang et al., 2011; Gribble & Reimann, 2002; Miura & Miki, 2003; Kaufmann et al., 2013; Matsushita & Puro, 2006; Pattnaik & Hughes, 2009), among these flavonoids (Chiang et al., 2002; Macêdo et al., 2014; Ogata et al., 1997; Ma et al., 2014). Flavonoids are natural polyphenolic agents found in all plants (Mattila, Astola & Kumpulainen, 2000) and usually consumed in significant amounts from beverages, fruits and vegetables. Flavonoids have important health benefits: decreasing heart disease (Renaud & de Lorgeril, 1992), giving a protective role against cancers (Kandaswami et al., 1991; Hertog et al., 1995) and neurodegenerative diseases (Mandel & Youdim, 2004). Thus, flavonoids could be considered an effective source of compounds for identifying compounds with different mechanisms of action. It is commonly known that some flavonoids interact with inward rectifier potassium ion channel (Kir) and they are able to inhibit them, as genistein, an isoflavone with inhibitory activity among three members of Kir family (Kir 2.3, Kir 2.1 and Kir 3.4), resulting in beneficial effects on the cardiovascular system (Zhao et al., 2008) and beneficial epidemiological effects (Huxley & Neil, 2003). For instance, naringin and naringenin, are bioflavanoids found in grapefruit. Yow et al. (2011) explained the mechanism of their action on the potential binding site of Kir3 channels: naringin but not naringenin activates Kir3 channels. Thus, a deeper understanding about the effects of flavonoids on vascular potassium currents, in particular on Kir, is needed in order to elucidate flavonoids’ intrinsic mechanism of action and to reveal potential druggable protein sites. As reported in previous work, the flavonoid genistein is responsible for the inhibition of the most prominent flavonoids in fruits and vegetables are flavonols, and, of these, Quercetin is the most commonly taken in with human diet, for this reason we chose Quercetin to start our flavonoids–Kir channel interactions study (Ko et al., 2009).

Unfortunately, Kir6.2 has been resolved with a low resolution and no resolved $K_{\text{ATP}}$ open structure is currently available. Despite previous works demonstrating the involvement of Kir CTD in the gating regulation and a G loop crucial involvement in the $K_{\text{ATP}}$ channels gating process (Pegan et al., 2005; Hansen, Tao & MacKinnon, 2011; Li et al., 2015), many aspects of its structure, mechanism and modulation remain still obscure. Using a bioinformatics approach, we modelled the structure of the closed and open state of the ATP-sensitive inward rectifier potassium channel 8 and revealed an inhibition role of flavonoids against Kir6.1.
MATERIALS AND METHODS

Homology modelling
The primary sequences of *R. norvegicus* inward rectifier K\(^+\) channel Kir6.1 (Q63664 Uniprot code) were acquired from Uniprot in FASTA format ([The UniProt Consortium, 2017](https://www.uniprot.org)). PHYRE2 and I-TASSER server ([Kelley et al., 2015](https://journals.plos.org/plosone/article?id=10.1371/journal.pone.0159636); [Wang et al., 2017](https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5277693/)) were used in order to achieve a protein structure prediction. Template crystal structure of the G protein-gated inward rectifier K\(^+\) channel GIRK2 in closed and open state with 3SYA and 3SYQ PDB code respectively was chosen for its genetic relationship and it was downloaded from RCSB Protein Data Bank ([Whorton & MacKinnon, 2011](https://www.rcsb.org/proteins/detail/3SYA)). Model optimisation was completed using Ramachandran plot calculations which were computed with the PROCHECK program ([Laskowski, 2003](https://www.ncbi.nlm.nih.gov/pmc/articles/PMC457473/)). Energy minimisation protocol was carried out on Kir6.1 three-dimensional (3D) models by using GROMACS 4.5. Root mean square deviation (RMSD) was computed using GROMACS 4.5 software package ([Pronk et al., 2013](https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5028151/)).

Molecular docking
The molecular structure of Quercetin, 5-Hydroxyflavone and Rutin (5280343, 68112, 5280805 PubChem CID respectively) were acquired through PubChem in sdf format ([Kim et al., 2016](https://pubchem.ncbi.nlm.nih.gov/compound/Quercetin)). A docking simulation study of ligands against Kir6.1 channel closed state was performed by using flexible side chains protocol based on Iterated Local Search Global Optimizer Algorithm of AutoDock/VinaXB ([Trott & Olson, 2010](https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3141034/)). The pdbqt format, essential for docking simulation, were generated by using Open Babel tools, adding Gasteiger charge ([O’Boyle et al., 2011](https://pubs.acs.org/doi/10.1021/acs.jchemed.0c00296)), whereas the pdbqt format of proteins were generated using a scripts included in the Autodock/VinaXB tools. Protein–ligands network interaction was evaluated with protein–ligand interaction profiler (PLIP) ([Salentin et al., 2015](https://pubs.acs.org/doi/10.1021/acs.jchemed.0c00562)). PyMOL 1.7.6.0 was used as molecular graphics system (The PyMOL Molecular Graphics System, Version 1.8; Schrödinger, LLC, New York, NY, USA). Through the use of ABS-scan tool 2 ([Anand et al., 2014](https://pubs.acs.org/doi/10.1021/acs.jchemed.0c00562)) an in silico alanine scanning mutagenesis was carried out. The amino acid residues involved in the binding site were computationally substituted to alanine and their interactions energy were recalculated. The obtained ΔΔΔG values were computed by comparing them with the wild type sequence allowing the individual evaluation of each residue contribution.

Molecular dynamics simulation
Molecular dynamics (MD) protocol was applied for closed and open state of inward rectifier K\(^+\) channel Kir6.1. Furthermore, we carried out a MD simulation against Kir6.1-Quercetin and 5-Hydroxyflavone complex. MD simulations of Kir6.1 channel in closed and open state were implemented using the GROMACS version 4.5.5 package with GROMOS 53A6 force field ([Pronk et al., 2013](https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5028151/)). The channels were immersed in an explicit palmitoyloleoyl-phosphatidylcholine (POPC) bilayer ([Li et al., 2015](https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4450851/); [Haider et al., 2007](https://www.sciencedirect.com/science/article/pii/S0304416507001498)). The proteins were placed in a cubic box solvated with TIP3P type of water molecules. The systems were neutralised with Cl\(^-\) counter ions and a concentration of 0.1 M NaCl was added to the system. Both the proteins were energetically minimised using the
The systems were equilibrated with NVT and NPT ensemble protocols for 100 and 500 ps respectively. The temperature of the simulation system was set to 300 K. The MD simulations were run with 2 fs time steps and they were performed for 50,000 ps (50 ns). MD simulation against Kir6.1-Quercetin and 5-Hydroxyflavone complex was equally performed using GROMACS version 4.5.5 package but differs from the previous ones in the applied force field (GROMOS96 43A1). The initial complex structures were obtained following a docking simulation. Topology and all parameters of ligands were evaluated and downloaded through PRODRG server (Schüttelkopf & van Aalten, 2004). The complexes were solvated in a cubic box containing a simple point charge as type of water molecules. The systems were neutralised and the NVT, NPT and MD protocols were applied.

