Kir Channel Molecular Physiology, Pharmacology, and Therapeutic Implications

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Contents

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Abstract

For the past two decades several scholarly reviews have appeared on the inwardly rectifying potassium (Kir) channels. We would like to highlight two efforts in particular, which have provided comprehensive reviews of the literature up to 2010 (Hibino et al., Physiol Rev 90(1):291–366, 2010; Stanfield et al., Rev Physiol Biochem Pharmacol 145:47–179, 2002). In the past decade, great insights into the 3-D atomic resolution structures of Kir channels have begun to provide the molecular basis for their functional properties. More recently, computational studies are beginning to close the time domain gap between in silico dynamic and patch-clamp functional studies. The pharmacology of these channels has also been expanding and the dynamic structural studies provide hope that we are heading toward successful structure-based drug design for this family of K^+ channels. In the present review we focus on placing the physiology and pharmacology of this K^+ channel family in the context of atomic resolution structures and in providing a glimpse of the promising future of therapeutic opportunities.

Keywords

Cytosolic G-loop gate · GIRK · Helix bundle crossing gate · K^+ transport channel · K_{ATP} · Phosphoinositides · Potassium inward rectifiers · Resting potential

1 Historical Perspective: The Pre-structure Era

Inward Rectification and Dependence of Conductance on $[K^+]_o$: Kir currents were first described in skeletal muscle fibers, where Bernard Katz observed (Katz 1949a, b) that when these cells were immersed in solutions containing high

potassium the membrane conductance was larger at hyperpolarizing potentials. This behavior was the opposite from what Cole and Curtis had reported in squid giant axon (Cole and Curtis 1941), where the depolarization-induced non-linear conductance had been described as rectification, borrowing from electrical engineering terminology used for diodes. The fact that the first example of outward rectification, demonstrated by the delayed rectifier K_v current, was also in Katz's laboratory with Hodgkin and Huxley (Hodgkin et al. 1952; Katz 1949a, b), led Katz to describe the skeletal muscle conductance in high K^+ as "anomalous rectification" ("propriétés détectrices anormales," from the French description). This anomalous rectification was later (Adrian et al. 1970) renamed "inward rectification," the term most used at the present. Another difference between the Kir and the outwardly rectifying K_v currents was their respective voltage dependence. Kir currents were voltage-independent but instead their activation depended on the driving force (V_m-E_K, i.e. the distance of the membrane potential V_m from the equilibrium potential E_K for K^+ ions). Activation was shown to depend on (V_m-E_K) if $[K^+]_o$, but not $[K^+]_i$, was changed (Hagiwara and Yoshii 1979; Leech and Stanfield 1981; Stanfield et al. 2002). The dependence of Kir current rectification and conductance on $[K^+]_o$ is shown in Fig. 1a (Hagiwara et al. 1976). Early on, the inward rectification was attributed to block of the channel by particles from the cytosol (Armstrong 1969; Hille and Schwarz 1978; Standen and Stanfield 1978), and later the intracellular blocking substances were identified to be the millimolar levels of Mg^{2+} as well as polyamines that exist in sub-millimolar concentrations inside of cells (Lopatin et al. 1994; Matsuda et al. 1987). Upon hyperpolarization, the inward Kir current showed a time-independent increase due to fast Mg^{2+} unblock, followed by a time-dependent increase due to slow polyamine unblock (Lopatin et al. 1995).

1.1 Tissue Distribution of Kir Family Members

Kir channels have been found in a wide variety of cells, including: cardiac myocytes (Beeler and Reuter 1970; Kurachi 1985; McAllister and Noble 1966; Rougier et al. 1967), neurons (Brown and Carpentier 1990; Gahwiler and Brown 1985; Lacey et al. 1988; North et al. 1987; Takahashi 1990; Williams et al. 1988), blood cells (Lewis et al. 1991; McKinney and Gallin 1988), osteoclasts (Sims and Dixon 1989), endothelial cells (Silver and DeCoursey 1990), glial cells (Kuffler and Nicholls 1966; Newman 1984), epithelial cells (Greger et al. 1990; Hebert et al. 2005; Lorenz et al. 2002; Lu et al. 2002), and oocytes (Hagiwara et al. 1978; Hagiwara et al. 1976; Hagiwara and Takahashi 1974). Figure 2 shows the tissue/organ expression of each Kir subfamily throughout the body. The Kir tissue expression has been comprehensively reviewed (de Boer et al. 2010; Hibino et al. 2010). The role of Kir channels lies in contributing to the cellular resting potential and keeping V_m near E_K. This serves to reduce action potential firing in excitable cells, to control K^+ transport in non-excitable cells or to transduce extracellular (by external stimuli) to intracellular (by internal metabolites) communication and vice versa. In 1993 the first 3 of the 16 members of the Kir channel family were cloned (Dascal et al. 1993;

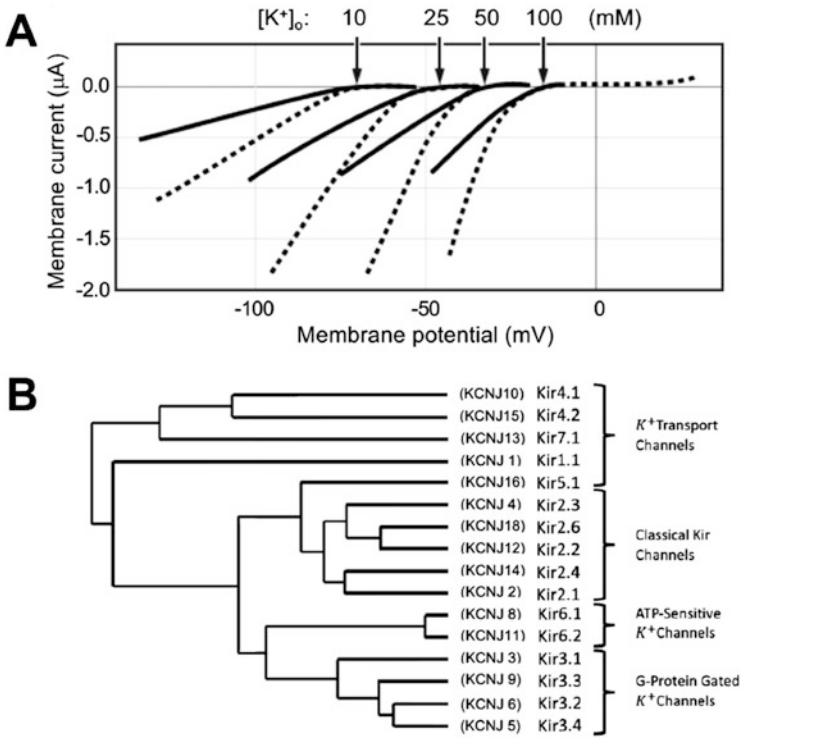

Fig. 1 Kir channel inward rectification and phylogenetic/functional classification. (**a**) I-V relationship of the starfish Kir at four different $[K^+]_o$. Continuous and broken lines indicate instantaneous and steady-state currents, respectively (from Hagiwara et al. 1976) (**b**) Phylogenetic analysis of the 16 known subunits of human Kir channels (for identity/similarity see Fig. 10). These subunits have been classified into four functional groups

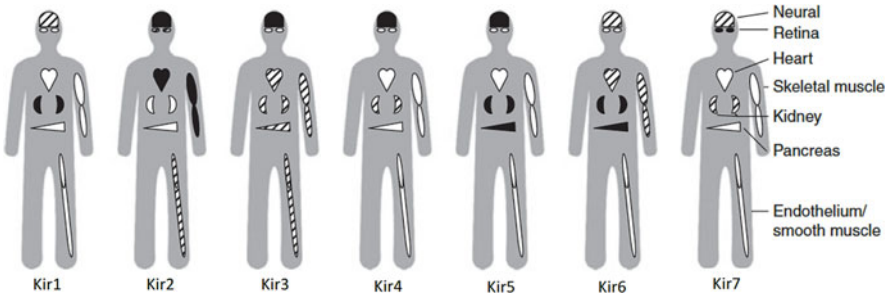

Fig. 2 Expression in indicated organs and tissues of Kir channel members of each of the seven subfamilies. Blank indicates no, striations intermediate, and black prominent levels of expression. Adapted (permission obtained) from de Boer et al. (2010)

Ho et al. 1993; Kubo et al. 1993a; Kubo et al. 1993b). Based on sequence alignment (see Fig. 9) and phylogenetic analysis, the 16 human isoforms have been classified into seven subfamilies (Kir1-7) and four functional groups (a. the K^+ transport, b. the classical, c. the ATP-sensitive, and d. the G protein-gated K^+ channels) (Fig. 1b). The strong sequence similarity among family members (30–99%) (Fig. 10) allows for formation of heteromeric assemblies along with homomeric ones, in one case across subfamilies (Kir4.1 and Kir5.1) and in most cases within subfamilies (e.g., Kir3.1/3.2 in neuronal tissues and Kir3.1/3.4 in atrial cells of the heart). The resulting heteromers display unique functional properties compared to the homomers that will be discussed individually for each subfamily.

1.2 Kir Channel Gating

The signaling phospholipid, phosphatidylinositol 4,5-bisphosphate [$PI(4,5)P_2$ or PIP_2] found in the inner leaflet of the plasma membrane plays an essential role in supporting Kir channel gating (Hilgemann and Ball 1996; Huang et al. 1998; Logothetis et al. 2015; Petit-Jacques et al. 1999; Sui et al. 1998). Excision into inside-out patches in ATP-free solutions showed a typical gradual decline in channel activity, referred to as "run-down" (Hilgemann and Ball 1996). Run-down activity could be restored by hydrolyzable forms of ATP or by PIP_2 (Hilgemann and Ball 1996; Huang et al. 1998; Petit-Jacques et al. 1999; Sui et al. 1998). The seven Kir subfamily members could be classified into four groups depending on the degree of stereospecificity and affinity to $PI(4,5)P_2$ (over $PI(3,4,5)P_3$ and $PI(3,4)P_2$) (Rohacs et al. 2003): *Highest* or Group 1 (Kir2.1, Kir2.4, Kir4.1), *Moderate* or Group 2 (Kir1.1, Kir2.2, Kir2.3, Kir4.2, Kir7.1), *Weak* or Group 3 (Kir3.4, Kir3.1/3.4), *Lowest* or Group 4 (Kir6.2, Kir6.2/SURs). Mutagenesis studies identified channel regions critical for the effects of PIP_2 and linked basic and non-basic residues to sensitivity to PIP_2 and Kir channelopathies (Lopes et al. 2002).

1.3 Kir Channel Trafficking

Kir channel mutations that lead to channelopathies, especially those that affect trafficking, have been recently reviewed (Zangerl-Plessl et al. 2019). As other transmembrane proteins, Kir channels are translated in the endoplasmic reticulum (ER) and transported through the Golgi apparatus and the trans-Golgi network to the plasma membrane through the process known as forward trafficking. Upon removal from the plasma membrane, channel proteins enter the degradation pathway through trafficking to the early endosome and the multivesicular body to the lysosome, through a process known as backward trafficking. Kir channels could instead of becoming degraded be recycled back to the plasma membrane from the early endosome to the trans-Golgi network. Kir channel mutations resulting in loss (LOF) or gain (GOF) of function are associated with a variety of human diseases, including Bartter syndrome type II (Kir1.1 LOF), Andersen-Tawil syndrome (Kir2.1

LOF and GOF), thyrotoxic hypokalemic periodic paralysis (Kir2.6 LOF), Keppen-Lubinsky syndrome (Kir3.2 LOF), familial hyperaldosteronism type III and long QT syndrome 12 (Kir3.4 LOF), EAST/SeSAME syndrome (Kir4.1 LOF), Cantú syndrome (Kir6.1 or SUR2 GOF) and hyperinsulinism and hypoglycemia (Kir6.2 LOF) and diabetes (Kir6.2 GOF), Leber congenital amaurosis type 16 and Snowflake vitreoretinal degeneration (Kir7.1 LOF). In many of the diseases mentioned above LOF has been associated with defective forward trafficking, while GOF mutations or loss of specificity mutations (e.g., some Kir3.4 mutations) are not likely to be related to trafficking abnormalities. The likely causes of trafficking defects are either (1) defects in trafficking motifs compromising interactions with the proteins involved in the trafficking machinery, or (2) protein structure defects leading to channel misfolding, destabilization and ER-associated degradation (Zangerl-Plessl et al. 2019).

1.4 The Structural Era

1.4.1 Kir Structures by 2020

The first Kir channel high-resolution crystal structure to be solved in 2002 was the cytoplasmic domain (CTD) of Kir3.1 (Nishida and MacKinnon 2002). In this crystal structure, the transmembrane domain of the channel was removed, and the N-terminus was fused to the CTD and expressed in bacteria (PDBID: 1N9P). Soon after this structure, the CTDs of Kir2.1 (PDBID: 1U4F, 2005), (Pegan et al. 2005) pointing to the potential of a cytosolic constriction, coined as the G-loop gate, and Kir3.2 (PDBID: 2E4F, 2007) (Inanobe et al. 2007) were solved. Subsequently, in 2007, a crystal structure of a Kir3.1 prokaryotic Kir channel chimera was solved (PDBID: 2QKS) (Table 1). In this structure, two thirds of the transmembrane domains (TMDs) were replaced with the corresponding region of a homologous prokaryotic Kir channel, revealing the continuity of the permeation pathway from the membrane (2/3 prokaryotic, 1/3 mammalian) to the cytosolic (mammalian) domains of the Kir3.1 channel. Two distinct conformations of the G-loop gate were captured, in one structure the apex of the CTD was open (or dilated), and in the other it was closed (or constricted) (Nishida et al. 2007). The transmembrane gate, referred to as the helix bundle crossing (HBC) gate was captured in the closed conformation in both structures. Even though Nishida and colleagues were not able to show that the chimera they constructed was functional (Nishida et al. 2007), a subsequent paper showed that the purified chimera once reconstituted in planar lipid bilayers with PIP2 was indeed functional (Leal-Pinto et al. 2010). In 2009, a crystal structure of the chicken Kir2.2, 90% identical to the human Kir2.2, was solved (PDBID:3JYC), where large structured turrets and an unusual selectivity filter entryway were seen, which may explain the relative insensitivity of eukaryotic Kir channels to toxins (Tao et al. 2009). In 2011, the crystal structure of Kir2.2 channel in complex with a short-chain derivative of PIP_2 was solved [PDBID:3SPI] (see Fig. 3b). This structure showed that PIP_2 binds at an interface between the TMD and CTD of the Kir2.2 channel. Upon PIP_2 binding, a flexible linker between the TMD and CTD transforms to a helical structure, which causes a 6 Å translation of the CTD

Table 1 Selected functional Kir channel structures solved by X-ray crystallography (X-ray) or cryo-electron microscopy (Cryo-EM)

PDBID	Channel	Resolution	Method	Year	References
2QKS	Kir3.1-prokaryotic Kir channel chimera	2.2 Å	X-ray	2007	Nishida et al. (2007), Leal-Pinto et al. (2010)
3JYC	Kir2.2	3.11 Å	X-ray	2009	Tao et al. (2009)
3SPI	Kir2.2/PIP_2	3.31 Å	X-ray	2011	Hansen et al. (2011)
3SYO	Kir3.2/sodium	3.54 Å	X-ray	2011	Whorton and MacKinnon (2011)
3SYA	Kir3.2 in complex with sodium and PIP_2	2.98 Å	X-ray	2011	Whorton and MacKinnon (2011)
4KFM	Kir3.2/βγ G protein subunits	3.45 Å	X-ray	2013	Whorton and MacKinnon (2013)
5WUA	ATP-sensitive K^+ channel	5.6 Å	Cryo-EM	2017	Li et al. (2017)
5YW8/ 5YWC/ 6YKE	Pancreatic ATP-sensitive potassium channel bound with ATPγS	4.4 Å	Cryo-EM	2017	Wu et al. (2018)
6BAA	Kir6.2/SUR1/Glibenclamide, ATP	3.63 Å	Cryo-EM	2017	Martin et al. (2017)
6C3O/ 6C3P	Kir6.2/SUR1/ATP(ADP), Mg^{2+}	3.9 Å/ 5.6 Å	Cryo-EM	2017	Lee et al. (2017)
6JB1	Pancreatic ATP-sensitive K^+ channel bound with repaglinide and ATPγS	3.3 Å	Cryo-EM	2019	Ding et al. (2019)

toward the TMD, as the HBC gate begins to open (Hansen et al. 2011). In the same year (2011), the crystal structure of Kir3.2 in complex with sodium and PIP_2 was solved (PDBID:3SYA). The structures suggested that the presence of PIP_2 couples the G loop and HBC gates to open in a coordinated manner. The intracellular Na^+ binding site was also confirmed in this structure (Whorton and MacKinnon 2011). In 2013, the crystal structure of Kir3.2-βγ G-protein (Gβγ) complex was solved (PDBID:4KFM). In this structure, the Gβγ subunit bound to the interfaces between four pairs of adjacent Kir3.2 channel CTD subunits that were also bound to sodium and a PIP_2 analog. The structure was thought to represent a "pre-open" state, an intermediate between the closed and open but still a non-conducting conformation of the channel (Whorton and MacKinnon 2013) (shown later in Fig. 7b). In 2017, a hetero-octameric pancreatic K_{ATP} channel in complex with the non-competitive inhibitor glibenclamide was solved by cryo-electron microscopy (EM) at a 5.6-Å resolution [PBDID:5WUA]. This structure showed four SUR1 regulatory subunits located in the periphery of a centrally located Kir6.2 channel tetramer (Li et al.