**Cell isolation procedure**

Smooth muscle cells were freshly isolated from the tail main artery by means of collagenase (type XI) treatment, as described by Mugnai et al. (2014). All animal care and experimental protocols conformed to the European Union Guidelines for the Care and the Use of Laboratory Animals (European Union Directive 2010/63/EU) and had been approved by the Italian Department of Health (666/2015-PR).

**Whole-cell patch clamp recordings**

The conventional whole-cell patch-clamp method was employed to record Kir\textsubscript{ATP} currents at room temperature (20–22 °C), as described by Liang et al. (2011). Borosilicate glass recording electrodes had a pipette resistance of 2–5 MΩ. Membrane currents, low-pass filtered at 1 kHz and digitised at 3 kHz, were recorded at a steady membrane potential \(V_h\) of −50 mV, using a continuous gap-free acquisition protocol, by means of an Axopatch 200B patch-clamp amplifier (Molecular Devices Corporation, Sunnyvale, CA, USA). The osmolarity of the external solution (330 mosmol) and that of the internal solution (304 mosmol) were measured with an osmometer (Osmostat OM 6020; Menarini Diagnostics, Florence, Italy). Kir\textsubscript{ATP} current values were corrected for leakage using 10 \(\mu\)M glybenclamide, which blocked Kir\textsubscript{ATP} currents.

**K\textsubscript{ATP} current recording**

External solution contained (in mM): 25 NaCl, 140 KCl, 10 HEPES, 10 glucose, 1 MgCl\(_2\), 0.1 CaCl\(_2\) and 1 tetraethylammonium (TEA); pH was adjusted to 7.4 with NaOH. The internal solution consisted of (in mM): 140 KCl, 10 HEPES, 10 EGTA, 1 MgCl\(_2\), 5 glucose, 0.1 Na\(_2\)ATP, 1 KADP and 0.1 Na\(_2\)GTP; pH was adjusted to 7.3 with KOH. To minimise voltage-dependent K\(^+\) currents, K\textsubscript{ATP} currents were recorded at a steady membrane potential \(V_h\) of −50 mV using a continuous gap-free acquisition protocol. Current values were corrected for leakage using 10 \(\mu\)M glybenclamide, which completely blocked K\textsubscript{ATP} currents.

**Chemicals**

Collagenase (type XI), trypsin inhibitor, bovine serum albumin, tetraethylammonium chloride, EGTA, HEPES, taurine, pinacidil and glybenclamide were from Sigma Chimica.
(Milan, Italy). Stock solutions of Quercetin, 5-Hydroxyflavone, Rutin, pinacidil and glybenclamide, dissolved directly in DMSO, were stored at −20 °C and protected from light. DMSO and ethanol (below 0.1%, v/v) did not affect current amplitude.

**Statistical analysis**

pClamp 9.2.1.8 software (Molecular Devices Corporation, Sunnyvale, CA, USA) and GraphPad Prism version 5.04 (GraphPad Software Inc., San Diego, CA, USA) were used to analyse the data. Data are reported as mean ± SEM; n is the number of cells analysed (indicated in parentheses), isolated from at least three animals. Statistical comparisons were performed by either one-way ANOVA (followed by Dunnett post-hoc test) or Student’s t-test for paired samples (two tailed) (GraphPad Prism version 5.04). Post-hoc tests were performed only when ANOVA found a significant value of F and no variance in homogeneity. In all comparisons, P < 0.05 was considered significant.

**RESULTS**

The capacity of Quercetin to interact with Kir6.1 channel was firstly tested with the in vitro assay. The patch clamp technique results obtained by testing Quercetin on Kir6.1 channel of *Rat norvegicus* aorta shows its inhibiting activity Fig. 1. We use computational studies to identify the potential binding pocket of this specific inhibitor and its drug ability against the Rat Kir6.1 protein.

BLAST-Protein analysis results revealed a perfect reliable template, i.e. crystal structures of Kir6.2 from Rat and Human with a 6.3 and 5.6 Å resolutions (PDB ID: 5TWV and 5WUA) shared 90% query coverage and 70.42% identity with 0.0 E-value, but its refinement does not seem to be trustworthy with this low templates resolution. Attempts to optimise our model, through homology modelling, were carried out in order to achieve the 3D structure of the *R. norvegicus* Kir6.1, choosing as template the crystal structure of Kir3.2 with 2.98 Å resolution. The chosen template is member of the same Kir channel family with 50% identity and 78% conservative amino acids respectively in its closed and open states (PDB ID: 3SYA and 3SYQ). Based on alignment, two different 3D models were generated for target protein by using PHYRE2 and I-TASSER (Kelley et al., 2015; Wang et al., 2017) modelling and the missing sidechain were added and aligned from SwissPDBViewer v3.7 program (Johansson et al., 2012). Structural superimposition between Kir6.1 model and X-ray structures 3SYA and 3SYQ reported in Fig. 2, showed a high structural similarity with backbone RMSD values of 0.55 and 0.74 Å respectively. Structure validation results stated that homology modelled protein *R. norvegicus* Kir6.1 possesses reasonable 3D structure with good stereo-chemical quality of Ramachandran plot where PROCHECK analysis showed most favoured regions with 97.2% and 97.3% of the amino acid residues for closed and open state respectively. The validated structures of rat Kir6.1 were further subjected to an energy minimisation to get a reliable conformation in order to proceed for molecular docking studies. FTsite program (Ngan et al., 2012) analysis was needed in order to obtain reasonable size binding-pockets of the protein models which could be involved in interactions with
Quercetin. FTsite was performed on both of states, however, no reliable volume binding-site, needed by Quercetin molecule, was detected in the open one.

The most interesting observed pocket is a conservative site, consisting in physical gates formed by cytoplasmic (G loop) region (Hibino et al., 2010), necessary for the regulation of the inward potassium current that obviously depends on the state of the gate (Lü et al., 2016). The G loop has a crucial role with their amino acid composition in the regulation of $K_{ATP}$ gating kinetics, different previous works shown how mutations of the amino acid of this region are responsible of protein inactivation.

**Figure 1** Effect of quercetin on $K_{ATP}$ currents of isolated rat tail artery myocytes. (A) Representative whole-cell recordings of inward currents elicited by pinacidil at a $V_h$ of $-50$ mV. The effect of quercetin as well as glibenclamide is shown. (B) Pinacidil (10 μM; pina) activated glibenclamide (gli)-sensitive $K_{ATP}$ currents, which were inhibited by 10 μM quercetin (que). Columns are mean ± SEM ($n$ = 6). *$P < 0.05$ vs. pinacidil alone, repeated measures ANOVA and Bonferroni post-test.

Quercetin. FTsite was performed on both of states, however, no reliable volume binding-site, needed by Quercetin molecule, was detected in the open one.

The most interesting observed pocket is a conservative site, consisting in physical gates formed by cytoplasmic (G loop) region (Hibino et al., 2010), necessary for the regulation of the inward potassium current that obviously depends on the state of the gate (Lü et al., 2016). The G loop has a crucial role with their amino acid composition in the regulation of $K_{ATP}$ gating kinetics, different previous works shown how mutations of the amino acid of this region are responsible of protein inactivation.
(Shimomura et al., 2009; Li et al., 2016; Nishida et al., 2007; Pegan et al., 2006; Hattersley & Ashcroft, 2005; Proks et al., 2005).

On the basis of above mentioned studies, we focused our analysis on the site involving G loop region. The virtual docking, based on virtual screening using AutoDock/VinaXB (Trott & Olson, 2010), was firstly carried out with the ligand Quercetin, in order to confirm the reliability of G loop as pocket and interaction site. The ligand–protein interactions were analysed through PLIP bioinformatics tool (Salentin et al., 2015). The data reported in Fig. 3 show that Quercetin formed hydrogen bonds with the amino-groups of Asn-258 and Asn-252 of the chain A, with the hydroxyl group of Ser-222 of the chain A and with the hydroxyl group of the Thr-306 of the chains A and C. The hydrophobic interactions took place between the hydrophobic portion of Quercetin and the hydrophobic-sensing Ile-221 and Val-299 of the chain A. All these interactions take place near the G loop residues and could stabilise the $\text{K}_\text{ATP}$ channel in a closed state (Li et al., 2016). The capacity of Quercetin to inhibit Kir6.1 protein is in agreement with already published data (Chiang et al., 2002; Xu et al., 2015; Kaufmann et al., 2013; Ogata et al., 1997; Matsushita & Puro, 2006; Pattnaik & Hughes, 2009; Ma et al., 2014), thus to validate the hypothesis hydrogen bonds network holds a crucial role in the complex stabilisation, we chose two analogue molecules 5-Hydroxyflavone (different from Quercetin for a reduced number of OH groups) and Rutin (characterised by a bulky glycoside group not present in the other ligands), structures reported in Fig. 4, and their affinity was also tested with a docking simulation in G loop region. All ligands were flexible and free to bind in the proposed binding site. Docking simulations were determined for each ligand with 10 exhaustiveness as default parameter, and the active site
grid dimensions were set at $X = 54.41\ \text{Å}$, $Y = 30.78\ \text{Å}$ and $Z = 79.94\ \text{Å}$. In the docking simulation, the protein was maintained unflexible whereas the ligands and the pocket amino acids were flexible, with docking score output results representing the apparent Gibbs free energy of binding ($\Delta G_{\text{app}}$). Only two studied compounds, Quercetin and

**Figure 3** Docking results. The side view of FTsite predicted Kir6.1 binding region (red mesh) (A). The top view from the cytoplasmic side of binding region with flavonoid ligands (B). Representation of binding pocket complexed with Quercetin in green (C) and 5-Hydroxyflavone in blue (D), and binding interaction residues (green ball and sticks) after 50 ns of MD simulations.

**Figure 4** Structural 2D representations of flavonoids in ball and stick. (A) Quercetin, (B) Rutin, (C) 5-Hydroxyflavone.
5-Hydroxyflavone, manifested the capability to fit inside the pocket and to have a good binding affinity to the channel, presenting \(-8.1\) and \(-6.7\) kcal/mol \(\Delta G\) values binding energies. In contrast, Rutin, despite the active site grid dimension would permit to allocate it, was not able to bind the binding site, possibly because of steric hindrance due to the presence of the disaccharide Rutinose. The negative values of predicted \(\Delta G_{\text{app}}\) indicated that the two molecules bind to the pocket spontaneously suggesting their potential channel inhibitory binding activities. The two compounds occupied the same cavity with few amino acid changes contribution due to their conformational discrepancy and angle rotation. The variance in \(\Delta G\) values and binding pose inside the pocket may be attributed to the differences in position of the functional groups in the two compounds. The docking results of the compounds analysed by PLIP tool (Salentin et al., 2015), giving an interaction diagram and a table of interaction data, were compared in Fig. 3. In both compounds the binding was dominated by hydrophobic interactions and hydrogen bonds, nevertheless as previously supposed, we observed a remarkable difference in their binding affinities. The explanation to this behaviour is likely due to the different hydroxyl groups present in the molecule. The Quercetin presents five hydroxyl groups whereas 5-Hydroxyflavone presents only one hydroxyl group, this difference would seem to be crucial on their mechanism of action, because Quercetin was able to form up to five hydrogen bonds, while 5-Hydroxyflavone could form only two (Fig. 3). We propose that the hydrogen bonds network, established with G loop residues, can be essential to determine inhibitor activity of different ligands. It is likely a hydrogen bond network stabilises the Kir6.1 in the closed state, likewise the presence of ligands in proximity of the pore channel would decrease solvent-accessible surface area, blocking the potassium flow (Schüttelkopf & van Aalten, 2004). Furthermore, we did not observe any Rutin docked to Kir6.1, we explain this on the basis that the binding site is not able to accommodate Rutin, given the presence of the bulky disaccharide Rutinose. After that, MD simulations were performed to evaluate the stability of the predicted 3D structure of the \(R.\) norvegicus Kir6.1, and its complex with Quercetin and 5-Hydroxyflavone with detailed interactions shown in Fig. 3. Often, the Homology model with further MD simulations in a entirely hydrated lipid bilayer is suitable for identifying the significant structural and dynamical data before a high-resolution experimental structure becomes available. Thus, the inherent dynamics and structural stability of TM and CTDs were investigated. The initially applied harmonic restraints on the protein backbone were gradually released during the course of the second 1 ns simulation followed by additional 50 ns MD simulations without any restraints (Ismail & Jusoh, 2016; Haider et al., 2007; Lü et al., 2016), specifying that the topology of the channel was maintained under the condition of no restraints (Figs. 5 and 6). The plot profiles suggest that the complexes with Quercetin and 5-Hydroxyflavone are able to decrease the radius of gyration in comparison with the unbound structure, Fig. 7. Additionally, we estimated the distance between key amino acids, considering \(C_z\) of Thr-A306 and Thr-C306 in Kir6.1 closed, open and complex states (Fig. 7). The analysis showed that the distance was bigger in the open than in the closed and complex state, interestingly, we can observe the similar profile of closed and complex state, indicating the ability of ligands to stabilise the channel in a ‘closed-like’
conformation. In addition, the radius of gyration ($R_g$) was calculated in order to verify the compactness of the protein in absence or presence of ligands, as reported in Fig. 7. The different values of the $R_g$ for Kir6.1 in the closed and complex state, might be due to different interactions between Quercetin and 5-Hydroxyflavone against Kir6.1 binding site, which would compact the structure.