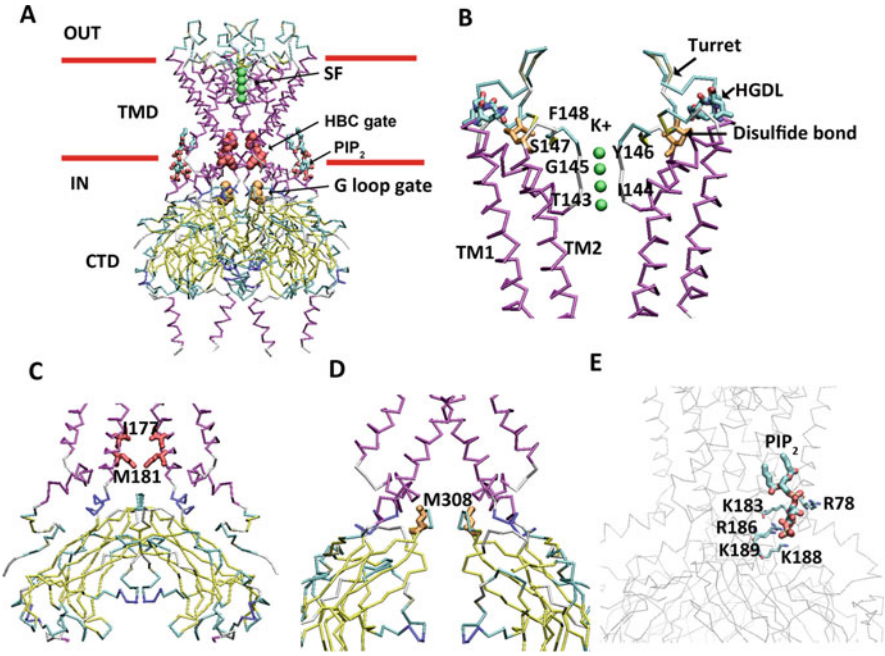

Fig. 3 (**a**) Crystal structure of Kir2.2 (PDBID: 3SPI) in complex with PIP_2. (**b**) Selectivity Filter. (**c**) Helix bundle crossing gate. (**d**) G-loop gate. (**e**) PIP_2 binding site

2019). In 2017, Wu and colleagues solved complexes of the K_{ATP} channel (KIR6.2/SUR1) with ATP, Mg-ADP, and ATP/glibenclamide, respectively, by using cryo-EM (PDBID: 5YW8, 5YWC, 5YKE). These structures depict the binding site of glibenclamide, ATP, and suggested a mechanism of how Mg-ADP binding on nucleotide-binding domains drives a conformational change of the SUR1 subunit (Wu et al. 2018). Martin and colleagues solved a cryo-EM structure of SUR1/Kir6.2 channel bound to glibenclamide and ATP at 3.63 Å resolution (PDBID: 6BAA). The structure showed that the drug bonded to the transmembrane bundle of SUR1, and mutation of the interacting residues in the binding site reduced the channel sensitivity to the drug (Martin et al. 2017). Lee and colleagues solved two cryo-EM structures of the human K_{ATP} channel (Kir6.2/SUR1) in complex with Mg^{2+} and nucleotides, referred to as the quatrefoil (PDBID: 6C3O) and propeller (PDBID: 6C3P) forms. In both forms, ATP binds to the inhibitory site in Kir6.2. Mg^{2+}-ATP and Mg^{2+}-ADP bind to the degenerate and consensus sites, respectively, in the nucleotide-binding domains of SUR1. A lasso extension interface between Kir6.2 and SUR1 formed in the propeller form but was disrupted in the quatrefoil form (Lee et al. 2017). In 2019, a higher resolution (3.3 Å) cryo-EM structure of the same pancreatic K_{ATP} channel was achieved in complex with the short-acting insulin secretagogue repaglinide and adenosine-5′-(γ-thio)-triphosphate (ATPγS) (PDBID: 6JB1) (Ding et al. 2019) (shown later in Fig. 8). Table 1 summarizes crystal and cryo-EM Kir channel structures for which functional expression has been demonstrated.

1.4.2 Structural Features of Kir Channels

The Kir channel structures have provided new insights into the mechanism of channel function, such as channel gating, selectivity, rectification, and modulation by PIP_2, sodium ions, alcohol, G proteins, ATP, etc. Figure 3 shows the classical Kir2.2 crystal structure with which we will illustrate some key features of this channel family. Kir channels are either homotetramers or heterotetramers formed by four subunits. Each subunit contains a transmembrane region, the TM1 (or outer) and TM2 (or inner) helices (see Fig. 3b), and a cytoplasmic domain (Fig. 3d).

Selectivity Filter (SF)

The selectivity filter (SF) of the Kir channel is similar to that of other potassium channels (Fig. 3b), but instead of the canonical filter sequence TXGYGDX (X: aliphatic amino acid), the corresponding sequence in Kir channels is TIGYGXR (X: Y/F/H, V/L, T, G), with very few exceptions (Kir2.4-S147, Kir6.1/6.2-F143/133, Kir7.1-M125) (see Fig. 9). The conserved Kir2.2(R149) forms an ionized hydrogen bond with E139 in the pore helix. Kir channels also contain a highly conserved disulfide bond flanking the pore region (C123-C155) (see Figs. 3b and 9). Between the outer helix and pore region "turret," there is the conserved 3–10 helical HGDL signature sequence for Kir channels (Tao et al. 2009), with very few exceptions: Kir3.1/3.3/3.4 (RGDL), Kir3.2 (RGDM), Kir1.1 (HKDL), and Kir7.1 (NGDL) (see Figs. 3b and 9).

Gates

The residues I177 and M181 on the TM2 helix in Kir2.2 form two hydrophobic seals that close the path from the pore to the CTD, which is referred to as the helix bundle crossing (HBC) gate (Fig. 3c). In other K^+ channels, such as KcsA and Kv channels, a small or polar amino acid exists at the position corresponding to I177. Alternate residues can be utilized in other Kirs at these positions [at I177: V/L/T/C and at M181: Kir2 (M), Kir3/6 (F), Kir1/4/5 (L), Kir7.1 (V)] (see Figs. 3c and 9). In addition to the HBC gate, Kir channels have a second unique cytoplasmic gate (G loop) at the apex of the cytoplasmic domain (see Figs. 3d and 9) (Tao et al. 2009).

PIP_2 Binding Site

PIP_2 binds to the interface between TMD and CTD and is thought to cause a 6 Å translation of the CTD toward the TMD (Fig. 3a, e). The negatively charged phosphate groups interact with the Kir channel through highly conserved salt bridge interactions. The 1′ phosphate interacts with the R78 and R80 of the RWR highly conserved sequence motif with a few exceptions (Kir2.2 numbers, unless otherwise specified): at R78 – Kir2/7 (R), Kir1/3.1/3.2/3.4/4.2/5/6 (K), Kir3.3/4 (Q) and at R80 – Kir6.2(P69) (see Figs. 3e and 9) at the N-terminus of the outer helix. The 4′ and 5′ phosphates interact with the TM2 residue K183 (absolutely conserved) and the highly conserved residues of the B-loop region sequence motif – 186-RPKKR-190 – with the following exceptions: R186 (Kir3/6 (Q), Kir5.1(T174)); K188 (Kir6 (H), Kir3.3 (N)); and K189 (Kir6 (R); Kir7.1(N165)) (see Figs. 3e and 9). These

basic amino acids are critical for PIP_2 activation of Kir channels (Hansen et al. 2011; Tao et al. 2009).

Cholesterol Regulation

Cholesterol enrichment or depletion was shown to decrease or increase, respectively, Kir2.1 currents in endothelial cells (Romanenko et al. 2002). Through mutagenesis structural determinants could be identified, suggesting strongly that specific interactions of cholesterol with the Kir proteins were responsible for the observed effects (Epshtein et al. 2009; Rosenhouse-Dantsker 2019). Cholesterol enrichment caused significant inhibition to several active Kir channels (Kir1.1, Kir2.1, Kir3.1 (F137S), Kir6.2Δ36) and activation (rather than inhibition) to others (Kir3.2, Kir3.4 (S143T)). Modeling studies and site-directed mutagenesis suggested that the interaction sites were located between α-helices of two adjacent channel subunits and involved hydrophobic and aromatic residues (Rosenhouse-Dantsker 2019). Even though some key determinant residues for cholesterol sensitivity overlapped with those for PIP_2 sensitivity, others did not, making the interrelationship between cholesterol and PIP_2 influences on Kir channels unclear. A recent cryo-EM structure of Kir3.2 with a cholesterol analog (in the presence and absence of PIP_2) suggests that cholesterol binds near PIP_2 potentiating its effects (Mathiharan et al. 2020).

Rectification Determinants

Kir channels can be classified as strong, intermediate, and weak rectifiers based on their rectification properties (Table 2; Yellow – Kir2, Blue – Kir3, Green – Transport, Orange – Kir6). The rectification features of Kir channel are generally believed to occur through blocking of the channels by Mg^{2+} and polyamines (spermine, spermidine, and putrescine) (Nichols and Lee 2018). Key acidic residues involved in the rectification of Kir channels have been identified through mutagenesis. A TM2 aspartate residue (corresponding to D173 in the Kir2.2 channel) was the first residue identified as critical for inward rectification. Mutation of this residue affected both polyamine-blocking affinity and voltage dependence (Fakler et al. 1996; Lopatin et al. 1994; Wible et al. 1994; Yang et al. 1995). The corresponding residue in intermediate/weak rectifiers such as Kir1.1 and Kir6.2 is an asparagine. Mutation of this asparagine to an aspartate residue converted these channels to strong rectifier channels (Lopatin et al. 1994; Lu and MacKinnon 1994; Shyng et al. 1997). Thus, this residue has been termed as the "rectification controller." Several other negatively charged residues in the pore-lining region of the CTD in Kir channels are important for rectification. For example, mutation of E224, E229, D259, and E299 of Kir2.1 (numbers are one less than Kir2.2) to a neutral residue reduced the intensity of inward rectification (Fujiwara and Kubo 2006; Guo and Lu 2003; Kubo and Murata 2001; Taglialatela et al. 1995; Xie et al. 2002; Yang et al. 1995). Figure 4 shows a comparison of these residues between the strong rectifier Kir2.2 and the weak rectifier Kir6.2. Table 2 shows that the number of acidic residues per Kir channel subunit correlates well with the functional rectification properties of each subfamily member, such that 4–5 negatively charged residues per subunit (Q/s) result in strong, 3 Q/s in intermediate and 1 Q/s in weak rectification.

Table 2 Kir channel critical residues for inward rectification

R	Channel	173	225	256	260	300	Q/s
S	Kir2.1	D	E	D	D	E	5
S	Kir2.2	D	E	D	D	E	5
S	Kir2.4	D	E	D	D	E	5
S	Kir2.3	D	E	D	D	E	5
S	Kir2.6	D	E	D	D	E	5
NF	Kir3.1	D	S	S	D	E	
S	Kir3.2	N	E	Y	D	E	3
S	3.1/3.2	D/N	S/E	S/Y	D	E	4
NF	Kir3.3	N	E	D	D	E	
S	Kir3.4	N	E	D	D	E	4
S	3.1/3.4	D/N	S/E	S/D	D	E	5
I	Kir4.1	E	G	D	D	S	3
I	Kir4.2	E	Q	D	E	N	3
NF	Kir5.1	N	E	K	D	I	
S	4.1/5.1	E/N	G/E	D/K	D	S/I	4
S	4.2/5.1	E/N	Q/E	D/K	E/D	N/I	4
I	Kir1.1	N	G	D	E	D	3
I	Kir7.1	E	S	D	E	S	3
W	Kir6.1	N	S	P	N	E	1
W	Kir6.2	N	S	G	N	E	1

Yellow – Kir2 family, blue – Kir3 Family, green – Transport family, orange – Kir6 family
R Rectification, *S* Strong, *I* Intermediate, *W* Weak, *NF* Non-functional, *Q/s* number of charges per subunit for each Kir channel
Residue numbers from Kir2.2 channel

Recent microsecond MD simulation studies on polyamine blocking of the mKir3.2 channel were conducted by Chen et al. (2020). Two binding sites for putrescine were identified, one located close to the E236 (hE234) in the CTD, and another located close to the T154 (hT152) from the SF. These observations were consistent with previous experimentally identified residues. By applying a cross-membrane electric field, putrescine was transferred from the CTD binding site to the SF site. In contrast to putrescine (+2 charges), spermine (+4 charges) did not transfer to the SF site, but remained bound to the CTD site. An additional force was needed to be applied to make spermine transfer from the CTD to the SF binding site (Chen et al. 2020). These results contribute toward a dynamic molecular insight of the rectification mechanism through polyamines. In future studies, additional dynamic simulations will be needed to provide further molecular insight into the dynamic rectification mechanisms of Kir channels, such as how polyamines limit potassium ion permeation.

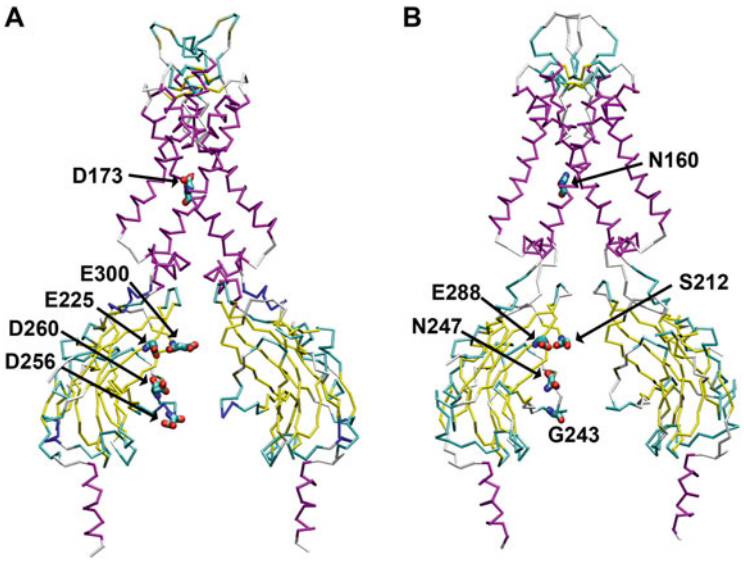

Fig. 4 Critical residues for Kir channel rectification. (**a**) Kir2.2 (PDBID: 3SPI); (**b**) Kir6.2 (PDBID: 6C3O)

2 Classical Kir2 Channels

2.1 Historical Perspective

The classical (also sometimes referred to as "canonical") rectifiers display strong inward rectification, with little outward current at depolarizing potentials. As we mentioned already in Sect. 1, they were differentiated early on from voltage-gated K^+ (Kv) channels (outward rectifiers) in that at membrane potentials around the K^+ equilibrium potential (E_K), where K_v channels are closed, they are constitutively open, albeit conducting small but physiologically relevant outward currents. They conduct very little to not at all at depolarized membrane potentials, where K_v channels are open and conduct the most. This property of strong inward rectifiers is critical for setting a background K^+ conductance in excitable tissues, such as in muscle (both skeletal and cardiac, where Kir2 channels are expressed – see below), driving the resting potential near E_K and enabling rapid conduction of excitation and coordinated contraction (Nichols et al. 1996). As already mentioned, the molecular mechanisms underlying Kir rectification, such as the blockade by polyamines, have been studied extensively (Fujiwara and Kubo 2006; Guo and Lu 2003; Ishihara and Ehara 2004; Kubo and Murata 2001; Nishida et al. 2007; Taglialatela et al. 1995; Xie et al. 2003; Yang et al. 1995). Yet, several aspects of the mechanisms involved remain unclear (Liu et al. 2012; Xu et al. 2009). One of these is the voltage dependence of inward rectification (i.e., polyamine block) displaying shifts that occur with changes in E_K when $[K^+]_o$ but not $[K^+]_i$ is varied (see Fig. 1a) (Hagiwara and Takahashi 1974; Hagiwara and Yoshii 1979; Hestrin 1981; Kubo 1996; Kubo

et al. 1993a; Lopatin and Nichols 1996; Nishida et al. 2007). Thus, the probability of channels being open (P_{open}) shifts along the voltage axis by 25 mV for an e-fold change in $[K^+]_o$ and the open channel conductance (inward or outward) is proportional to $[K^+]_o$ with a power of 0.2–0.6, referred to as a "square root" proportionality (Hagiwara and Takahashi 1974; Hille and Schwarz 1978; Kubo et al. 1993a; Matsuda 1988; Ohmori 1978; Sackmann et al. 1984). It has been argued that this dependence of the Kir conductance on $[K^+]_o$, particularly the outward conductance, plays a significant role in regulating the myocardial action potential duration (Matsuoka et al. 2003; Nichols et al. 1996; Shimoni et al. 1992), cardiac contractility (Bouchard et al. 2004), and arrhythmogenicity (Asakura et al. 2014; Ishihara et al. 2009; Maruyama et al. 2011). Ishihara has recently shown (2018) that in the absence of Na^+ in the external solutions, the open conductance of Kir2.1 does not change over a wide $[K^+]_o$ range but in its presence, Na^+ competitively inhibits K^+ conductance in a voltage-dependent manner. Thus, it is an impermeant physiological cation that mediates the apparent $[K^+]_o$ dependence of the open Kir channel conductance (Ishihara 2018). The structural determinants of the Na^+ binding site have not yet been elucidated. A guiding clue comes from Kir7.1, that differs from other Kir channels in lacking to a great extent in the property of being activated by $[K^+]_o$. We will revisit this clue of the structural determinants for the dependence of the K^+ conductance on $[K^+]_o$ and how Na^+ may be involved when we discuss Kir7.1 in the "K^+ transport Kir channels" in Sect. 5.3.