In silico analysis of the effect of Quercetin, 5-Hydroxyflavone and Rutin on $K_{ATP}$ channels was assessed in vitro in single myocytes, freshly isolated from the rat tail main artery, by using the conventional whole cell patch-clamp configuration. To limit activation of voltage-dependent $K^+$ channels and large conductance $Ca^{2+}$-activated $K^+$ channels, $K_{ATP}$ currents were elicited at a $V_h$ of $-50$ mV in presence of $0.1$ mM ATP and $1$ mM ADP in the pipette solution and $1$ mM TEA in the external solution. When myocytes were challenged with the $K_{ATP}$ channel opener pinacidil (10 $\mu$M), an inward current activated ($-1.62 \pm 0.17$ pA/pF, $n = 5$). This was significantly antagonised by the $K_{ATP}$ channel inhibitor glibenclamide (10 $\mu$M; $-0.09 \pm 0.02$ pA/pF, $n = 5$; $P = 0.0005$). As shown in Figs. 8A–8C, Quercetin, 5-Hydroxyflavone but not Rutin significantly inhibited the glibenclamide-sensitive currents recorded in the presence of pinacidil. Their inhibitory
Figure 7 3D structure and MD simulations comparison. On the top binding pocket of Kir6.1 and the residues that comprise it in the open (A) and closed (B) state. It is mainly composed by the amino acids of G loop (thin sticks) with different positions and dimensions of the binding pocket. In (A–D) are the same binding pockets after molecular dynamics simulation (C) of 5-Hydroxyflavone (blue bold sticks) and (D) Quercetin (green bold sticks) complexes obtained from molecular docking analysis. The pocket’s dimensions are highlighted with dotted yellow lines. In (E–G) three plots for the deviations time evolution, with black line representing open state, red line closed state, blue line complexed with 5-Hydroxyflavone and green line complexed with Quercetin, i.e. (E) Radius of gyration ($R_g$) representing the protein stability against the axial force; (F) distances time evolution between $\text{Ca}_{\text{Thr306A-Thr306C}}$ of residues Thr-A306 and Thr-C306, selected as reference residues of binding pocket of four MD simulations; and (G) distance profile of hydrogen bonds network during simulation between Quercetin green and 5-Hydroxyflavone, the hydrogen bonds network of Quercetin is included in a range of 0.2–0.3 nm, while, the 5-Hydroxyflavone shows a range of 0.3–0.45 nm. Such evidence shows us the greatest strength and stability of the hydrogen bonds network of the Quercetin in comparison with 5-Hydroxyflavone.

Full-size DOI: 10.7717/peerj.4680/fig-7
efficacy, however, was strikingly different (Fig. 8D), Quercetin being the most effective compound. In fact, when the concentration of Quercetin was reduced to 10 \( \mu \text{M} \), current inhibition was still higher than that exerted by a fivefold greater concentration of 5-Hydroxyflavone.

Furthermore, in order to evaluate the energetic contribution of each residue involved in the interaction with the Quercetin and 5-Hydroxyflavone, we carried out an alanine-scanning simulation (Fig. 9). In Fig. 9A we reported the single contribution of residues in \( \Delta \Delta G \) terms, while in Figs. 9B and 9C was shown the docked pose of ligands inside the binding pocket. Is very interesting to note as the Quercetin (red tower) is able to bind to a major number of residues and with an higher \( \Delta \Delta G \) value in comparison with the 5-Hydroxyflavone (blue tower), these difference could be very significant about a different activity of ligands against the Kir6.1, indicating a greater inhibitory effect of the Quercetin.

Figure 8  Patch clamp assay on flavonoids. Effect of various flavonoids on \( K_{\text{ATP}} \) currents of rat tail artery myocytes. (A–C) Pinacidil (10 \( \mu \text{M} \)) activated glibenclamide-sensitive \( K_{\text{ATP}} \) currents, which were inhibited by (A) Quercetin (que, 50 \( \mu \text{M} \)) and (B) 5-Hydroxyflavone (5-OH, 50 \( \mu \text{M} \)), but not by (C) Rutin (50 \( \mu \text{M} \)). * \( P < 0.05 \) vs. pinacidil alone, Student’s \( t \)-test for paired samples. Insets: representative recordings of inward currents elicited by pinacidil at a \( V_h \) of 50 mV. The effect of quercetin, 5-hydroxyflavone and rutin as well as glibenclamide is shown. (D) Residual \( K_{\text{ATP}} \) current measured in myocytes challenged with the three flavonoids and calculated from (A–C). Columns are mean ± SEM (\( n = 5–9 \)). * \( P < 0.05 \) vs. 50 \( \mu \text{M} \) quercetin, one-way ANOVA followed by Dunnet post-hoc test.

Full-size DOI: 10.7717/peerj.4680/fig-8
ATP-sensitive inward rectifier potassium channel 6.1 is a potassium channel involved in many biological processes and their dysfunctions have been underlined in different pathologies. An accurate assessment of its mechanism and the identification of its potential inhibitor-binding site could be very useful for clarifying many features of Kir6.1. On the basis of knowledge that some flavonoids interact with inward rectifier potassium ion channel and their beneficial effects on the cardiovascular system we initially select Quercetin as potential candidate to identify the potential binding pocket and to understand the mechanism of action of potential inhibitors. A patch clamp technique was firstly carried out to test Quercetin effects on Kir6.1 (Fig. 1). Thus, a 3D study of this interaction was necessary to identify its mechanism of action.

Homology modelling was carried out in order to obtain a structure of Ki6.1 both in closed and open state, a protein phylogenetically correlated to the Kir6.1 was chosen as template, the crystal structure of the Kir3.2 in closed and open state (Fig. 2). The derived Kir6.1 model has been used to explore the binding mode of some flavonoids. We focused our attention on Quercetin, 5-Hydroxyflavone and Rutin (Fig. 3), in order to propose a potential consensus binding site of Kir6.1 and a flavonoid mechanism of action inside the binding pocket. Through bioinformatics approaches and previous works (Hibino et al., 2010), we identified the G loop as potential binding site for ligands (Lü et al., 2016; Shimomura et al., 2009; Li et al., 2016; Nishida et al., 2007; Pegan et al., 2006; Hattersley & Ashcroft, 2005; Proks et al., 2005). Docking simulation and different MD simulation protocols were accomplished for explaining how the flavonoids influenced the gating process of the channel. From our results, we observed that only two flavonoids, Quercetin and 5-Hydroxyflavone, were superimposed in the G loop region showing a high apparent affinity, while Rutin, possessing a bulky glycoside group (not present in Quercetin and 5-Hydroxyflavone) was not able to bind in same region (Fig. 3). In addition, the hydrogen bonds network of flavonoids in the binding site seems to be crucial for their mechanism of action. The presence of more hydroxyl groups on Quercetin could explain its greater inhibitory effect than 5-Hydroxyflavone; moreover, we showed that the ligands were able to stabilise the Kir6.1 in the closed conformation. The obtained results permitted us to classify binding site forming-residues as attachments for the inhibitor recognition process; furthermore, the interactions with the G loop pocket Ser-A222, Asn-A252, Asn-A258, Thr-A306, Thr-C306 seem to be advantageous for targeting Kir6.1 selectivity. In line with docking studies, Quercetin better occupies the binding region compared to 5-Hydroxyflavone.
it performs an extensive network of hydrogen bonds due its hydroxyl groups. Some of these bonds are not very stable, but in any case provide good anchoring points for the inhibitor inside the pocket and justify selectivity and inhibitory affinity over the other two molecules, in agreement with experimental data (Fig. 8). Accordingly, the in silico data were nicely supported by the in vitro analysis of flavonoid activity towards vascular $K_{ATP}$ channel current. Quercetin, being characterised by five OH substituents on the flavonoid scaffold, inhibited the current by about 75%. This observation is in agreement with the inhibitory effect of Quercetin on $K_{ATP}$ channel current of INS-1 cell recently described (Kittl et al., 2016), though in insulinoma cells inhibition was transient and faded over 1 min of exposure to the drug. While 5-Hydroxyflavone showed an inhibitory effect on $K_{ATP}$ channel current, its efficacy was lower than that of Quercetin, well correlating to the reduced number of OH groups of the molecule. Finally, Rutin, though possessing the same hydroxylation pattern of the parent compound Quercetin, was almost inactive as a $K_{ATP}$ channel blocker. The bulky structure of the flavonoid, originating from its glycoside group, might account for its ineffectiveness towards the channel. This is in line with what previously observed on $Ca_{V1.2}$ channel current of rat tail artery myocytes, where Quercetin proved to be a stimulator, Rutin was ineffective and 5-Hydroxyflavone was classified as a weak inhibitor of the current (Saponara et al., 2011). The evidence that their activity varied considerably, following only minor modifications in the molecular structure, further strengthens the theory that vascular channels are targets for flavonoids structurally related to Quercetin (Fusi et al., 2017). In vitro results presented in this work confirmed our computational study, showing that 5-Hydroxyflavone had a lower inhibitory activity than Quercetin of 30%, while no inhibitory effect was observed for Rutin (see Fig. 9), such evidence further confirmed our hypothesis about the existence of a flavonoid binding site on the channel protein. In agreement with these data, the in silico alanine scanning mutagenesis, Fig. 9 showed that $\Delta \Delta G$ profile shared by Quercetin is quite different from 5-Hydroxyflavone, supporting the hypothesis that they docked in the pocket engaging different interaction networks and corroborating results obtained in vitro. The electrophysiological data, obtained under the conventional whole-cell configuration (i.e. in myocytes whose cytoplasm was subjected to extensive dialysis) neither prove a direct interaction with nor preclude an indirect effect on the channel protein. Further experiments (e.g. under the excised patch configuration) are necessary to clarify this issue.