2.2 Subfamily Members and Tissue Distribution

The first subfamily member Kir2.1 (also referred to as IRK1) was cloned in 1993 from a mouse macrophage cell line (Kubo et al. 1993a). In the following 2 years three additional members were cloned, Kir2.2, Kir2.3, and Kir2.4 (Bond et al. 1994; Bredt et al. 1995; Morishige et al. 1994). Kir2.5 was cloned from fish in 2008 (Crucian carp; *Carassius carassius*) (Hassinen et al. 2008), while Kir2.6 was revealed as the fifth mammalian isoform in 2010 (Ryan et al. 2010).

In excitable cells, Kir2 channels are expressed in skeletal muscle (Kir2.1, Kir2.2, Kir2.6), brain (Kir2.1, Kir2.2, Kir2.3, Kir2.4), and heart (Kir2.1, Kir2.2, Kir2.3), while an intermediate level of expression is seen in smooth muscle tissue and the retina (Fig. 2) (de Boer et al. 2010; Hibino et al. 2010). Their role in non-excitable cells remains to be elucidated (e.g., in macrophages where Kir2.1 was first cloned from) (Kubo et al. 1993a). Not only these channel subunits assemble as homotetramers but also as heterotetramers, both in heterologous expression systems and in native tissues where they are expressed, endowing tissues with greater versatility in the physiological roles they play (Hibino et al. 2010). Only a small fraction of Kir2.6 manages to leave the endoplasmic reticulum to make it to the cell surface, whilst the almost identical subunit Kir2.2 (6 differences in the human clones Kir2.6-Kir2.2: L15S, A56E, V100I, H118R, L156P, G430E) is trafficked robustly. Dassau and colleagues showed that the L156P and P156L accounted for the majority of the endoplasmic reticulum rescue and retention of Kir2.6 and Kir2.2, respectively.

In addition, they showed that the wild-type Kir2.6 co-assembles with Kir2.1 and Kir2.2 in vitro and in skeletal muscle acting as a dominant negative subunit, limiting Kir2.1 and Kir2.2 localization to the cell surface (Dassau et al. 2011).

2.3 Physiology/Pathophysiology

The physiology and pathophysiology of Kir2 channels has been previously reviewed (de Boer et al. 2010; Hibino et al. 2010). Here, we will highlight the major roles of this channel subfamily members in the various tissues they are expressed.

Heart: Kir2 channels are critically involved in determining the shape of the cardiac action potential by (1) setting the resting potential, (2) permitting the plateau phase (through their Mg^{2+} and polyamine block at depolarized potentials), and (3) inducing a rapid final stage of repolarization. Contributions of different Kir2 subunits in comprising cardiac Kir currents have revealed that Kir2.1 knockout in mice abolishes ventricular Kir2 currents, while Kir2.2 knockout reduces the currents by 50% (Zaritsky et al. 2001). This suggests that Kir2.1 is the dominant expressing subunit and Kir2.2 contributes to the current by assembling with Kir2.1. Kir2.1 dysfunction due to 21 mutations identified in 30 families have been identified to cause Andersen-Tawil syndrome (ATS) by multiple mechanisms, including allosteric decreases in channel-PIP_2 interactions (Donaldson et al. 2004; Lopes et al. 2002). ATS is an autosomal-dominant disorder resulting in cardiac arrhythmias (long Q-T syndrome 7, LQT7), periodic paralysis, and dysmorphic bone structure in the face and fingers (Tawil et al. 1994). The cardiac symptoms entail a depolarized V_{REST} and this loss of the stabilization of V_m can trigger arrhythmias. The inability to contribute to the late phase of the repolarization of the action potential results in a prolonged action potential duration, hence the LQT7 arrhythmia. Differential expression is seen within tissues of the same organ, as in the heart for example, where dominant expression in the atria is seen for Kir2.3, while in the ventricles for Kir2.1 (Anumonwo and Lopatin 2010). Besides ATS, Kir2.1 has been implicated in atrial fibrillation (AF), the most prevalent arrhythmia that ranges from 1–2% in the general population to 9–10% in the elderly population (Dobrev et al. 2005). Kir3.1 and Kir3.4 have also been implicated in AF but will be discussed in the next section devoted to Kir3 channels. Enhanced expression of the Kir2.1 channel leads to AF and this has been linked with downregulation for two microRNAs: miR-1 (Girmatsion et al. 2009; Yang et al. 2007) and miR-26 (Luo et al. 2013). Additionally, the Kir2.1(V93I) is a gain-of-function mutation that has been linked to hereditary AF (Xia et al. 2005).

Skeletal muscle: The periodic paralysis symptom of ATS is due to the effects of the $I_{Kir2.1}$ reduction in skeletal muscle. There, the depolarized V_{REST} inactivates Na_v channels making them unavailable for initiation and propagation of the action potential, leading to paralysis. Kir2.1 has been reported to be essential for myoblast differentiation (Konig et al. 2004) and also for fusion of mononucleated myoblasts to form multinucleated skeletal muscle fibers (Fischer-Lougheed et al. 2001). Kir2.1 underlies a 60 mV hyperpolarization in the V_{REST} of myoblasts during development, which drives Ca^{2+} entry through Ca^{2+}-permeant ion channels that promotes

differentiation and fusion of myoblasts. Even though there are no gross skeletal muscle developmental defects in ATS patients, their "slender" built could be related to the mild developmental defects caused by the Kir2.1 dysfunction. Kir2.6 is also expressed in skeletal muscle and as already mentioned is nearly identical to Kir2.2 (Ryan et al. 2010). It was discovered in a screen for candidate genes responsible for thyrotoxic hypokalemic periodic paralysis, a serious complication of hyperthyroidism characterized by skeletal muscle paralysis and hypokalemia, affecting young adult male patients of Asian descent (Kung et al. 2006). The gene coding for Kir2.6 (*KCNJ18*) is transcriptionally regulated by thyroid hormone via a thyroid-responsive element in its promoter region. Thirty three percent of the patients afflicted by this condition bear mutations mainly localized in the C-terminus causing decreased current densities (de Boer et al. 2010). Destabilizing V_{REST} would inactivate Na_v channels yielding paralysis.

Bone: The dysmorphic facial bone structure defects point out the role of Kir2.1 channels in bone development. Indeed, it has been recently reported that Kir2.1 is important for efficient signaling by the bone morphogenic proteins in mammalian face development (Belus et al. 2018).

Blood vessels – Endothelial cells: Both the endothelial and smooth muscle cells that comprise the vasculature express Kir2 channels (Adams and Hill 2004; Nilius and Droogmans 2001). In fact, for vascular endothelial cells these are considered the most prominent channels expressed (Nilius and Droogmans 2001; Nilius et al. 1993; von Beckerath et al. 1996). By setting V_{REST} near E_K, they drive Ca^{2+} entry into endothelial cells (Kwan et al. 2003; Wellman and Bevan 1995) that triggers NO-mediated vasodilation. More recently, Kir2.1 channels were shown to boost the endothelial cell-dependent vasodilation generated by Ca^{2+}-dependent activation of small and intermediate conductance Ca^{2+}-activated K^+ channels in resistance-sized arteries (Sonkusare et al. 2016). In aortic endothelial cells evidence has suggested that Kir2.2 channels are the dominant Kir2 conductance (Fang et al. 2005).

Smooth muscle cells: A mild increase in $[K^+]_o$ (from 6 to 15 mM) hyperpolarizes the V_m of smooth muscle cells by 15 mV (from −45 to −60 mV) by increasing Kir conductance and vasodilating cerebral and coronary arteries (Knot et al. 1996; McCarron and Halpern 1990; Nelson et al. 1995). The hyperpolarization closes Ca_v channels reducing $[Ca^{2+}]_i$ leading to vasodilation (Knot and Nelson 1998). In cerebral arteries, evidence has been presented arguing for astrocyte secretion of K^+ upon neuronal stimulation, suggesting a way to couple neuronal activity to local blood flow in the brain (Filosa et al. 2006). Kir2.1, rather than Kir2.2 or Kir2.3, is thought to underlie these hyperpolarization effects in vascular smooth muscle cells (Bradley et al. 1999; Zaritsky et al. 2000). This capillary-to-arteriole coupling, whereby neuronal activity results in a small increase in extracellular K^+, is sensed by the Kir2.1 channels of capillaries to increase Kir2.1 outward current and hyperpolarization that in turn vasodilates the arterioles to cause an increase in local cerebral blood flow (Longden et al. 2017). Interestingly, this is a regulated process, as signaling through Gq-protein coupled receptors (GqPCRs) that hydrolyzes PIP_2 prevents activation of Kir2.1 by the $[K^+]_o$ increase and uncouples this intricate sensing mechanism (Harraz et al. 2018).

Neurons: Kir2 channels are abundantly and differentially expressed in somata and dendrites of neurons in the brain: diffusely and weakly in the whole brain (Kir2.1),

moderately throughout the forebrain and strongly in the cerebellum (Kir2.2), mainly in the forebrain and olfactory bulb (Kir2.3), and in the cranial nerve motor nuclei in the midbrain, pons (Kir2.4) (Hibino et al. 2010). PIP_2 depletion, once again, modulates activity, this time of striatopallidal neurons, as stimulation of their dendritic spines that contain Kir2.3 channels results in enhancement of dendritic excitability (Shen et al. 2007). The microvilli of Schwann cells at the node of Ranvier express Kir2.1 and Kir2.3 which may be serving a buffering role to maintain $[K^+]_o$ by absorbing excess K^+ released by excited neurons as done by astroglia (Mi et al. 1996).

2.4 Pharmacology

Kir2 channel inhibitors: A number of small molecule inhibitors for Kir2 channels have been reported. *Tamoxifen*, an estrogen receptor antagonist for breast cancer treatment, inhibits Kir2.1, Kir2.2, and Kir2.3. The experimental results suggested the compound inhibits the channels by interfering with channel–PIP_2 interactions (Ponce-Balbuena et al. 2009). Chloroquine, an important anti-malaria drug, inhibits the Kir2.1 channel and can induce lethal ventricular arrhythmias. Molecular modeling and mutagenesis suggested that chloroquine blocks the Kir2.1 channel by plugging the cytoplasmic conduction pathway, interacting with the negatively charged and aromatic residues within the central pocket (Rodriguez-Menchaca et al. 2008).

Gambogic acid (GA) was discovered as a Kir2.1 inhibitor through a library screening of 720 naturally occurring compounds. GA acts slowly at nanomolar concentrations to abolish Kir2.1 but not $K_v2.1$, HERG or Kir1.1 channel activity. GA could interfere with Kir2.1 channel trafficking to the cell surface (Zaks-Makhina et al. 2009). VU573 was discovered through a thallium (Tl^+) flux-based high-throughput screen of a Kir1.1 inhibitor library. The compound inhibits Kir3, Kir2.3, and Kir7.1 over Kir1.1 and Kir2.1 channels (Raphemot et al. 2011). ML133 was discovered through a high-throughput screen (HTS) of a library (>300,000 small molecules). It inhibits Kir2.1 with little selectivity against other Kir2 family channels. However, ML133 has no effect on Kir1.1, and a weak activating effect on Kir4.1 and Kir7.1 channels. Using a chimera between Kir2.1 and Kir1.1, the molecular determinants were identified to be residues D172 (the "rectification controller" residue) and I176 (one of the two HBC gates, I177 in Kir2.2) of the TM2 segment in Kir2.1 (Wang et al. 2011). Chloroethylclonidine (CEC) has been reported as an agonist for α_2-adrenergic receptors and an antagonist for α_{1x}-receptors. It inhibits Kir2.1 by directly blocking the channel pore, possibly at an intracellular polyamine binding site. Mutation of E172 in Kir2.1 to asparagine abolished CEC inhibition (Barrett-Jolley et al. 1999). Celastrol has been discovered as an inhibitor for the Kir2.1 and hERG channels and causes QT prolongation. It also alters the rate of channel transport and causes a reduction of channel density at the cell surface (Sun et al. 2006a). 3-bicyclo [2.2.1] hept-2-yl-benzene-1,2-diol was discovered as an inhibitor of the Kir2.1 and $K_v2.1$ channels through screening of

10,000 small molecules from a combinatorial chemical library. It is a weak inhibitor of Kir2.1 compared to Kv2.1 channels (Zaks-Makhina et al. 2004).

Kir2 channel activators: Pregnenolone sulfate (PREGS) belongs to the neurosteroid family and has been shown to be a Kir2.3 activator from the extracellular side of the membrane with no effect from the intracellular side. The activation was not affected by changes in the external pH. Other Kir channels, such as, Kir1.1, Kir2.1, Kir2.2, and Kir3 channels, were insensitive to PREGS (Kobayashi et al. 2009). Tenidap was discovered as a potent activator of Kir2.3 channels using an $^{86}Rb^{+}$ efflux assay. The action of tenidap was from the extracellular side of the membrane. It had little or no effect on Kir2.1, Kv1.5, and Na_v channels. Tenidap could serve as a pharmacological tool, an opener of Kir2.3 channels (Liu et al. 2002) (Table 3).

Table 3 Small molecule inhibitors and activators of Kir2 channels

Drug name	Species system	Potency (IC_{50}/EC_{50})	References
Inhibitor			
ML133	HEK-293 cells	Kir2.1 (IC_{50} = 1.8 μM) Kir2.6 (IC_{50} = 2.8 μM) Kir2.2 (IC_{50} = 2.9 μM) Kir2.3 (IC_{50} = 4.0 μM)	Wang et al. (2011)
VU573	HEK-293 cells	Kir2.3 (IC_{50} = 4.7 μM)	Raphemot et al. (2011)
Tamoxifen (antiestrogens)	HEK-293 cells	Kir2.1 (IC_{50} = 0.93 μM) Kir2.2 (IC_{50} = 0.87 μM) Kir2.3 (IC_{50} = 0.31 μM)	Ponce-Balbuena et al. (2009)
Gambogic acid (xanthonoid)	HEK-293 cells	Kir2.1 (IC_{50} = 27 nM)	Zaks-Makhina et al. (2009)
Chloroquine (4-aminoquinoline)	HEK-293 cells	Kir2.1 (IC_{50} = 8.7 μM	Rodriguez-Menchaca et al. (2008)
Chloroethylclonidine (imidazoline)	Skeletal muscle	Kir2.1 (IC_{50} = 37 μM)	Barrett-Jolley et al. (1999)
Celastrol (dienonephenolic triterpene)	HEK-293 cells	Kir2.1 (IC_{50} = 20 μM)	Sun et al. (2006a)
3-Bicyclo[2.2.1] hept-2-yl-benzene-1,2-diol	HEK-293 cells	Kir2.1 (IC_{50} = 60 μM)	Zaks-Makhina et al. (2004)
Activator			
Pregnenolone sulfate (neurosteroids)	Xenopus oocytes	Kir2.3 (EC_{50} = 1.43 μM)	Kobayashi et al. (2009)
Tenidap (indoles)	CHO cells	Kir2.3 (EC_{50} = 402 nM)	Liu et al. (2002)

2.5 Structural Studies

Channel activation of all Kir channels requires PIP_2, but Kir2 channels have been shown to require an additional secondary non-specific phospholipid (PL-) (i.e., PA, phosphatidic acid; PG, phosphatidylglycerol; PS, phosphatidylserine; PI, phosphatidylinositol) for high PIP_2 sensitivity. Lee et al. (2013) using molecular docking simulations and experimental validation identified a putative anionic phospholipid [PL(−)] binding site, which is located adjacent to the PIP_2 binding site formed by two lysine residues, K64 and K219. Neutralization of either of these residues to Cys reduces channel activity, but lipid-tethering K64C with decyl-MTS (methanethiosulfonate) induces high-affinity PIP_2 activation even in the absence of PL(−). These results suggest a molecular mechanism for a PL(−) synergistic effect on PIP_2-dependent activation of Kir2 channels. Interestingly, the residues K64 and K219 in Kir2.1 are not highly conserved in the Kir channel family. For example, the corresponding residues in Kir 3.2 are E71 and N229 (Fig. 9). Thus, for Kir3 channels, instead of PL(−), it is the regulators $G_{\beta\gamma}$ and Na^+ that play a critical role in stimulating channel activity by enhancing channel-PIP_2 interactions (Lee et al. 2013). Lee and colleagues further characterized the PL(−) site by introducing a Trp mutation at the K62 position in Kir2.2 channel (corresponding to the K64 in Kir2.1), which enhances PIP_2 sensitivity even in absence of PL(−). High-resolution crystal structures of Kir2.2(K62W) were solved in the presence and absence of PIP_2. The mutation caused a tight tethering of the CTD to the TMD of the channel regardless of the presence of PIP_2 and mimicked the PL(−) binding to the second site, inducing formation of the high affinity primary PIP_2 site. MD simulations have revealed a more extensive hydrogen bonding to basic residues at the PIP_2 binding site in the Kir2.2 (K62W) mutant compared to the wild-type channel (Lee et al. 2016). To elucidate the open conformation and mechanism of K^+ ion permeation, Zangerl-Plessl et al. (2020) introduced an additional G178D mutation in the Kir2.2 (K62W) mutant channel to generate the "forced open" mutant channel (KW/GD). Crystal structures of the KW/GD mutant channel were solved in the presence and absence of PIP_2. The PIP_2 bound form of the KW/GD structure showed a slight widening of the HBC gate (∼1.5 Å) compared to the PIP_2 bound K62W structure. However, microsecond MD simulations for KW/GD in lipid bilayer showed opening of the HBC gate and K^+ permeation. Simulation results showed K^+ ion permeation through the SF via a strict ion-ion knock-on mechanism with rates comparable to the experimentally measured ion conductance (Zangerl-Plessl et al. 2020).

3 G Protein Kir3 Channels

3.1 Historical Perspective

In the mid-1950s after the advent of intracellular microelectrodes and voltage-clamp techniques Burgen and Terroux (1953) revisited the vagal induced hyperpolarization of heart muscle reported by Gaskell in the mid-1880s (Gaskell 1886; Gaskell 1887).

They first confirmed that hyperpolarization is induced by ACh application (Burgen and Terroux 1953) and then by measuring the effect of external K^+ concentration on resting potential in the absence and presence of ACh, they also demonstrated that an increased cell permeability to potassium may underlie hyperpolarization. Del Castillo and Katz used microelectrodes to directly show hyperpolarization of the sinus node upon vagal stimulation (Del Castillo and Katz 1955). Voltage clamp allowed Trautwein and Dudel to directly confirm changes in cell potassium permeability by measuring K^+ reversal potentials (Trautwein and Dudel 1958). Noma and Trautwein studied activation kinetics of ACh-induced K^+ currents and concluded that ACh binding activates a specific ion channel, K_{ACh} (Noma and Trautwein 1978). The introduction of the patch-clamp technique (Hamill et al. 1981) led to the first single-channel recordings of K_{ACh} currents ($I_{K\text{-}ACh}$) (Sakmann et al. 1983), which clearly demonstrated kinetic properties distinct from other background potassium channels. In the mid-1980s five studies shed light as to how ACh activated $I_{K\text{-}ACh}$. First, following the advent of the patch-clamp technique, Soejima and Noma demonstrated the membrane-delimited nature of $I_{K\text{-}ACh}$ activation, when in cell-attached recordings they showed that ACh could only activate the channels if it was perfused (or loaded) directly in the pipette but not in the bath, arguing that no second messenger was involved in activating $I_{K\text{-}ACh}$ (Soejima and Noma 1984). The following year two reports published back-to-back provided evidence that pertussis toxin- (PTX-) sensitive $G_{i/o}$ proteins coupled muscarinic receptors to $I_{K\text{-}ACh}$, either by showing that PTX treatment disabled ACh stimulation of channel activity (Pfaffinger et al. 1985) or showing that non-hydrolyzable GTP analogs activated $I_{K\text{-}ACh}$ constitutively (Breitwieser and Szabo 1985). The following year using inside-out patches from atrial cells Kurachi and colleagues showed that in the presence of adenosine (or ACh) in the pipette GTP perfusion in the bath supported activation of $I_{K\text{-}ACh}$ (Kurachi et al. 1986). In 1987, Logothetis and colleagues showed that purified Gβγ (but not Gα subunits) activated $I_{K\text{-}ACh}$ in a manner similar to non-hydrolyzable GTP analogs, identifying $I_{K\text{-}ACh}$ as the first direct effector of the Gβγ subunits (Fig. 5a – top). A controversy arose as Birnbaumer and Brown argued that the activated Gα (i.e., Gα-GTPγS) and not the Gβγ subunits were the activators of $I_{K\text{-}ACh}$ (Birnbaumer and Brown 1987). The controversy was resolved 7 years later when Reuveny and colleagues confirmed with recombinant channels in *Xenopus* oocytes that Gβγ subunits were the G protein activators of $I_{K\text{-}ACh}$ (Reuveny et al. 1994). Intracellular Na^+ ions were also shown to activate Kir3.4 and Kir3.2 homo- and heterotetramers in a G protein-independent manner (Ho and Murrell-Lagnado 1999; Sui et al. 1996). Ethanol was also shown to activate Kir3 currents (Kobayashi et al. 1999; Lewohl et al. 1999). In 1998, Kir3 currents activated by Gβγ or Na^+ were shown to require PIP_2 in the plasma membrane for activation (Huang et al. 1998; Petit-Jacques et al. 1999; Sui et al. 1998). Although for classical Kir channels, PIP_2 was sufficient to stimulate activity, Kir3 channels required a gating molecule (e.g., Na^+, Gβγ, alcohol, etc.) together with PIP_2 to stimulate activity (Huang et al. 1998; Sui et al. 1998). These gating molecules seemed to strengthen the interactions of Kir3 channels with PIP_2, as a single point mutant (Kir3.4-I229L) removed the

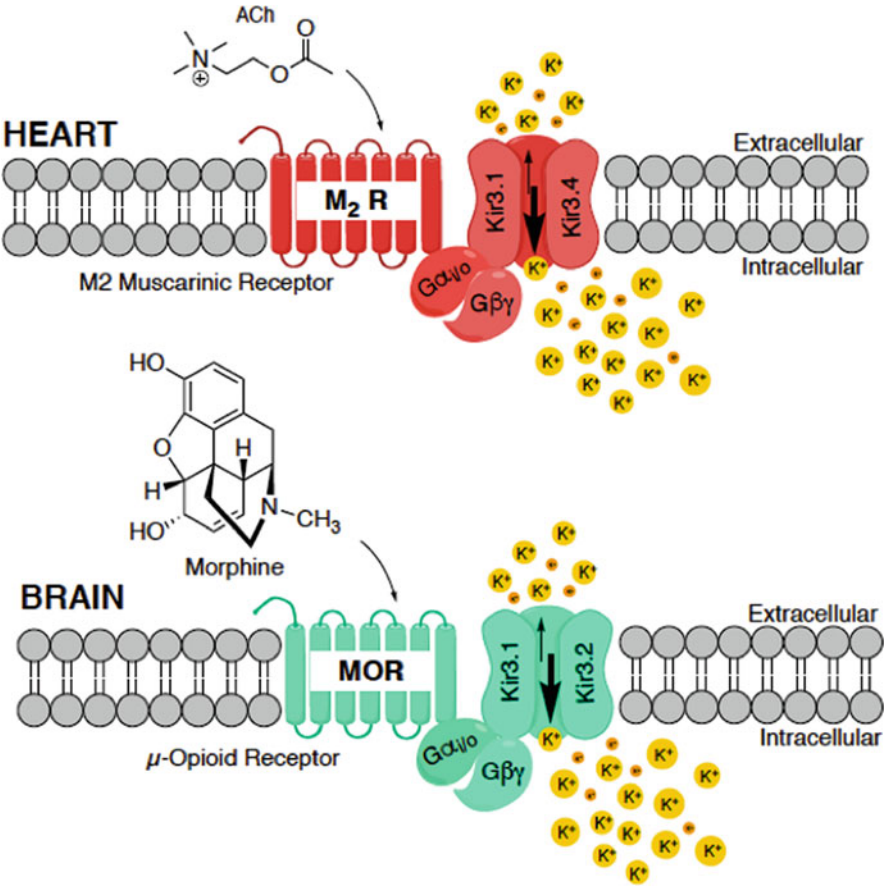

Fig. 5 The Kir3 GEMMA ($G_{i/o}$pcr-Effector-Macromolecular-Membrane-Assembly) before activation (name/concept adapted from Ferré et al. 2021)

requirement for gating molecules, allowing PIP_2 to activate on its own (Zhang et al. 1999).

3.2 Subfamily Members and Tissue Distribution

It wasn't until 1993 that the first member Kir3.1 of this subfamily was cloned (Dascal et al. 1993; Kubo et al. 1993b). The following year the neuronal Kir3.2 and Kir3.3 subunits were identified (Lesage et al. 1994). Finally, in 1995 the Kir3.4 subunit was cloned and when co-expressed together with Kir3.1 was shown to produce the biophysical properties of $I_{K\text{-}ACh}$ expressed in heterologous cells (Chan et al. 1996; Krapivinsky et al. 1995). Kir3.2 and Kir3.4 are capable of conducting currents through homotetramers, while Kir3.1 and Kir3.3 need to heteromerize with

other subunits to produce functional channels. In the brain, multiple signals including ACh, adenosine, dopamine, opioids, GABA, etc. bind their respective $G_{i/o}$ protein-coupled receptors ($G_{i/o}$PCRs) to activate Kir3 channels using the Gβγ subunits of PTX-sensitive G proteins (Fig. 5b – bottom). Expression of different subunits within distinct neuronal populations defines their responsiveness to neurotransmitters. The ventral tegmental area (VTA) in the reward centers of the brain illustrates well the relevance of the Kir3 subunit composition (Lujan et al. 2014). Dopaminergic neurons within the VTA do not express Kir3.1 but do express Kir3.2 and Kir3.3, producing Kir3.2 homotetramers as well as Kir3.2/3.3 heterotetramers (Cruz et al. 2004; Jelacic et al. 2000; Kotecki et al. 2015). GABAergic neurons within the VTA also express Kir3.2 and Kir3.3 along with Kir3.1 producing also the dominating Kir3.1/3.2 heterotetramers. VTA dopaminergic neurons show less sensitivity to GABA than GABAergic neurons do (Cruz et al. 2004; Labouebe et al. 2007). Genetic ablation of Kir3.3 in the dopaminergic neurons enhances their sensitivity to GABA, suggesting an inhibitory role by Kir3.3. Thus, low concentrations of agonists preferentially inhibit GABAergic neurons and thereby disinhibit dopaminergic neurons. This disinhibition might confer reinforcing properties on addictive $GABA_B$ receptor agonists (Labouebe et al. 2007).

Three major alternatively spliced isoforms of Kir3.2 are differentially expressed in the brain Kir3.2a-c. Kir3.2c is longer than Kir3.2a, which is longer than Kir3.2b and also shows differences in 8 C-terminal aa (Hibino et al. 2010). In the periphery, Kir3 channels show moderate expression in the heart and pancreas (Fig. 2) as well as in the testis and the pituitary and adrenal glands.

Heart: Kir3.1 and Kir3.4 subunits are predominantly expressed in the atria, in nodal cells and pulmonary vein myocardial sleeves (Ehrlich et al. 2004; Greener et al. 2011; Krapivinsky et al. 1995). Expression in mouse ventricles has also been reported but it is not thought to impact cardiac physiology or ventricular arrhythmogenesis (Anderson et al. 2018).

Pancreas: Four Kir3 isoforms have been reported to be expressed and play important roles in the physiology of the pancreas. Kir3.4 subunits exist in α, β, and δ cells of pancreatic islets and in the exocrine pancreas, whereas the Kir3.2c isoform is expressed in α and δ cells (Ferrer et al. 1995; Iwanir and Reuveny 2008; Vaughn et al. 2000; Yoshimoto et al. 1999).

Testis: Kir3.1 and Kir3.2d (another splice isoform, 18 aa shorter than Kir3.2c, that has been described only in the testis) have been reported to be expressed in the spermatids and in spermatogonia and spermatocytes (Inanobe et al. 1999).

Anterior pituitary lobe: Kir3.1, Kir3.2, and Kir3.4 have all been reported to be expressed in the anterior pituitary (Gregerson et al. 2001; Morishige et al. 1999).

Adrenal gland: Since 2011 it was reported that in patients with severe hereditary hypertension Kir3.4 mutations were found in adrenal aldosterone-producing adenomas to cause hyperaldosteronism. These findings brought a greater appreciation of the role of Kir3.4 in aldosterone production and disease (Choi et al. 2011; Gomez-Sanchez and Oki 2014). Other tissues including the eye, skin, and the colon have also been reported to express Kir3 channels (Huang et al. 2018a; Yamada et al. 1998).

Central nervous system (CNS): Kir3.1-Kir3.3 are expressed throughout, with highest levels seen in the olfactory bulb, neocortex, hippocampus, and the granule cell layer of the cerebellum but notably also in the amygdala, thalamus, substantia nigra, ventral tegmental area, locus coeruleus, some nuclei of the brainstem, and the spinal cord (Kobayashi and Ikeda 2006; Lujan and Aguado 2015). In contrast, Kir3.4 is expressed in relatively fewer brain regions with highest levels in deep cortical pyramidal neurons, the endopiriform nucleus and claustrum of the insular cortex, the globus pallidus, the ventromedial hypothalamic nucleus, parafascicular and paraventricular thalamic nuclei, and a few brainstem nuclei, such as the inferior olive and vestibular nuclei (Wickman et al. 2000).

3.3 Physiology/Pathophysiology

Physiology: The Kir3 channels and the adenylyl cyclase enzymes have been studied extensively as prototypical effectors coupled to G proteins and their corresponding GPCRs. There is a vast body of literature supporting the existence of pre-coupled macromolecular complexes that have been coined as GEMMAs, GPCR-Effector-Macromolecular-Membrane-Assemblies (Ferré et al. 2021). Thus, the various $G_{i/o}$PCRs-$G\alpha_{i/o}\beta\gamma$-Kir3 channel tetramers constitute unique GEMMAs (Fig. 5). The GEMMA concept is significant in that it extends the collision coupling model by which receptors, G proteins, and effectors communicate and transduce signals. In a GEMMA, agonists set a series of conformational changes in the pre-coupled elements that communicate with each other like clock gears rather than billiard balls. Clearly, the best evidence for GEMMAs is yet to come from high-resolution structures capturing them in their pre-coupled inactive and active states and elucidating their interactions at atomic resolution. With cryogenic electron microscopy (cryo-EM) having achieved technological breakthroughs in the past decade, structures of complexes are becoming a reality and it is only a matter of time before we obtain high-resolution images of GEMMAs. Computational power to perform dynamic simulations of large systems is also in the midst of technological breakthroughs, such that the workings of GEMMAs can be captured in the course of a computer simulation. This unprecedented progress in our ability to make reliable dynamic structure-based models of macromolecular membrane assemblies of proteins promises to illuminate our understanding of the molecular physiology and pharmacology of GEMMAs.