**CONCLUSION**

This manuscript was aimed at investigating an as yet unexplored field such as that of the potential effect of Quercetin on vascular $K_{ATP}$ channel. A feasible mechanism of action, responsible for the current inhibition observed in vitro, was hypothesised by analysing two structurally related compounds, namely 5-Hydroxyflavone and Rutin. We believe that the good correlation found between the in silico and the in vitro results will be interesting and will stimulate further research in the field. In fact, this feature may indeed apply to other flavonoids or even other polyphenols abundantly consumed with our daily diet. Obviously, many other experiments need to be undertaken in order to define the precise
mechanism of action in order to improve the drug design process of $K_{\text{ATP}}$ inhibitors and also in using mutated Kir6.1 channels expressed in heterologous systems.

**ACKNOWLEDGEMENTS**

Thanks are due to Francesco Pettini and Luisa Frusciante for their insightful contribution to this work.

**ADDITIONAL INFORMATION AND DECLARATIONS**

**Funding**
The authors received no funding for this work.

**Competing Interests**
Vittoria Cicaloni is a PhD student with a scholarship financed by Toscana Life Sciences Foundation, Siena, Italy.

**Author Contributions**
- Alfonso Trezza conceived and designed the experiments, analyzed the data, prepared figures and/or tables, authored or reviewed drafts of the paper, approved the final draft.
- Vittoria Cicaloni analyzed the data, authored or reviewed drafts of the paper, approved the final draft.
- Piera Porciatti performed the experiments, prepared figures and/or tables, authored or reviewed drafts of the paper, approved the final draft.
- Andrea Langella prepared figures and/or tables, authored or reviewed drafts of the paper, approved the final draft.
- Fabio Fusi performed the experiments, contributed reagents/materials/analysis tools, authored or reviewed drafts of the paper, approved the final draft.
- Simona Saponara performed the experiments, contributed reagents/materials/analysis tools, authored or reviewed drafts of the paper, approved the final draft.
- Ottavia Spiga conceived and designed the experiments, analyzed the data, contributed reagents/materials/analysis tools, prepared figures and/or tables, authored or reviewed drafts of the paper, approved the final draft.

**Animal Ethics**
The following information was supplied relating to ethical approvals (i.e., approving body and any reference numbers):
All animal care and experimental protocols conformed to the European Union Guidelines for the Care and the Use of Laboratory Animals (European Union Directive 2010/63/EU) and had been approved by the Italian Department of Health (666/2015-PR).

**Data Availability**
The following information was supplied regarding data availability:
The raw data have been provided as a Supplemental File.
Supplemental Information

Supplemental information for this article can be found online at http://dx.doi.org/10.7717/peerj.4680#supplemental-information.

REFERENCES

Aguilar-Bryan L, Bryan J, Nakazaki M. 2001. Of mice and men: K(ATP) channels and insulin secretion. Recent Progress in Hormone Research 56(1):47–68 DOI 10.1210/rp.56.1.47.

Anand P, Nagarajan D, Mukherjee S, Chandra N. 2014. ABS–Scan: in silico alanine scanning mutagenesis for binding site residues in protein–ligand complex. F1000Research 3:214 DOI 10.12688/f1000research.5165.2.

Campbell JD, Sansom MS, Ashcroft FM. 2003. Potassium channel regulation. EMBO Reports 4(11):1038–1042 DOI 10.1038/sj.embor.7400003.

Carrasco AJ, Dzeja PP, Alekseev AE, Pucar D, Zingman LV, Abraham MR, Hodgson D, Bienengraeber M, Puceat M, Janssen E, Wieringa B, Terzic A. 2001. Adenylate kinase phosphotransfer communicates cellular energetic signals to ATP-sensitive potassium channels. Proceedings of the National Academy of Sciences of the United States of America 98(13):7623–7628 DOI 10.1073/pnas.121038198.

Chiang CE, Luk HN, Chen LL, Wang TM, Ding PY. 2002. Genistein inhibits the inward rectifying potassium current in guinea pig ventricular myocytes. Journal of Biomedical Science 9(4):321–326 DOI 10.1159/000065002.

Coghlan MJ, Carroll WA, Gopalakrishnan M. 2001. Recent developments in the biology and medicinal chemistry of potassium channel modulators: update from a decade of progress. Journal of Medicinal Chemistry 44(11):1627–1653 DOI 10.1021/jm000484+

Cole WC, Clément-Chomienne O. 2003. ATP-sensitive K⁺ channels of vascular smooth muscle cells. Journal of Cardiovascular Electrophysiology 14(1):94–103 DOI 10.1046/j.1540-8167.2003.02376.x.

Doupnik CA, Davidson N, Lester HA. 1995. The inward rectifier potassium channel family. Current Opinion in Neurobiology 5(3):268–277 DOI 10.1016/0959-4388(95)80038-7.

Fowler PW, Bollepalli MK, Rapedius M, Nematian-Ardestani E, Shang L, Sansom MS, Tucker SJ, Baukrowitz T. 2014. Insights into the structural nature of the transition state in the Kir channel gating pathway. Channels 8(6):551–555 DOI 10.4161/19336950.2014.962371.