GEMMAs use a core assembly, as shown in Fig. 5 with the examples of the K_{ACh} and K_{MOR} GEMMAs, to carry out their basic function but can accommodate in a dynamic manner the coming and going of multiple other partners to fine-tune effector function in response to external and internal signals. Each member of the core GEMMA can recruit additional regulatory partners or scaffold proteins that orchestrate a symphony of regulatory partners with the GEMMA core proteins. RGS (regulator of G protein signaling) proteins, for example, enhance the GTPase activity of G proteins and as such they accelerate receptor-induced Kir3 current deactivation kinetics and inhibit GPCR-Kir3 signaling (Doupnik 2015; Doupnik et al. 1997;

Sjogren 2011). Molecular platforms to achieve subcellular localization of membrane proteins or post-translational modifications needed to serve the physiology of the GEMMA have been reviewed by Ferré and colleagues (2021).

Utilization of motifs in the C-termini of any of the core proteins of the GEMMA can recruit specific modulatory proteins. As an example, let us consider the Kir3 channels using their PDZ domains to recruit scaffold proteins. The PDZ domain is a common structural domain of 80–90 amino acids found in signaling proteins from bacteria to animals. Proteins containing PDZ domains play a key role in anchoring receptor proteins in the membrane to cytoskeletal components. Proteins with these domains help hold together and organize signaling complexes at cellular membranes. PDZ got its name by combining the first letters of the first three proteins discovered to share the domain – post synaptic density protein (PSD95), Drosophila disc large tumor suppressor (Dlg1), and zonula occludens-1 protein (zo-1). Scaffold proteins of the PSD-95 and Shank families recognize class I PDZ motifs (x-S/T-x-F: F is a hydrophobic aa and x is any aa) and can bind the C-termini of these channel subunits. Kir3.2c and Kir3.3 possess a C-terminal motif for class I PDZ domain-containing proteins (E**S**K**V**) that is absent in Kir3.2a. PDZ-scaffold proteins are considered to be one of the major determinants of postsynaptic localization of various proteins (Sheng and Sala 2001). Thus, Kir3.2c and Kir3.3 channels could be localized post-synaptically through direct or indirect interactions with PDZ-scaffold proteins. Classical Kir2 channels, for which there is no evidence that they are part of a GEMMA, also contain class I PDZ motifs. Interestingly, Kir2, but not Kir3 subunits, has been found to interact directly with PSD-95 (Nehring et al. 2000), indicating that the mere presence of the binding motif does not necessarily imply a direct binding of the scaffold protein. Instead, the scaffold protein sorting nexin 27 (SNX27) binds the Kir3.2c and Kir3.3 channels but not the Kir2 (or a Kir4 channel that also contains the motif (Balana et al. 2011; Lunn et al. 2007)). SNX27 association with the Kir3 subunits leads to a reduction of signaling at the plasma membrane, likely by promoting internalization of the channel (Lunn et al. 2007). This example points to how subunit composition (in this case Kir3.2c and Kir3.3) makes the channel and potentially its GEMMA prone to another regulatory control of channel density at the plasma membrane to control cellular function. Interactions of Kir3.1/3.2 with NCAM (neural cell adhesion molecule) in lipid raft microdomains of hippocampal neurons have been reported to decrease Kir3.1/3.2 cell surface density (Delling et al. 2002).

Finally, let us consider post-translational modifications of Kir3 channels, such as phosphorylation. Kir3 (as well as several other Kir) channels have been reported to be modified in a functionally meaningful manner by phosphorylation, e.g. by protein kinases A and C (PKA, PKC): (Keselman et al. 2007; Leaney et al. 2001; Lei et al. 2001; Lopes et al. 2007; Mao et al. 2004; Medina et al. 2000; Mullner et al. 2000; Sharon et al. 1997; Sohn et al. 2007; Stevens et al. 1999; Witkowski et al. 2008; Gada and Logothetis 2021). Another large (more than 50-member) class of scaffolding proteins called AKAPs (A kinase anchoring proteins) binds to a common motif on effectors or GPCRs to organize recruitment of specific kinases and phosphatases (e.g., AKAP5 anchors PKA, PKC, calcineurin to proteins it binds) to modify the

protein(s) of interest (Ferré et al. 2021). Is phosphorylation of Kir3 channels involving the use of scaffolding partner proteins, such as AKAPs? Although direct evidence is not yet available for Kir channels, GPCRs have been studied extensively and as parts of a GEMMA they could provide the organization needed for phosphorylation/dephosphorylation of Kir channels. *Pathophysiology* Studies on mutant mice lacking specific Kir3 subunits have provided evidence for physiological/pathophysiological effects suggesting these channels as therapeutic targets and highlighting the usefulness of potential Kir3 activators/inhibitors (Fig. 6).

Cardiac arrhythmias: As we have discussed above, stimulation of M_2R in supraventricular tissues by ACh released by the vagus nerve activates $I_{K\text{-}ACh}$ slowing down heart rate and endowing the heart with the adaptability needed to respond to rate adjustments (heart rate variability or HRV) and meet the demands of the body,

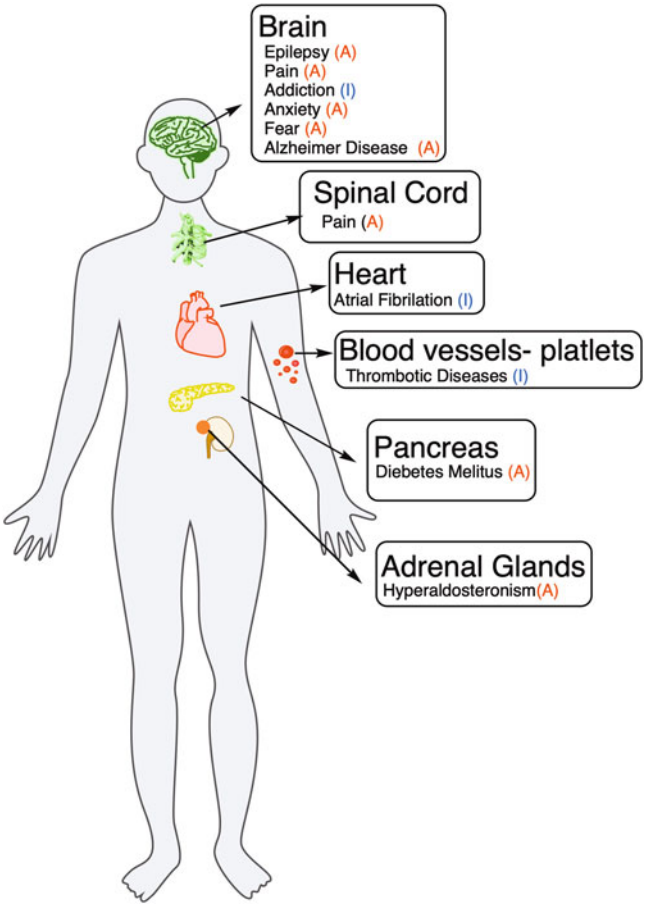

Fig. 6 Pathophysiological Kir3 conditions and drug regulators needed as potential therapeutics (A) Activators (I) Inhibitors. *CB*, cannabinoids, *ETOH* ethanol, *MOR* μ-opioid receptor, *PSVT* paroxysmal supraventricular tachycardia

as in exercise. Adenosine has similar effects on $I_{K\text{-}ACh}$ (Kurachi et al. 1986), acting through adenosine 1 receptors (A1Rs). Although Kir3.4 or Kir3.1 knockout in mice resulted at best in mild resting tachycardia, these deletions strongly decreased chronotropic response and HRV (Bettahi et al. 2002; Wickman et al. 1998). Kir3.4 loss of function mutations in people have been described resulting in Long QT (LQT) syndrome (Yang et al. 2010). Similarly, in cases of paroxysmal supraventricular tachycardia (PSVT), adenosine and other A1R receptor agonists have been used successfully for its acute treatment (Prystowsky et al. 2003). Vagal denervation (or knockout of the Kir3.4 channel) prevents the induction of atrial fibrillation (AF), the most common arrhythmia in clinical practice (Kovoor et al. 2001). On the other hand, gain of function conditions, such as vagal stimulation, predispose to AF. In AF, a constitutive increase in I_{KACh}, in the absence of channel stimulation by acetylcholine, promotes the shortening of action potential duration (Carlsson et al. 2010; Dobrev et al. 2005; Kovoor et al. 2001). This increase in basal activity of the channel is ATP dependent and is enhanced in the presence of phosphatase inhibitors (Makary et al. 2011), suggesting kinase-mediated activity. Conventional PKC isoforms inhibit, while novel PKC isoforms stimulate $I_{K\text{-}ACh}$ (Makary et al. 2011). A decrease in PKCα and a concomitant increase in PKCε expression have been reported from patients with AF (Voigt et al. 2014; Gada and Logothetis 2021).

Hyperaldosteronism: As mentioned above, Kir3.4 mutations were found in adrenal aldosterone-producing adenomas causing hyperaldosteronism in patients with severe hereditary hypertension (Choi et al. 2011).

Hyperalgesia and decreased analgesia: Kir3.1 and/or Kir3.2 knockout mice exhibit hyperalgesia (Marker et al. 2004). Moreover, $G_{i/o}$PCR ligands for opioid, M2 muscarinic, α_2 adrenergic, $GABA_B$, and cannabinoid receptors show reduced analgesic effects in Kir3.2 and/or Kir3.3 knockout mice (Blednov et al. 2003; Cruz et al. 2008; Lotsch et al. 2010; Lujan et al. 2014; Luscher and Slesinger 2010; Mitrovic et al. 2003; Nishizawa et al. 2009; Smith et al. 2008; Tipps and Buck 2015). Gene polymorphisms in *KCNJ6* (coding of Kir3.2) in humans have been associated with analgesia and pain sensitivity (Bruehl et al. 2013; Nishizawa et al. 2014). Thus, a plethora of studies converge on the mechanism that Kir3 channel activation induces analgesia accounting for the action of many drugs that reduce pain.

Drug addiction: As discussed earlier, Kir3 channels are thought to play an important role in the communication between the midbrain GABAergic and VTA dopaminergic neurons in the mesolimbic reward system, resulting in disinhibition of the VTA dopaminergic neurons which have been connected to reward behaviors (Kotecki et al. 2015; Marron Fernandez de Velasco et al. 2015; Tipps and Buck 2015). Kir3 channels play a role in mediating the rewarding effects of ethanol and addictive drugs that target GPCRS, such as the MOR and cannabinoid type 1 receptor (Blednov et al. 2003; Blednov et al. 2001a; Blednov et al. 2001b; Marron Fernandez de Velasco et al. 2015; Nagi and Pineyro 2014; Nassirpour et al. 2010; Rifkin et al. 2017; Tipps and Buck 2015). Kir3.2 and Kir3.3 knockout mice showed reduced self-administration of cocaine compared to wild-type mice indicating that in the absence of Kir3 channels the behavioral response to drugs of abuse is altered (Morgan et al. 2003). It has been shown in multiple cell lines that exposure to these addictive drugs

reduces Kir3 currents (Marron Fernandez de Velasco et al. 2015; Rifkin et al. 2017; Tipps and Buck 2015). Recent studies have attempted to generate cell-specific Kir3 channel knockouts with cre-lox methodologies (Kotecki et al. 2015). Whether specific Kir3 subunit composition selective modulators could become useful pharmacotherapies for managing addiction remains to be tested.

Epilepsy: Unlike Kir3.4 (Wickman et al. 2000), Kir3.2 knockout mice exhibit spontaneous seizures and increased susceptibility to a convulsant agent (Signorini et al. 1997). Adenosine-induced hyperpolarization in hippocampal brain slice recordings could be blocked by a Kir3 blocker, which induced seizure activity (Hill et al. 2020). In temporal lobe epilepsy, the most common epilepsy in adults (Tellez-Zenteno and Hernandez-Ronquillo 2012) that progresses to drug-resistant seizures, N-methyl-D-aspartate receptor (NMDAR) hyperactivity triggers apoptotic mechanisms, including activation of cysteinyl aspartate-specific proteases (caspases) that characterize the severity of epileptic seizures. It has been recently shown that caspase-3 targets the Kir3.1 and Kir3.2 proteins, cleaving their C-terminal ends to compromise Gβγ activation and cell surface localization of these channels, implicating them strongly in the progression of this disease (Baculis et al. 2017).

Down syndrome: The KCNJ6 that encodes for Kir3.2 is located in human chromosome 21. In Down syndrome there is a trisomy of chromosome 21, resulting in a duplication of the KCNJ6 gene that alters synaptic transmission (Reeves et al. 1995; Sago et al. 1998). Full or partial duplication of the corresponding mouse gene that contains KCNJ6 produced hallmark characteristics of the disease (Reeves et al. 1995; Sago et al. 1998). Kir3.2 protein upregulation resulted in altered reward mechanisms, cognitive functions, and synaptic plasticity (Reeves et al. 1995; Sago et al. 1998; Siarey et al. 1999). To control for the fact that there are several gene duplications that make the pathophysiology more complex in this disorder, a single trisomy of the KCNJ6 gene was produced in mice recapitulating deficits characteristic of the syndrome, suggesting that triplication of only this gene is key in some of the abnormal neurological phenotypes seen in Down syndrome (Cooper et al. 2012).

Alzheimer's disease (AD): AD is characterized by progressive cognitive decline accompanied by formation of amyloid-beta (Aβ) plaques, neurofibrillary tangles, and aggregates of hyperphosphorylated tau protein. The main brain regions affected by AD are the hippocampus and amygdala, where early onset AD pathogenesis is seen (Arriagada et al. 1992; Haass and Selkoe 2007; Hardy and Selkoe 2002; Huang and Mucke 2012; Swanberg et al. 2004; Zald 2003). Imbalances of excitatory/inhibitory synaptic transmission occur early in the pathogenesis of AD, leading to hippocampal hyperexcitability and causing synaptic, network, and cognitive dysfunction. Neuronal Kir3 channels control neuronal excitability contributing to the inhibitory signaling in the hippocampus. The tonic Kir3 currents related to $GABA_B$ receptors contribute critically to the balancing of membrane excitability and synaptic transmission (Lujan et al. 2009; Nava-Mesa et al. 2013; Sanchez-Rodriguez et al. 2020). Intracerebroventricular injections of Aβ impaired inhibitory signaling and disrupted synaptic plasticity (Sanchez-Rodriguez et al. 2017). $A\beta_{25-35}$ significantly decreased Kir3 channel conductance in the hippocampus (Mayordomo-Cava et al. 2015). RT-qPCR revealed that in CA3-CA1 hippocampal neurons application of Aβ

decreased mRNA transcripts for Kir3.2, Kir3.3, and Kir3.4 subunits (Mayordomo-Cava et al. 2015). Contradictory results have also been published suggesting that a Kir3-mediated potassium efflux triggers apoptosis in hippocampal neurons (May et al. 2017). These conflicting results will need to be resolved in future studies.

Fear: Kir3.2 knockout mice showed impairments in both hippocampal-dependent contextual fear conditioning and hippocampal-independent cue fear conditioning (Victoria et al. 2016). Mice subjected to auditory cue-induced fear extinguish the fear by activating pathways between basolateral amygdala (BLA) and the nucleus accumbens (NAc) in the ventral striatum. BLA expresses all Kir3 isoforms, except Kir3.4, and displays baclofen-induced Kir3 currents through $GABA_B$ receptors (Xu et al. 2020).

3.4 Pharmacology

Kir3 channel inhibitors: Table 4 lists a number of inhibitors of Kir3 currents that have been studied in supraventricular cardiac arrhythmias and AF in particular.

3.4.1 Inhibitors in Disease

Atrial fibrillation (AF): The discovery that potent low nanomolar inhibition of Kir3.1/3.4 could be achieved by tertiapin (TPN), the 21 aa peptide extracted from the honey bee venom that also inhibits similarly Kir1.1 channels (Jin and Lu 1998), provided a useful tool to explore the role of this channel in AF. A non-oxidizable variant of TPN(M13Q) (or TPNQ) shows a similar Ki value to TPN and is often used instead (Jin and Lu 1999). Its specificity limitation, however, encouraged exploration for small molecule inhibitors.

Three compounds in the benzopyran class emerged, NIP-142, NIP-151, and NTC-801 with outstanding potency but not high enough specificity, especially against the neuronal Kir3.1/3.2. NTC-801 in particular inhibited Kir3.1/3.4 channels with a sub-nanomolar IC_{50} but inhibited Kir3.1/3.2 with an IC_{50} of 24 nM (Machida et al. 2011).

Although NTC-801 was able to convert AF to normal sinus rhythm in canine tachypacing models (Yamamoto et al. 2014), in clinical trials it failed to reduce AF burden in 20 patients with paroxysmal AF (Podd et al. 2016). The reason for this failure was likely that due to lack of specificity between Kir3.1/3.2 and Kir3.1/3.4 it was not possible to reach concentrations required for antiarrhythmic effect without producing CNS side effects. Cui and colleagues have revealed the mechanism of action of this class of compounds by the prototypic Benzopyran-G1 (likely to be NTC-801) revealing its binding site in Kir3.1 and showing that it acts non-specifically in Kir3.1 heteromeric channels (Cui et al. 2021). Recently, XAF-1407 was reported to inhibit Kir3.1/3.4 with an IC_{50} of 1 nM and to decrease the AF rate in an equine model of persistent AF (Fenner et al. 2020) but whether due to specificity issues it will have a similar fate as NTC-801 remains to be seen.

Pain: TPNQ (tertiapin-Q) was shown to reduce MOR agonist activity in the immersion tail flick test (Marker et al. 2004). Additionally, intrathecal administration

Table 4 Small molecule inhibitors and activators of Kir3 channels

Drug name	Species system	Kir3.1/3.2 ~IC_{50}/EC_{50} or Tc (Tc: typical concentration) *NR: not reported*	Kir3.1/3.4 ~IC_{50}/EC_{50} Tc (Tc: typical concentration) *N/A: not applicable*	References
Inhibitor				
Tertiapin-Q (peptide)	Xenopus oocyte	NR	0.0082 μM	Jin and Lu (1998)
SCH3390 (D_1R antagonist)	CHO cells	7.78 μM	NR	Kuzhikandathil and Oxford (2002)
NIP-151 (tricyclic benzopyran)	HEK-293 cells	NR	0.0016 μM	Hashimoto et al. (2008)
NIP-142 (tricyclic benzopyran)	Xenopus oocyte HEK293 cells	NR	10.0 μM 0.64 μM	Matsuda et al. (2005, 2006)
NTC-801 (BMS914392) BP-G1 (tricyclic benzopyran)	Xenopus oocytes Guinea pig atrial cells	0.024	0.0007 μM	Machida et al. (2011) Cui et al. (2021)
Halothane (general anesthetic)	Xenopus oocyte	Tc = 2MAC	NR	Yamakura et al. (2001)
Haloperidol (antipsychotic)	Xenopus oocyte	75.5 μM	40.9 μM	Kobayashi et al. (2000)
Desipramine (tricyclic antidepressant)	Xenopus oocyte	36.4 μM	53.9 μM	Kobayashi et al. (2004)
Methadone (MOR agonist)	Xenopus oocyte	53.3 μM	NR	Ulens et al. (1999)
Ifenprodil (α-1R antagonist NMDAR inhibitor)	Xenopus oocyte	7.01 μM	2.83 μM	Kobayashi et al. (2006)
Fluoxetine (prozac -SSRI)	Xenopus oocyte	16.9 μM	18.4 μM	Kobayashi et al. (2003)
Falcatin-A (terpenoid)	HEK-293 cells	NR	2.5 μM	Vasas et al. (2016)
VU573	HEK-293 cells	1.3 μM	1.3 μM	Raphemot et al. (2011)
XAF-1407	HEK-293 cells	NR	0.0011 μM	Fenner et al. (2020)

(continued)

Table 4 (continued)

Drug name	Species system	Kir3.1/3.2 ~IC_{50}/EC_{50} or Tc (Tc: typical concentration) *NR: not reported*	Kir3.1/3.4 ~IC_{50}/EC_{50} Tc (Tc: typical concentration) *N/A: not applicable*	References
Activator				
Ethanol (n-alcohol)	Xenopus oocyte	Tc = 100 mM	Tc = 100 mM	Kobayashi et al. (1999), Lewohl et al. (1999)
Naringin (flavonoid)	Xenopus oocyte	111.0 μM	120.9 μM	Yow et al. (2011)
ML297 (N-phenyl pyrazole urea)	HEK-293 cells	0.16 μM	1.8 μM	Kaufmann et al. (2013)
VU0466551 (N-benzyl pyrazole urea)	HEK-293 cells	0.07 μM	0.11 μM	Wen et al. (2013)
VU464 (N-cyclohexyl pyrazole acetamide)	HEK-293 cells	0.165 μM	0.72 μM	Wieting et al. (2017)
GAT1508 (N-phenyl pyrazole urea)	Xenopus oocyte HEK-293 cells	0.37 μM 0.075 μM	N/A	Xu et al. (2020)
VU331	HEK-293 cells	5.2 μM (Kir3.2 = 5.1 μM)	N/A	Kozek et al. (2019)
Ivermectin (antiparasitic)	Xenopus oocyte	3.5 μM	7.5 μM	Chen et al. (2017)
GiGA1 (urea)	HEK-293 cells	31 μM	NR	Zhao et al. (2020)

of TPNQ attenuated oxycodone-induced antinociceptive effects in mice (Nakamura et al. 2014).

Epilepsy: TPNQ administered intrathecally induced seizures (Mazarati et al. 2006). Several drugs used clinically as antipsychotics and antidepressants in the early 2000s (e.g., haloperidol, clozapine, desipramine, fluoxetine) were identified to inhibit Kir3 channels at higher concentrations and to induce seizures as a side effect (Kobayashi et al. 2000; Kobayashi et al. 2003; Kobayashi et al. 2004; Kobayashi et al. 2006; Luscher and Slesinger 2010; Ulens et al. 1999; Yamakura et al. 2001).

Kir3 channel activators: Table 4 also lists a number of Kir3 activators. Specifically, there is a highly potent urea scaffold class that gave rise to a completely neuronal specific Kir3.1/3.2 activator with no effect on the cardiac Kir3.1/3.4 channels (i.e., GAT1508 – see below – Xu et al. 2020).

Ethanol was the first direct Kir3 channel drug activator, as a series of N-alkyl alcohols were shown to activate Kir3.1/3.2 and Kir3.1/3.4 channels with 1-propanol

being the most effective and potent activator (Kobayashi et al. 1999; Lewohl et al. 1999). In 2011 it was identified that at high micromolar concentrations the flavonoid Naringin from grapefruit could activate Kir3.1/3.2 channels (Yow et al. 2011). In 2013, data mining and development of the thallium flux fluorescent assay for Kir channels allowed for early identification of CID736191 (nF-ML297), revealing an urea core scaffold that was connecting a left side benzene ring, to the N-phenyl pyrazole on the right side (see Fig. 11, Kir3 Channel Activators, ML297) (Kaufmann et al. 2013; Weaver et al. 2004; Wen et al. 2013). Structure-activity relationship studies produced compound ML297 [1-(3,4-difluorophenyl)-3-(3-methyl-1-phenyl-1*H*-pyrazol-5-yl) urea]. ML297 has a Kir3.1/3.2 EC_{50} of 0.160 μM and a Kir3.1/3.4 EC_{50} of 1.8 μM and did not show any local motor impairments at efficacious doses in the rotor rod behavioral test in mice. ML297 was effective in two models of epilepsy in mice (Kaufmann et al. 2013). A slight chemical modification of the ML297 scaffold changing a phenyl group to a benzyl group produced compound VU0466551, 1-(1-benzyl-3-methyl-1*H*-pyrazol-5-yl)-3-(3,4-difluorophenyl) urea. VU0466551 has a Kir3.1/3.2 EC_{50} of 0.07 μM and Kir3.1/3.4 EC_{50} of 0.11 μM and was efficacious in two rodent models of pain (Abney et al. 2019; Wen et al. 2013). A second scaffold was data mined and screened for Kir3.1-containing channel activity by Weaver and colleagues, VU0259369, (2-(2-chlorophenyl)-*N*-(3-(*N*,*N*-dimethylsulfamoyl)-4-methylphenyl) acetamide) with an acetamide core, similar to the Urea-pyrazole class of compounds. However, this compound lacked selectivity and potency and was not pursued (Ramos-Hunter et al. 2013). The following SAR reported in 2017 was a scaffold merging of ML297's pyrazole core and replacing the urea with the acetamide core from VU0259369 to yield the hybrid compound VU0810464, 2-(3-chloro-4-fluorophenyl)-*N*-(1-cyclohexyl-3-methyl-1*H*-pyrazol-5-yl) acetamide. VU0810464 was synthesized to improve blood brain barrier (BBB) penetration (Wieting et al. 2017). In 2018, VU0810464 was evaluated in cultured hippocampal neurons and a stress induced hyperthermia model for anxiety and was found to be effective at reducing induced hyperthermia and activated Kir3 currents in hippocampal neurons (Vo et al. 2019). However, VU0810464 was not efficacious in the elevated plus maze model of anxiety in mice, similar to its parent compound ML297 (Vo et al. 2019). Recent studies identified GAT1508, a highly selective potent and efficacious Kir3.1/3.2 activator, with an EC_{50} of 0.075 μM, which is devoid of cardiac Kir3.1/3.4 channel activity (Xu et al. 2020). GAT1508 was evaluated in a rat auditory cue-induced fear extinction model of post-traumatic stress disorder (PTSD) that was found to be effective at extinguishing conditioned fear (Xu et al. 2020). GAT1508 also was found to induce Kir3 currents in basolateral amygdala (BLA) brain slice recordings where at low ineffective concentrations it potentiated baclofen-induced current showing synergism (Xu et al. 2020). Additionally, in 2017 a larger macrocyclic antiparasitic drug, Ivermectin was also shown to activate Kir3.1/3.2 channels with an EC_{50} of 3.5 μM and Kir3.1/3.4 channel with an EC_{50} of 7.5 μM (Chen et al. 2017).

3.4.2 Activators in Disease

Pain: Flupirtine, a non-opioid analgesic that non-selectively activates potassium channels and indirectly antagonizes NMDA receptors, activated Kir3 channels in rat hippocampal neurons and in cultured retinal ganglion cells (Jakob and Krieglstein 1997; Sattler et al. 2008). When given in combination with morphine it potentiated antinociception in two rat models of pain, suggesting a role for Kir3 in flupirtine-induced analgesia (Devulder 2010; Jakob and Krieglstein 1997).

Epilepsy: $G_{i/o}$PCR agonists, like a selective A1 adenosine receptor ligand, could be effective in suppressing seizures in a model of pharmaco-resistant epilepsy in mice (Gouder et al. 2003). *Urea-based activators* (e.g., ML297) of Kir3.1/3.2 heteromers have been shown to exert antiepileptic efficacy both in maximal electric shock-induced and in chemically-induced models of epilepsy in rodents (Huang et al. 2018b; Kaufmann et al. 2013; Zhao et al. 2020).

Alzheimer's disease (AD): Activation of Kir3 channels by ML297 was able to rescue all hippocampal deficits induced by intracerebroventricular injection of $A\beta_{1-42}$, restoring proper excitability in CA3-CA1 synapses (Sanchez-Rodriguez et al. 2017). ML297 prevented increased excitability, restored long-term potentiation (LTP) hindered by Aβ, and recovered hippocampal oscillatory activity (Sanchez-Rodriguez et al. 2017). ML297 was also able to restore long-term potentiation and novel object recognition deficits (Sanchez-Rodriguez et al. 2017). These findings suggest that Kir3 channel activation may represent a novel therapeutic strategy to recover excitation imbalances conferred by Aβ in early onset AD.

3.5 Structural Studies

Numerous MD simulation studies have been performed with Kir3 channels. Pioneering MD simulations were conducted based on homology models of Kir1.1, Kir3.1, and Kir6.2 (Haider et al. 2007; Stansfeld et al. 2009). The results provided insights into the nature of Kir channel–lipid interactions, in particular the importance of the slide helix and linker of the TMD and CTD in forming interactions with the headgroups of lipids. However, the timescale of the simulations at these early studies was limited to 10 ns that is a relatively short time period for an adequate sampling of protein motions (Haider et al. 2007). Using a combination of coarse-grained (1.5 μs) and atomistic MD simulations (20 ns), the interactions of PIP_2 and KirBac1.1, Kir3.1-KirBac1.3 chimera, and Kir 6.2 channels were explored. The PIP_2-binding site was identified at the N-terminal end of the slide helix and interface between adjacent subunits of Kir channels (Stansfeld et al. 2009). Meng et al. (2012) provided the first dynamic molecular view of PIP_2-induced channel gating by conducting MD simulations on the Kir3.1-KirBac1.3 chimera in the presence and absence of PIP_2 (100 ns). Simulations of the closed state with PIP_2 revealed an intermediate state between the closed and open conformations of the channel. A PIP_2-driven movement of the N-terminus and C-linker led to CD-loop stabilization of the G-loop gate in the open state (Meng et al. 2012). Due to timescale limitations of the MD simulations, the opening of the HBC gate of Kir channels had not been observed

in any of these early studies. A highly conserved glycine residue in the middle of TM2 plays an important pivoting role in channel gating. Replacement of the residue immediately following this glycine by a proline in Kir3 channels leads to constitutively active channels (Jin et al. 2002; Sadja et al. 2001). Meng et al. (2016) conducted MD simulations on the Kir3.1 chimera (M170P) mutant (2QKS) in the presence of PIP_2 (100 ns). Interestingly, the HBC gate of the channel mutant was opened within 30 ns of the simulations. The open HBC gate reached 6 Å in diameter, which allowed partial hydrated K^+ ions to pass through. During the gating process, a cooperative rotation of TM1 and TM2 in counterclockwise direction (viewed from the extracellular side) was observed. Three K^+ ions passed through the HBC gate during a 100 ns simulation. Introduction of the proline mutation decreased the free energy barrier for opening the channel by 1.4 kcal/mol (Meng et al. 2016).

Lacin et al. (2017) studied the role of a conserved basic residue, Kir3.2(K200) at the tether helix region, combining functional studies with MD simulations (400 ns). Both experiments and simulations demonstrated that K200 in Kir3.2 supports a dynamic interaction with PIP_2. When K200 was mutated to a Tyr, it activated the channel by enhancing the interaction with PIP_2. The mutant K200Y opened the HBC gate during a 400 ns simulation (Lacin et al. 2017). Bernsteiner et al. (2019) conducted multi-microsecond–timescale MD simulations based on the crystal structures of Kir3.2 bound to PIP_2. The simulations provided detailed insights into the channel's gating dynamics, as well as the movement of K^+ ions through the channel under an electric field across the membrane (Bernsteiner et al. 2019). Li et al. (2019) also conducted microsecond-scale MD simulations based on the Kir3.2 crystal structure (PDB code 4KFM) in complex with PIP_2, Na^+, and $G_{\beta\gamma}$ to understand which gates are controlled by Na^+ and $G_{\beta\gamma}$ and how each regulator uses the channel domain movements to control gate transitions. The simulation results suggested that Na^+ ions control the cytosolic gate of the channel through an anticlockwise rotation, whereas $G_{\beta\gamma}$ stabilizes the transmembrane gate in the open state through a rocking movement of the cytosolic domain. Both effects alter the way in which the channel interacts with PIP_2 and thereby stabilize the open states of the respective gates (Fig. 7) (Li et al. 2019). Li et al. (2016a, b) predicted Tertiapin and Kir3.2 channel interactions using MD simulations and PMF (potentials of mean force) calculations. The residue K17 of TPN was predicted to protrude into the pore of the channel and form a hydrogen bond with carbonyl group of T157, and R7 of TPN to form a salt bridge with the E127 from the channel turret (Li et al. 2016b). As discussed under the "Kir3 Channel Activators" (Sect. 3.4), ML297 and particularly its derivative GAT1508 proved to be potent and specific activators of Kir3.1/3.2 channels. Molecular docking and MD simulations predicted a GAT1508 binding site validated by mutagenesis experiments, providing molecular insights into how GAT1508 interacts with the channel and allosterically modulates channel–PIP_2 interactions (Xu et al. 2020).