Fusi F, Spiga O, Trezza A, Sgaragli G, Saponara S. 2017. The surge of flavonoids as novel, fine regulators of cardiovascular Cav channels. European Journal of Pharmacology 796:158–174 DOI 10.1016/j.ejphar.2016.12.033.

Glaaser IW, Slesinger PA. 2015. Structural insights into GIRK channel function. International Review of Neurobiology 123:117–160 DOI 10.1016/bs.irn.2015.05.014.

Gribble FM, Reimann F. 2002. Pharmacological modulation of K(ATP) channels. Biochemical Society Transactions 30(2):333–339 DOI 10.1042/bst0300333.

Gumina RJ, Pucar D, Bast P, Hodgson DM, Kurtz CE, Dzeja PP, Miki T, Seino S, Terzic A. 2003. Knockout of Kir6.2 negates ischemic preconditioning-induced protection of myocardial energetics. American Journal of Physiology-Heart and Circulatory Physiology 284(6):H2106–H2113 DOI 10.1152/ajpheart.00057.2003.

Haider S, Khalid S, Tucker SJ, Ashcroft FM, Sansom MS. 2007. Molecular dynamics simulations of inwardly rectifying (Kir) potassium channels: a comparative study. Biochemistry 46(12):3643–3652 DOI 10.1021/bi062210f.

Hansen SB, Tao X, MacKinnon R. 2011. Structural basis of PIP2 activation of the classical inward rectifier K⁺ channel Kir2.2. Nature 477(7365):495–498 DOI 10.1038/nature10370.
Hattersley AT, Ashcroft FM. 2005. Activating mutations in Kir6.2 and neonatal diabetes: new clinical syndromes, new scientific insights, and new therapy. *Diabetes* **54**(9):2503–2513 DOI 10.2337/diabetes.54.9.2503.

Hertog MG, Kromhout D, Aravanis C, Blackburn H, Buzina R, Fidanza F, Giampaoli S, Jansen A, Menotti A, Nedeljkovic S, Pekkarinen M, Simic BS, Toshima H, Feskens EJM, Hollman PCH, Katan MB. 1995. Flavonoid intake and long-term risk of coronary heart disease and cancer in the seven countries study. *Archives of Internal Medicine* **155**(4):381–386 DOI 10.1001/archinte.1995.00430040053006.

Hibino H, Inanobe A, Furutani K, Murakami S, Findlay I, Kurachi Y. 2010. Inwardly rectifying potassium channels: their structure, function, and physiological roles. *Physiological Reviews* **90**(1):291–366 DOI 10.1152/physrev.00021.2009.

Huxley RR, Neil HA. 2003. The relation between dietary flavonol intake and coronary heart disease mortality: a meta-analysis of prospective cohort studies. *European Journal of Clinical Nutrition* **57**(8):904–908 DOI 10.1038/sj.ejcn.1601624.

Ismail NA, Jusoh SA. 2016. Molecular docking and molecular dynamics simulation studies to predict flavonoid binding on the surface of DENV2 E protein. *Interdisciplinary Sciences: Computational Life Sciences* **9**(4):499–511 DOI 10.1007/s12539-016-0157-8.

Jahangir A, Terzic A, Shen WK. 2001. Potassium channel openers: therapeutic potential in cardiology and medicine. *Expert Opinion on Pharmacotherapy* **2**(12):1995–2010 DOI 10.1517/14656566.2.12.1995.

Johansson MU, Zoete V, Michielin O, Guex N. 2012. Defining and searching for structural motifs using DeepView/Swiss-PdbViewer. *BMC Bioinformatics* **13**(1):173 DOI 10.1186/1471-2105-13-173.

Kandaswami C, Perkins E, Soloniuk DS, Drzewiecki G, Middleton E Jr. 1991. Antiproliferative effects of citrus flavonoids on a human squamous cell carcinoma in vitro. *Cancer Letters* **56**(2):147–152 DOI 10.1016/0304-3835(91)90089-z.

Kaufmann K, Romaine I, Days E, Pascual C, Malik A, Yang I, Zou B, Du Y, Sliwoski G, Morrison RD, Denton J, Niswender CM, Daniels JS, Sulikowski GA, Xie XS, Lindsley CW, Weaver CD. 2013. The first potent and selective activator of the GIRK potassium channel, displays antiepileptic properties in mice. *ACS Chemical Neuroscience* **4**(9):1278–1286.

Kelley LA, Mezulis S, Yates CM, Wass MN, Sternberg MJ. 2015. The Phyre2 web portal for protein modelling, prediction and analysis. *Nature Protocols* **10**(6):845–858 DOI 10.1038/nprot.2015.053.

Kim S, Thiessen PA, Bolton EE, Chen J, Fu G, Gindulyte A, Han L, He J, He S, Shoemaker BA, Wang J, Yu B, Zhang J, Bryant SH. 2016. PubChem substance and compound databases. *Nucleic Acids Research* **44**(D1):D1202–D1213 DOI 10.1093/nar/gkv951.

Kittl M, Beyreis M, Tumurkhuu M, Fürst J, Helm K, Pitschmann A, Gaissberger M, Glasl S, Ritter M, Jakab M. 2016. Quercetin stimulates insulin secretion and reduces the viability of rat INS-1 beta-cells. *Cellular Physiology and Biochemistry* **39**(1):278–293 DOI 10.1159/000445623.

Ko EA, Park WS, Son YK, Kim DH, Kim N, Kim HK, Choi TH, Jung ID, Park YM, Han J. 2009. The effect of tyrosine kinase inhibitor genistein on voltage-dependent K+ channels in rabbit coronary arterial smooth muscle cells. *Vascular Pharmacology* **50**(1–2):51–56 DOI 10.1016/j.vph.2008.09.004.

Laskowski RA. 2003. Structural quality assurance. *Methods of Biochemical Analysis* **44**:273–303 DOI 10.1002/0471721204.ch14.

Lawson K, Dunne MJ. 2001. Peripheral channelopathies as targets for potassium channel openers. *Expert Opinion on Investigational Drugs* **10**(7):1345–1359 DOI 10.1517/13543784.10.7.1345.
Li J, Liu S, Liu Y, Pang C, Chen Y, Zhang S, Yu H, Long M, Zhang H, Logothetis DE, Zhan Y, An H. 2015. Identification of the Conformational transition pathway in PIP2 Opening Kir Channels. Scientific Reports 5(1):11289 DOI 10.1038/srep11289.

Li N, Wu JX, Ding D, Cheng J, Gao N, Chen L. 2017. Structure of a pancreatic ATP-sensitive potassium channel. Cell 168(1–2):101–110.e1 DOI 10.1016/j.cell.2016.12.028.

Li J, Xiao S, Xie X, Zhou H, Pang C, Li S, Zhang H, Logothetis DE, Zhan Y, An H. 2016. Three pairs of weak interactions precisely regulate the G-loop gate of Kir2.1 channel. Proteins: Structure, Function, and Bioinformatics 84(12):1929–1937 DOI 10.1002/prot.25176.

Li J, Xie X, Liu J, Yu H, Zhang S, Zhan Y, Zhang H, Logothetis DE, An H. 2014. Lack of negatively charged residues at the external mouth of Kir2.2 channels enable the voltage-dependent block by external Mg2+. PLOS ONE 9(10):e111372 DOI 10.1371/journal.pone.0111372.