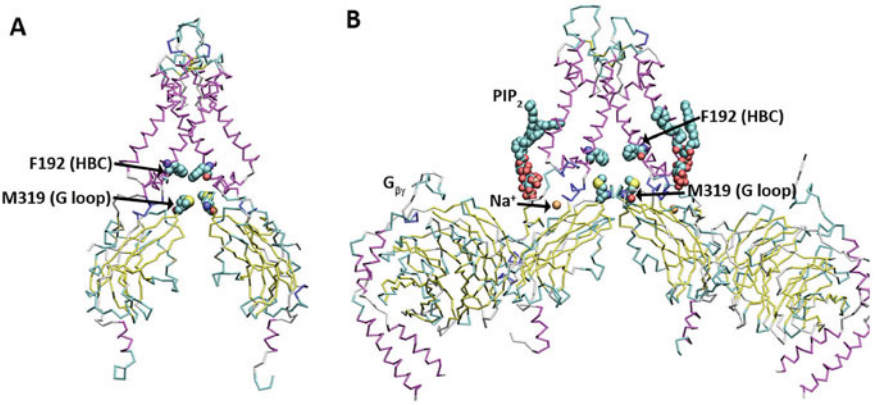

Fig. 7 Modulators, PIP_2, Na^+, and $G_{\beta\gamma}$ stabilize Kir3.2 channel in open state conformation. (**a**) Kir3.2 (Apo) is in a close conformation. (**b**) Kir3.2 (Holo, with PIP_2, Na^+ and $G_{\beta\gamma}$) is in an open conformation. Residues F192 form HBC gate, and M319 form G loop gate. The structural coordinates were obtained from MD simulations (Li et al. 2019)

4 ATP-Sensitive Kir6 Channels

4.1 Introduction

The pharmacology of ATP-sensitive K^+ channels (K_{ATP}) is in part addressed by Li and colleagues (2021, in this issue chapter). K_{ATP} channels are comprised of four pore-forming subunits (Kir6.1 or Kir6.2) and four sulfonylurea receptors subunits (SUR1, SUR2A or SUR2B) creating an octomeric structure (eight total subunits). Here we complement (Li et al. 2021) with emphasis on the Kir6 subunits of the K_{ATP} channels, as members of the Kir channel family at large. K_{ATP} channels (different combinations of SUR/Kir6 subunits) are found in cardiomyocytes, pancreatic β cells, neurons, skeletal muscle, smooth muscle, endothelial cells, and in membranes of mitochondria (Rodrigo and Standen 2005; Li et al. 2021). They couple metabolic changes (ATP to ADP ratio) to cellular excitability. They are regulated by a variety of physiological regulators including H_2S, long chain CoA esters, phosphatidylinositol phosphates (PIPs), carbon monoxide (CO), and changes in intracellular pH. As the intracellular pH acidifies, the channel activates, increasing rectification (Baukrowitz et al. 1999; Li et al. 2016a; Tinker et al. 2018; Rohacs et al. 2003; Shumilina et al. 2006). Like most other ion channels, K_{ATP} is regulated by post-translational modifications, such as S-palmitoylation (Yang et al. 2020) and phosphorylation affecting open probability and functional channel cell surface expression (Beguin et al. 1999). Analogous to all other Kir channels, K_{ATP} channels are ultimately regulated by phosphatidylinositol (4,5) bisphosphate (PIP_2). The sensitivity of the channel to ATP is regulated by PIP_2. PIP_2 decreases the ATP sensitivity resulting in a reversal of channel closure by ATP and stabilizing the open

state (Shyng et al. 2000). However, these channels are the least stereospecific to regulation by $PI(4,5)P_2$ relative to other PIPs. The electrostatic interactions, dictated by the number of negative charges in the head group of PIPs, and the stereoselective nature, length of acyl chain, is less important for K_{ATP} channels, allowing $PI(4,5)P_2$, $PI(3,4,5)P_3$, $PI(3,4)P_2$, and PI(4)P to regulate the channel equally (Rohacs et al. 2003). Furthermore, long chain CoA esters activate and regulate the K_{ATP} channel by binding residues 311–332 on the C-terminus, a site distinct to both PIP_2 and ATP binding sites (Branstrom et al. 2007; Shyng et al. 2000). The metabolic sensing capability and its coupling to the electrical activity of K_{ATP} channels along with its distinct tissue localization has put much of the clinical emphasis on insulin regulation, cardiac rhythm, and neurological function.

4.2 Channel Regulation and Trafficking

ATP binds directly to Kir6 while MgADP binds to SUR. As ATP levels increase, ATP binds to the Kir6 subunits causing closure of the channel gates leading to a depolarization of the membrane and thus an increase in electrical activity. Conversely, as ATP levels fall and the ADP levels increase, MgADP binds to the SUR subunits causing the channels to open leading to hyperpolarization of the membrane and reduction in electrical activity (Ashcroft 2005).

Trafficking: Both SUR and Kir have a putative ER retention signal, RKR, in the distal portion of the C-termini. The SUR and Kir subunits co-assemble and in doing so mask each other's ER retention signals. This allows for the octameric complex to exit the ER and localize to the plasma membrane (Zerangue et al. 1999). The truncated Kir6.2 channel (Kir6.2ΔC36) is a functional channel in the absence of any SUR subunits due to its lack of ER retention motif and is an experimentally useful tool (Reimann et al. 2001; Zerangue et al. 1999). K_{ATP} channels may also be post-translationally trafficked to specific excitable membrane domains by the cytoskeletal adapter ankyrin-B (AnkB) (Kline et al. 2009). SUR and Kir6 are regulated in the ER by proteasome-mediated degradation as well as through protein recognition and translocation by Derlin-1 and folding by the chaperone protein Hsp90 (Wang et al. 2012; Yan et al. 2010).

4.3 Physiology/Pathophysiology and Tissue Distribution

We will briefly summarize major points of channel characteristics, disease implications, and tissue distribution. For a more detailed discussion of channel characteristics and disease implications, refer to (Li et al. 2021) as well as to more in-depth reviews (e.g., Tinker et al. 2018).

Pancreatic: The K_{ATP} channels of pancreatic β cells are comprised of SUR1/Kir6.2. These channels couple the metabolic state of the cell to the release of insulin. In the presence of elevated blood glucose levels, ATP levels rise, bind directly to the Kir6 subunit, and cause channel closure. In β cells this closure depolarizes the cell leading to activation of voltage-gated Ca^{2+} channels, thus increasing levels of

intracellular Ca^{2+}, which results in insulin vesicle fusion to the plasma membrane and secretion (Hibino et al. 2010). There are a range of polymorphisms in the SUR1 and Kir6 subunit that leads to insulin secretory disorders. Hyperinsulinemia, neonatal diabetes, and DEND syndrome have all been linked to loss of function or gain of function mutations in the Kir6.2 subunit (Ashcroft 2005).

Cardiac: Cardiac K_{ATP} channels were initially thought to be an assembly of SUR2A and Kir6.2 due to most studies being conducted with ventricular cells. However, there has been discovery of other subunit assemblies within various tissues in the heart (for a list see Table 3 in Li et al. 2021). Cardiac K_{ATP} channels play an important cardioprotective role. Depending on their location in the heart, conductance and Ki of ATP do vary. K_{ATP} channels appear to be predominantly cardiac adaptive/protective in the presence of stressors (Tinker et al. 2018). Activation of cardiac K_{ATP} channels plays a significant role in ischemic preconditioning, and by shortening the action potential duration during myocardial ischemia increases cell survival (Tamargo et al. 2004). There is also evidence of K_{ATP} channels having altered expression patterns due to exercise leading to physiological adaptation (Tinker et al. 2018; Zingman et al. 2011).

Skeletal muscle: The classical skeletal muscle K_{ATP} channel assembly is SUR2A/Kir6.2, however skeletal muscles have also been shown to contain SUR1/Kir6.2. This has been found in fast twitch muscle, where the current density is larger (Tinker et al. 2018). Like cardiac K_{ATP} channels, in the skeletal muscle these channels contribute little to the membrane potential at rest. Their roles in skeletal muscle are still being investigated but it is evident they prevent muscle fatigue and muscle damage as well as regulate glucose uptake and cellular metabolism (Tinker et al. 2018). These findings suggest potential roles in muscle disorders, weight gain, and glucose regulation.

Vasculature and smooth muscle: Vascular smooth muscle, smooth muscle, and endothelial K_{ATP} channels are all comprised of Kir6.1/SUR2B (Suzuki et al. 2001; Aziz et al. 2017). Regulation of vascular smooth muscle through K_{ATP} channels is due to the ability of channel activity to regulate membrane potential. When hyperpolarization occurs through activation, intracellular Ca^{2+} is reduced causing dilation. This can occur through the G_s/PKA phosphorylation pathway as well as indirectly through hypoxic conditions via the mitochondria. Contraction occurs through the G_i/PLC$\beta_{2/3}$/PKC pathway that inhibits channel activity, causes depolarization and therefore contraction due to increased intracellular Ca^{2+} (Tinker et al. 2018). Smooth muscle K_{ATP}, like cardiac and neuronal K_{ATP}, plays a protective role in hypoxia and ischemic reperfusion (Aziz et al. 2017). However, there may be variation in expression depending on the location of the smooth muscle. K_{ATP} channels have been found in smooth muscle in the GI tract, bladder, uterus, urethra, and respiratory tract.

Neuronal: There is extensive expression of K_{ATP} channels in neuronal populations including glial cells (SUR1/Kir6.1 or Kir6.2) and neurons in the cortex, hippocampus, dorsal root ganglion (Kir6.2/SUR1 or SUR2), hypothalamus (Kir6.2/SUR1), and substantia nigra pars reticulata (Kir6.2/SUR1) (Liss et al. 1999; Sun et al. 2006b). The differential expression of Kir6 and SUR in neuronal populations leads to distinct roles in metabolic and non-metabolic sensing (Sun et al. 2006b;

Tinker et al. 2018). K_{ATP} channels have been implicated in pain, locomotion and behavior, nerve function, immune activity of glial cells, and most notably neuroprotection against ischemia in hypoxic conditions (Sun and Feng 2013; Tinker et al. 2018). In hypoxic conditions, K_{ATP} channels open leading to hyperpolarization which dampens neuronal firing and reduces metabolic demands. Depolarization increases Ca^{2+} entry which can lead to neuronal death. The neuroprotective nature of K_{ATP} appears to be linked directly to the Kir6 subunit (Sun et al. 2006b; Heron-Milhavet et al. 2004; Tinker et al. 2018).

4.4 Pharmacology

Therapeutic compounds that regulate K_{ATP} channels belong to a few main classes, the sulfonylureas and the general class of potassium channel openers (KCOs) or potassium channel blockers (KBOs). These drug classes exert their effects on K_{ATP} channels by binding to the sulfonylurea receptors (SURs) and allosterically inhibiting or activating the channel or by directly engaging with the channel pore.

Small molecule drugs may exert their regulatory properties affecting gating by allosteric modulation or direct interaction with the binding pockets of PIP_2, ATP, or the channel pore. Experimental and MD studies have uncovered other specific chemical scaffolds that bind directly to the Kir6 channels to exert regulatory effects (see Fig. 11). The exact mechanism of action by these chemicals varies and, in some cases, remains elusive. Therapeutic compounds that bind directly to the Kir6 channels, which have been experimentally confirmed, include the imidazoline compounds, thiazolidinedione ("glitazone") compounds, morpholinoguanidines, quinine, the antiarrhythmic, cibenzoline, biguanides and various anesthetics such as propofol and bupivacaine (Cui et al. 2003; Grosse-Lackmann et al. 2003; Kawahito et al. 2011; Kawano et al. 2004a; Kawano et al. 2004b; Kovalev et al. 2004; Mukai et al. 1998; Proks and Ashcroft 1997; Rui Zhang et al. 2010; Yu et al. 2012).

Computational simulations, including MD, have suggested travoprost, betaxolol, and ritodrine as other drugs that bind directly to the Kir6 subunit to exert their effects (Chen et al. 2019). These latter drugs, along with others uncovered through in silico screens and dynamic studies, will need to be experimentally validated for their binding affinity for the Kir6 subunit and the mechanistic impact on channel gating. However, they provide important insights into new therapeutic chemical scaffolds for Kir6 subunits. It remains an open question whether Kir6 targeting drugs control activity either directly by altering channel-PIP_2 interactions or indirectly by altering channel-ATP interactions. With the differential expression of both SUR and Kir6 subunits, drugs that bind each with varying affinities offer substantial advantage to subtype specific targeting and reduced off target effects.

Another mechanism of K_{ATP} channel regulation is the rescue of surface expression impairments causing biogenesis or trafficking defects, through pharmacological correctors (Shyng et al. 2000). There are well-established mutations in SUR domains that cause trafficking defects of the K_{ATP} complex to the cell surface (Zhou et al.

2014). Carbamazepine is a clinically approved anticonvulsant drug, approved for the treatment of epilepsy and bipolar disorder, known to bind to Na_V channels, Ca_V channels, and $GABA_A$ receptors (Chen et al. 2013). Carbamazepine rescued trafficking of K_{ATP} with trafficking mutations occurring in the TMD0 of SUR1 (Zhou et al. 2014). The TMD0 is part of the N-terminal region of the SUR which binds and couples to the Kir6 channel to form the K_{ATP} complex (Chan et al. 2003; Chen et al. 2013). Interestingly, carbamazepine also appears to affect MgADP binding to the NBD domains of SUR, so while it rescues trafficking and expression can inhibit the channel by abolishing the MgADP activation effect (Zhou et al. 2014). It has also been established that sulfonylurea drugs may also be able to rescue trafficking and folding in specific SUR mutants (Yan et al. 2004). Again, they also have an impact on K_{ATP} activity and conductance once folding and cell surface expression is rescued, increasing the complexity of the pharmacology of such drugs.

The following listed drugs (Table 5 and Fig. 11) were validated with the functional channels Kir6.2ΔC26 or Kir6.2ΔC36 that do not require the presence of SUR subunits, therefore suggesting they act directly on the Kir6 subunit. While they all provide valuable insights into structure-based drug discovery, they all require further computational and experimental investigation to better understand the binding sites and mechanisms of gating.

4.4.1 Drugs Acting Through the Kir6 Subunits

Imidazolines: Imidazolines, including clinically relevant clonidine and phentolamine, and others not on the clinical market, such as idazoxan, efaroxan, and RX871024, bind directly to Kir6 channels (Kawahito et al. 2011; Proks and Ashcroft 1997; Rui Zhang et al. 2010). Clonidine and phentolamine made it to the market based on their ability to bind α-adrenergic receptors but later discovered to bind endogenous imidazoline receptors (Bousquet et al. 2020). Computational studies have elucidated various putative binding sites on the Kir6 channels including the PIP_2 binding site (Idazoxan) and near the slide helix (clonidine and efaroxan).

Despite such differences, they all bind close to the interface between the transmembrane domain and cytosolic domain of the Kir6 subunit (Rui Zhang et al. 2010). Functional studies of clonidine and phentolamine (Kir6.2ΔC36 channel in HEK293 cells and *Xenopus* oocyte macropatches, respectively) suggest they do not act through SUR (Grosse-Lackmann et al. 2003; Kawahito et al. 2011; Proks and Ashcroft 1997). This finding has been further validated (Kawahito et al. 2011). RX871024 binds in the channel pore forming two hydrogen bonds with interior pore-lining residues. This causes direct block of the channel (Rui Zhang et al. 2010). Aside from RX871024, imidazoline drugs are likely candidates to be working through direct or allosteric alteration of channel–PIP_2 interactions. The ATP-insensitive mutant (Kir6.2ΔC36-K185Q) showed no reduction in current in the presence of ATP but both clonidine (1 mM) and phentolamine (1 μM) were still able to block the current as effectively as in the Kir6.2ΔC36 channel, suggesting the binding site of these two imidazolines is not directly at the ATP binding site or involving key ATP binding residues (Kawahito et al. 2011; Proks and Ashcroft 1997).