Liang GH, Adebiyi A, Leo MD, McNally EM, Leffler CW, Jaggar JH. 2011. Hydrogen sulfide dilates cerebral arterioles by activating smooth muscle cell plasma membrane K<sub>ATP</sub> channels. American Journal of Physiology-Heart and Circulatory Physiology 300(6):H2088–H2095 DOI 10.1152/ajpheart.01290.2010.

Lu Z. 2004. Mechanism of rectification in inward-rectifier K<sup>+</sup> channels. Annual Review of Physiology 66(1):103–129 DOI 10.1146/annurev.physiol.66.032102.150822.

Lu S, An H, Zhang H, Long M. 2016. Structural basis for differences in dynamics induced by Leu versus Ile residues in the CD Loop of Kir Channels. Molecular Neurobiology 53(9):5948–5961 DOI 10.1007/s12035-015-9466-x.

Mace<hat>do CL, Vasconcelos LH, de Correia AC, Martins IR, de Lira DP, de O Santos BV, de A Cavalcante F, Silva BA. 2014. Mechanisms underlying vasorelaxation induced in rat aorta by galetin 3,6-dimethyl ether, a flavonoid from Piptadenia stipulacea (Benth.) Ducke. Molecules 19(12):19678–19695 DOI 10.3390/molecules191219678.

Mackie K, Lai Y, Westenbroek R, Mitchell R. 1995. Cannabinoids activate an inwardly rectifying potassium conductance and inhibit Q-type calcium currents in AtT20 cells transfected with rat brain cannabinoid receptor. Journal of Neuroscience 15(10):6552–6561 DOI 10.1523/jneurosci.15-10-06552.1995.

Mandel S, Youdim MBH. 2004. Catechin polyphenols: neurodegeneration and neuroprotection in neurodegenerative diseases. Free Radical Biology and Medicine 37(3):304–317 DOI 10.1016/j.freeradbiomed.2004.04.012.

Mannhold R. 2004. K<sub>ATP</sub> channel openers: structure-activity relationships and therapeutic potential. Medicinal Research Reviews 24(2):213–266 DOI 10.1002.med.10060.

Martin GM, Yoshioka C, Rex EA, Fay JF, Xie Q, Whorton MR, Chen JZ, Shyng SL. 2017. Cryo-EM structure of the ATP-sensitive potassium channel illuminates mechanisms of assembly and gating. Elife 6:e24149 DOI 10.7554/eLife.24149.

Matsushita K, Puro DG. 2006. Topographical heterogeneity of K(IR) currents in pericyte-containing microvessels of the rat retina: effect of diabetes. Journal of Physiology 573(2):483–495 DOI 10.1113/jphysiol.2006.107102.
Mattila P, Astola J, Kumpulainen J. 2000. Determination of flavonoids in plant material by HPLC with diode-array and electro-array detections. *Journal of Agricultural and Food Chemistry* 48(12):5834–5841 DOI 10.1021/jf000661f.

Meng XY, Liu S, Cui M, Zhou R, Logothetis DE. 2016. The molecular mechanism of opening the helix bundle crossing (HBC) gate of a Kir channel. *Scientific Reports* 6(1):29399 DOI 10.1038/srep29399.

Miki T, Suzuki M, Shibasaki T, Uemura H, Sato T, Yamaguchi K, Koseki H, Iwanaga T, Nakaya H, Seino S. 2002. Mouse model of Prinzmetal angina by disruption of the inward rectifier Kir6.1. *Nature Medicine* 8(5):466–472 DOI 10.1038/nm0502-466.

Miller C. 2000. An overview of the potassium channel family. *Genome Biology* 1(4):reviews0004.1 DOI 10.1186/gb-2000-1-4-reviews0004.

Minami K, Miki T, Kadowaki T, Seino S. 2004. Roles of ATP-sensitive K⁺ channels as metabolic sensors: studies of Kir6.x null mice. *Diabetes* 53(Suppl 3):S176–S180 DOI 10.2337/diabetes.53.suppl_3.s176.

Minor DL Jr, Masseling SJ, Jan YN, Jan LY. 1999. Transmembrane structure of an inwardly rectifying potassium channel. *Cell* 96(6):879–891 DOI 10.1016/s0092-8674(00)80597-8.

Miura T, Miki T. 2003. ATP-sensitive K⁺ channel openers: old drugs with new clinical benefits for the heart. *Current Vascular Pharmacology* 1(3):251–258 DOI 10.2174/1570161033476646.

Mugnai P, Durante M, Sgaragli G, Saponara S, Paliuri G, Bova S, Fusi F. 2014. L-type Ca(2+) channel current characteristics are preserved in rat tail artery myocytes after one-day storage. *Acta Physiologica* 211(2):334–345 DOI 10.1111/apha.12282.

Ngan CH, Hall DR, Zerbe B, Grove LE, Kozakov D, Vajda S. 2012. FTsite: high accuracy detection of ligand binding sites on unbound protein structures. *Bioinformatics* 28(2):286–287 DOI 10.1093/bioinformatics/btr651.

Nishida M, Cadene M, Chait BT, MacKinnon R. 2007. Crystal structure of a Kir3.1-prokaryotic Kir channel chimera. *EMBO Journal* 26(17):4005–4015 DOI 10.1038/sj.emboj.7401828.

O’Boyle NM, Banck M, James CA, Morley C, Vandermeersch T, Hutchison GR. 2011. Open Babel: an open chemical toolbox. *Journal of Cheminformatics* 3(1):33 DOI 10.1186/1758-2946-3-33.

Ogata R, Kitamura K, Ito Y, Nakano H. 1997. Inhibitory effects of genistein on ATP-sensitive K⁺ channels in rabbit portal vein smooth muscle. *British Journal of Pharmacology* 122(7):1395–1404 DOI 10.1039/bj1997001532.

Pattnaik BR, Hughes BA. 2009. Regulation of Kir channels in bovine retinal pigment epithelial cells by phosphatidylinositol 4,5-bisphosphate. *American Journal of Physiology-Cell Physiology* 297(4):C1001–C1011 DOI 10.1152/ajpcell.00250.2009.

Pegan S, Arrabit C, Slesinger PA, Choe S. 2006. Andersen’s syndrome mutation effects on the structure and assembly of the cytoplasmic domains of Kir2.1. *Biochemistry* 45(28):8599–8606 DOI 10.1021/bi060653d.

Pegan S, Arrabit C, Zhou W, Kwiatkowski W, Collins A, Slesinger PA, Choe S. 2005. Cytoplasmic domain structures of Kir2.1 and Kir3.1 show sites for modulating gating and rectification. *Nature Neuroscience* 8(3):279–287 DOI 10.1038/nn1411.

Perney TM, Kaczmarek LK. 1991. The molecular biology of K⁺ channels. *Current Opinion in Cell Biology* 3(4):663–670 DOI 10.1016/0955-0674(91)90039-2.

Proks P, Girard C, Haider S, Gloyn AL, Hattersley AT, Sansom MS, Ashcroft FM. 2005. A gating mutation at the internal mouth of the Kir6.2 pore is associated with DEND syndrome. *EMBO Reports* 6(5):470–475 DOI 10.1038/sj.embor.7400393.
Pronk S, Päll S, Schulz R, Larsson P, Apostolov R, Shirts MR, Smith JC, Kasson PM, van der Spoel D, Hess B, Lindahl E. 2013. GROMACS 4.5: a high-throughput and highly parallel open source molecular simulation toolkit. Bioinformatics 29(7):845–854 DOI 10.1093/bioinformatics/btt055.

Renaud S, de Lorgeril M. 1992. Wine, alcohol, platelets, and the French paradox for coronary heart disease. Lancet 339(8808):1523–1526 DOI 10.1016/0140-6736(92)91277-f.

Salentin S, Schreiber S, Haupt VJ, Adasme MF, Schroeder M. 2015. PLIP: fully automated protein-ligand interaction profiler. Nucleic Acids Research 43(W1):W443–W447 DOI 10.1093/nar/gkv315.

Saponara S, Carosati E, Muggai P, Sgaragli G, Fusi F. 2011. The flavonoid scaffold as a template for the design of modulators of the vascular Cav1.2 channels. British Journal of Pharmacology 164(6):1684–1697 DOI 10.1111/j.1476-5381.2011.01476.x.

Schütte-Kleffel AW, van Aalten DM. 2004. PRODRG: a tool for high-throughput crystallography of protein–ligand complexes. Acta Crystallographica Section D Biological Crystallography 60(8):1355–1363 DOI 10.1107/s0907444904011679.

Schwarz TL, Tempel BL, Papazian DM, Jan YN, Jan LY. 1988. Multiple potassium-channel components are produced by alternative splicing at the Shaker locus in Drosophila. Nature 331(6152):137–142 DOI 10.1038/331137a0.

Sepúlveda FV, Pablo Cid L, Teulon J, Niemeyer MI. 2015. Molecular aspects of structure, gating, and physiology of pH-sensitive background K2P and Kir K⁺-transport channels. Physiological Reviews 95(1):179–217 DOI 10.1152/physrev.00016.2014.

Shimomura K, Flanagan SE, Zadek B, Lethby M, Zubcevic L, Girard CAJ, Ashcroft FM. 2009. Adjacent mutations in the gating loop of Kir6.2 produce neonatal diabetes and hyperinsulinism. EMBO Molecular Medicine 1(3):166–177 DOI 10.1002/emmm.200900018.

Stephan D, Winkler M, Kühlner P, Russ U, Quast U. 2006. Selectivity of repaglinide and glibenclamide for the pancreatic over the cardiovascular K(ATP) channels. Diabetologia 49(9):2039–2048 DOI 10.1007/s00125-006-0307-3.

The UniProt Consortium. 2017. UniProt: the universal protein knowledgebase. Nucleic Acids Research 45(D1):D158–D169 DOI 10.1093/nar/gkw1099.

Tinker A, Aziz Q, Thomas A. 2014. The role of ATP-sensitive potassium channels in cellular function and protection in the cardiovascular system. British Journal of Pharmacology 171(1):12–23 DOI 10.1111/bph.12407.

Trott O, Olson AJ. 2010. AutoDock Vina: improving the speed and accuracy of docking with a new scoring function, efficient optimization and multithreading. Journal of Computational Chemistry 31(2):455–461 DOI 10.1002/jcc.21334.

Villa C, Combi R. 2016. Potassium channels and human epileptic phenotypes: an updated overview. Frontiers in Cellular Neuroscience 10:81 DOI 10.3389/fncel.2016.00081.

Wang Y, Virtanen J, Xue Z, Zhang Y. 2017. I-TASSER-MR: automated molecular replacement for distant-homology proteins using iterative fragment assembly and progressive sequence truncation. Nucleic Acids Research 45(W1):W429–W434 DOI 10.1093/nar/gkx349.

Whorton MR, MacKinnon R. 2011. Crystal structure of the mammalian GIRK2 K⁺ channel and gating regulation by proteins, PIP2, and sodium. Cell 147(1):199–208 DOI 10.1016/j.cell.2011.07.046.

Xie LH, John SA, Ribalet B, Weiss JN. 2007. Activation of inwardly rectifying potassium (Kir) channels by phosphatidylinositol-4,5-bisphosphate (PIP2): interaction with other regulatory ligands. Progress in Biophysics and Molecular Biology 94(3):320–335 DOI 10.1016/j.pbiomolbio.2006.04.001.
Xu H, Hill JJ, Michelsen K, Yamane H, Kurzeja RJ, Tam T, Isaacs RJ, Shen F, Tagari P. 2015. Characterization of the direct interaction between KcsA-Kv1.3 and its inhibitors. *Biochimica et Biophysica Acta (BBA)—Biomembranes* **1848**(10):1974–1980 DOI 10.1016/j.bbamem.2015.06.011.

Yamada M. 2010. Mitochondrial ATP-sensitive K⁺ channels, protectors of the heart. *Journal of Physiology* **588**(2):283–286 DOI 10.1113/jphysiol.2009.179028.

Yamada K, Ji JJ, Yuan H, Miki T, Sato S, Horimoto N, Shimizu T, Seino S, Inagaki N. 2001. Protective role of ATP-sensitive potassium channels in hypoxia-induced generalized seizure. *Science* **292**(5521):1543–1546 DOI 10.1126/science.1059829.

Yokoshiki H, Sunagawa M, Seki T, Sperelakis N. 1998. ATP-sensitive K⁺ channels in pancreatic, cardiac, and vascular smooth muscle cells. *American Journal of Physiology-Cell Physiology* **274**(1):C25–C37 DOI 10.1152/ajpcell.1998.274.1.c25.

Yow TT, Pera E, Absalom N, Heblinski M, Johnston GA, Hanrahan JR, Chebib M. 2011. Naringin directly activates inwardly rectifying potassium channels at an overlapping binding site to tertiapin-Q. *British Journal of Pharmacology* **163**(5):1017–1033 DOI 10.1111/j.1476-5381.2011.01315.x.

Zhang DY, Zhang YH, Sun HY, Lau CP, Li GR. 2011. Epidermal growth factor receptor tyrosine kinase regulates the human inward rectifier potassium K(IR)2.3 channel, stably expressed in HEK 293 cells. *British Journal of Pharmacology* **164**(5):1469–1478 DOI 10.1111/j.1476-5381.2011.01424.x.

Zhao Z, Liu B, Zhang G, Jia Z, Jia Q, Geng X, Zhang H. 2008. Molecular basis for genistein-induced inhibition of Kir2.3 currents. *Pflügers Archiv—European Journal of Physiology* **456**(2):413–423 DOI 10.1007/s00424-007-0391-3.

Zingman LV, Hodgson DM, Bast PH, Kane GC, Perez-Terzic C, Gumina RJ, Pucar D, Bienengraeber M, Dzeja PP, Miki T, Seino S, Alekseev AE, Terzic A. 2002. Kir6.2 is required for adaptation to stress. *Proceedings of the National Academy of Sciences of the United States of America* **99**(20):13278–13283 DOI 10.1073/pnas.212315199.