Table 5 Small molecule inhibitors directly binding Kir6

Phentolamine (imidazoline)	Xenopus oocytes	KirΔC36: 0.77 μM	Kir6.2/SUR1: 1.22 μM	Proks and Ashcroft (1997)
Clonidine (imidazoline)	HEK293 cells Native HIT-T15 cells	KirΔC26: 40.1 μM	Kir6.1/SUR2B: 1.21 μM Native: 44.2 μM	Kawahito et al. (2011), Grosse-Lackmann et al. (2003)
PNU-37883A/ (morpholinoguanidine)	HEK293 cells Xenopus oocytes	KirΔC26: 4.6 μM	Kir6.1/SUR2B: 4.88 μM	Kovalev et al. (2004), Cui et al. (2003)
Rosiglitazone/ (thiazolidinedione)	HEK293 cells	KIR6.2ΔC36: 45 μM	KIR6.1/SUR2B: 10 μM KIR6.2/SUR1: 45 μM KIR6.2/SUR2A: 37 μM KIR6.2/SUR2B: 50 μM	Yu et al. (2012), Chen et al. (2019)
Cibenzoline/ (diphenylmethane)	COS-7 cells	Kir6.2ΔC36: 22.2 μM	Kir6.2/SUR1: 30.9 μM	Mukai et al. (1998)
Propofol (cumene)	COS-7 cells	Kir6.2ΔC36: 78 μM	Kir6.2/SUR1: 77 μM Kir6.2/SUR2A: 72 μM Kir6.2SUR2B: 71 μM	Kawano et al. (2004b)
Quinine	HEK293 cells	Kir6.2ΔC26: 14.6 μM	Kir6.2Δ26C/SUR1:29.8 μM	Grosse-Lackmann et al. (2003)
Bupivacaine/ (piperidinecarboxamides)	COS-7 cells	Kir6.2ΔC36: 366 μM	Kir6.2/SUR2A: 52 μM Kir6.2/SUR2B: 396 μM Kir6.2/SUR2B: 379 μM	Kawano et al. (2004a)
Phenformin (biguanide)	HEK293 cells	Kir6.2ΔC26: 1,780 μM	Kir6.1/SUR2B: 550 μM Kir6.2/SUR2B: 1,096 μM Kir6.2/SUR1: 9,330 μM	Aziz et al. (2010)
SpTx-1	Xenopus oocytes	Kir6.2ΔC26: 8.5 nM (Kd)	Kir6.2/SUR1: 8.42 nM (Kd)	Ramu et al. (2018)
Mitiglinide (benzylsuccinic acid derivative)	HEK293	KIR6.2ΔC36 > 1 mM	Kir6.2/SJUR1: (1) 3.8 nM, (2) 4.1 mM Kir6.2/SUR2A: (1) 3.2 nM, (2) 2.5 mM, Kir6.2/SUR2B: (1) 5 μM, (2) 2.9 mM	Reimann et al. (2001)

Thiazolidinediones: The thiazolidinediones (TZDs), also known as the glitazones, are a drug class whose members exert their clinical anti-diabetic effect by binding to the nuclear transcription factor, PPAR and as such they do have increased cardiovascular risks (Chen et al. 2020; Nanjan et al. 2018). Rosiglitazone (RSG) and pioglitazone are two important clinically approved anti-diabetic drugs of this class. TZDs are reported to have a direct and indirect mechanism of action in relation to diabetes. It is also well characterized that TZDs can directly bind to the Kir6 subunits of the K_{ATP} channel through functional studies (Yu et al. 2012). More interestingly, it has been shown computationally that RSG binds in close proximity to the helix bundle crossing (HBC) gate and PIP_2, without complete PIP_2 binding site occupation (Chen et al. 2020). The main impact of RSG on Kir6 channel current is increased mean closed times (Yu et al. 2012). Based on the putative binding site proposed by Stary-Weinzinger, RSG binds residues in or near the slide helix separating the putative binding site to be distinct from that of the ATP binding site (Chen et al. 2019; Martin et al. 2017).

Morpholinoguanidines: The most historically studied morpholinoguanidine in relation to K_{ATP} channels is PNU-37883A. It acts directly on the Kir6 subunit and has selectivity for Kir6.1 over Kir6.2 making it more selective for smooth muscle with some selectivity for the vascular K_{ATP} channels (Cui et al. 2003). The selectivity of PNU-37883A for K_{ATP} channels is largely impacted by the SUR that complexes with the Kir6.1 (Cui et al. 2003). This likely has to do with either obstruction of the drug binding pocket or an allosteric conformational change of Kir6 upon SUR binding (Humphrey 1999; Kovalev et al. 2004). The binding region for the PNU-38773A has been mapped to amino acids 200–280 on the C-terminus of Kir6 (Kovalev et al. 2004). It is still unclear if binding to the C-terminus allows the molecule to position into the channel pore or it causes a conformational rearrangement of the gating machinery to close the channel. The effect of PNU-37883A on the binding of ATP or PIP_2 to the Kir6 channel has not been adequately explored. Neither the precise binding site of PNU-37883A is determined nor how its binding results in current inhibition. Could it promote ATP binding? Could it disrupt MgADP binding to the SUR, since the SUR NBD interfaces with the Kir6.2 CTD? Could it work by disrupting channel–PIP_2 interactions directly or allosterically? Such questions would need to be further addressed in future studies (Lee et al. 2017). The non-K_{ATP} channel-specific nature of the drug leading to a variety of pharmacological actions does not make it a good drug candidate but its further study will provide a chemical and structural framework for developing more specific K_{ATP} channel inhibitors (Teramoto 2006).

Anesthetics (propofol, bupivacaine): Propofol is a general anesthetic that is used widely throughout the world. Its pharmacological action has been largely attributed to its action on the $GABA_AR$ (Tang and Eckenhoff 2018). Growing functional evidence has uncovered its action at K_{ATP} channels. Propofol is selective for K_{ATP} channels containing Kir6.2 over Kir6.1 and directly binds the Kir6 subunit of the K_{ATP} channel (Kawano et al. 2004b). Utilizing the ATP-insensitive mutant and the R31E mutation, inhibition was abolished indicating propofol has at least some partial overlap with the ATP binding site and with an N-terminal binding site

(Kawano et al. 2004b). There is also evidence to suggest that propofol affects the expression level of Kir6.1 (Zhang et al. 2016).

Bupivacaine is a local anesthetic with a chemical structure different and more complex than that of propofol (see Fig. 11). Bupivacaine was experimentally proven to have a potency on Kir6.2ΔC36 equal to that of Kir6.1/SUR2B. It has a greater affinity for the cardiac channel (Kir6.2/SUR2A). It had higher affinity over levobupivacaine and ropivacaine due to the stereoselective nature of the Kir6 binding pocket (Kawano et al. 2004a). It likely exerts its effects by binding residues at the cytosolic end of TM2 which are important in the channel gating properties likely relinquishing any interference with ATP binding to the channel (Kawano et al. 2004a). Bupivacaine and other similar local anesthetics bind to Na_V channels as well as Ca_V and K_V channels leading to their major cardiotoxic effects. It has been proposed that local anesthetics inhibit Kir3 channels through antagonism of channel–PIP_2 interactions (Zhou et al. 2001). If local anesthetics work at the same conserved site on K_{ATP} as they do on Kir3 channels, PIP_2 binding residues may be important candidates to explore.

Biguanides: Metformin and phenformin are the most well-known, clinically relevant biguanides. They are used to increase insulin sensitivity in type 2 DM (Hansen 2006). Phenformin was withdrawn from the market in the 1970s. Biguanides activate AMPK with several downstream implications of the metabolic state of the cell as well as potentially increasing K_{ATP} cell surface expression with evidence that they directly affect K_{ATP} channels independently of AMPK signaling (Aziz et al. 2010). Aziz et al. (2010) were able to show that there is differential activity of phenformin based on SUR subunit composition but equivalent activity of Kir6 in the absence of SUR subunits. While they appear to bind Kir6 directly, SUR does play a role in modulating the affinity of biguanides to the channel (Aziz et al. 2010).

Drugs acting through the SURs: K_{ATP} channel openers and inhibitors acting through the SURs of the K_{ATP} channel complexes have been discussed in chapter "The pharmacology of ATP-sensitive K^+ channels (K_{ATP})" (Li et al. 2021).

Miscellaneous drugs: Quinine, chloroquine, and quinacrine are all structurally related drugs containing the quinolone bicyclic ring. These drugs have been shown to bind and alter the conductance of Kirs (Lopez-Izquierdo et al. 2011; Grosse-Lackmann et al. 2003). Quinolones are used as one of the most common antibacterial in the world and their mechanism may limit their potential use on K_{ATP} regulation for the current clinically used quinolones (Aldred et al. 2014).

The well-known antiarrhythmic agent, cibenzoline, is a diphenylmethane with inhibitory properties on the Kir6 subunit shown by functional studies. There is evidence that cibenzoline binds the K^+-recognition site on K_{ATP} channels similar to how it binds the H^+, K^+ -ATPase (Tabuchi et al. 2001). This may prove to be a novel mechanism of binding but would decrease its selectivity for Kir6 over other Kirs given the conservation of the putative binding site. In 2018 Ramu and colleagues uncovered a 54-residue protein toxin recovered from the venom of *S. polymorpha* (SpTx) that inhibited Kir6 from the extracellular side (Ramu et al. 2018). SpTx binds the Kir subunit and not the SUR subunit. Further investigation is

needed to determine the mechanism of channel closure but nonetheless this discovery opens a new possibility in the pharmacology of Kir6 channel gating. There is evidence to suggest that glinides or the benzylsuccinic acid derivative, mitiglinide, bind both to the SUR at a high affinity site and directly to the Kir6 subunit at a lower affinity site (Aziz et al. 2010; Reimann et al. 2001). Similarly, the repaglinide binding site is on the SUR, as resolved by cryo-EM, but it also has a partial contribution by the N-terminus of the Kir6.2 subunit. This was confirmed through binding studies in which co-expression of Kir6.2 enhanced binding affinity and deletion of the N-terminal peptide of Kir6.2 abolished the binding enhancement (Ding et al. 2019).

4.5 Structural Studies

Before atomic resolution structures of the K_{ATP} were available, in order to identify the PIP_2 binding site, Haider and colleagues conducted molecular docking and MD simulations (10 ns) based on a Kir6.2 homology model. The PIP_2 head group was predicted to interact with K39, N41, and R54 in the N-terminus, K67 in the transmembrane domain and R176, R177, E179, and R301 in the C-terminus. The model predictions are consistent with a large body of functional data, suggesting how PIP_2 binding may lead to an increase in Kir6.2 open probability and a reduction in ATP sensitivity (Haider et al. 2007). In order to understand the mechanism by which disease mutations exert their deleterious effects, in 2017, Cooper and colleagues worked on a Cantu syndrome (CS) mutant, where the Kir6.1(V65M)/Kir6.2(V64M) GoF mutations in the slide helix enhance the channel activity when co-expressed with the SUR1 or SUR2 regulatory subunits (Cooper et al. 2017). The Val to Leu mutation that does not cause the disease was used as a negative control. The disease mutations abolished the sensitivity of K_{ATP} to ATP and to the blocker glibenclamide, which acts on the SUR subunit. This finding suggested that sulfonylurea therapy may not be successful, at least for some CS mutations. Homology modeling and MD simulations (100 ns) were conducted on the Kir6.1(WT), Kir6.1(V65L), and Kir6.1(V65M) systems. The stabilization of the open state of the Val to Met mutations (but not to Leu) that was obtained experimentally could not be shown computationally in the 100 ns simulation time frame. The same year in 2017, near-atomic resolution structures were obtained from three independent labs (Chen in Beijing, Shyng in Oregon and MacKinnon in New York). These structures were solved for the Kir6.2/SUR1 in complex with ADP, ATP, and glibenclamide (Lee et al. 2017; Martin et al. 2017; Wu et al. 2018). In 2019, a higher resolution (3.3 Å) cryo-EM structure of Kir6.2/SUR1 in complex with ATPγS and repaglinide was solved by the Chen lab. Figure 8 shows the Kir6.2 channel surrounded by four SUR1 subunits, the two distinct binding sites for ATPγS (one in Kir6.2 and the other in SUR1), and the binding site for repaglinide in the transmembrane domain of SUR1

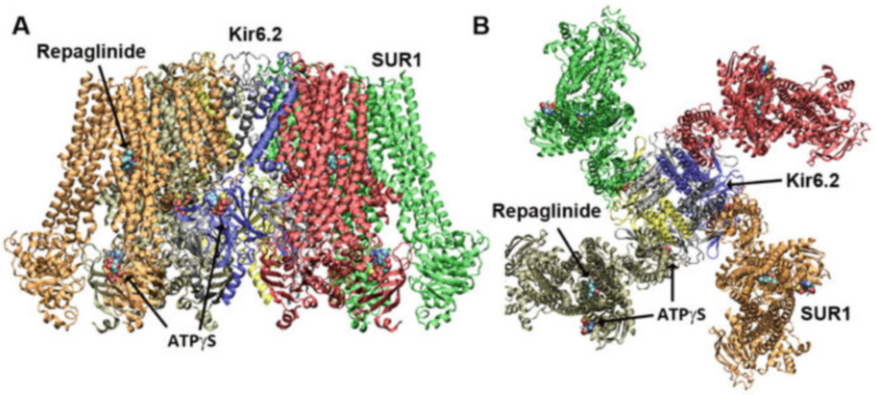

Fig. 8 Cryo-EM structure of K_{ATP} channel in complex with ATPγS and Repaglinide (PDBID:6JB1), (**a**) side view. (**b**) top view. The K_{ATP} channel and SUR1 are drawn in Newcartoon, the ATPγS and Repaglinide are drawn in VDW

(Ding et al. 2019). This study also suggested through cryo-EM and binding studies that the N-terminal portion of Kir6.2 adds to the binding pocket for repaglinide. This is further supported by earlier functional studies done by Reimann et al. (2001) in which a structurally similar drug, mitiglinide, had a biphasic dose-response curve, suggesting a high affinity binding site at SUR and a low affinity binding site at Kir6.2. Chen and colleagues conducted MD simulations (at a microsecond time scale) on Kir6.1 channel to investigate the blocking mechanism by rosiglitazone (RSG). The putative RSG binding site was identified through unbiased MD simulations of Kir6.1 channel with 20 RSG molecules randomly placed in the solvent, and followed by free energy calculations. Based on the predicted RSG binding site, dynamic pharmacophore models were constructed and used for screening of hits in the DrugBank database. Three new high affinity blockers, betaxolol, ritodrine, and travoprost, were identified and subsequently tested functionally. Using the inside-out patch-clamp mode in HEK293T cells expressing Kir6.2/SUR2A, travoprost, betaxolol, and ritodrine had IC_{50}s of 2.46 μM, 22.06 μM, and 7.09 μM, respectively (Chen et al. 2019). Given the computational identification of direct binding to Kir6, the expectation would be that these three drugs should have similar potency when tested on Kir6.2ΔC channels. Computational simulations to understand the dynamics of K_{ATP} channel structure and function are still lacking. The recent cryo-EM structures have provided us with the opportunity to characterize the channel dynamic function using long timescale MD simulations, and a molecular basis for structure-based drug discovery for these important drug targets.

5 K^+ Transport Kir (1, 4, 4/5, 7) Channels

5.1 Kir1.1: Historical Perspective

The first member of the Kir family to be cloned (see below) was initially referred to as the "rat outer medullary K^+" (ROMK1) channel for its prominent role in the kidney.

The task of renal epithelial cells is to maintain ionic homeostatic control of K^+, Na^+, and Cl^- in the urine and blood and Kir1 channels play a key role in achieving this task. The channels are specifically localized in the apical rather than the basolateral membrane of these cells. To appreciate the strategic importance of Kir1 channels let us consider their role in the thick ascending loop of Henle (TAL), the segment of the nephron responsible for 25–30% of the total Na^+ reabsorbed by the kidney and the site of action of loop diuretics. Here, K^+ efflux through Kir1 channels also maintains the Na^+-K^+-$2Cl^-$ symporter active by supplying K^+ to the extracellular site of the transporter and enabling the uptake of NaCl along with K^+ into the TAL cells. The Na^+ entering the cytoplasm from the apical (lumen or urine side) through the symporter fuels the basolateral Na^+-K^+ ATPase to continue transporting Na^+ to the blood side. K^+ channels (e.g., Kir1, Kir4, Kir7) co-expressed with the Na^+-K^+-ATPase in basolateral membranes supply K^+ to the extracellular side of the pump to maintain its activity. This is referred to as "K^+ recycling" (Hibino et al. 2010). Furthermore, the hyperpolarization caused by Kir1 channels accelerates Cl^- exit from basolateral Cl^- channels, establishing "the lumen positive transepithelial potential," which serves as the main driving force for paracellular Na^+, Ca^{2+}, and Mg^{2+} transport from the lumen to blood side. Deletion of Kir1 channels impairs renal NaCl absorption yielding "Barter's syndrome"-like phenotypes, as we will discuss below (Bleich et al. 1990; Greger et al. 1990). Moreover, Kir1 channels on the apical membrane of TAL epithelial cells are functionally coupled to Cl^- channels (the cystic fibrosis transmembrane regulator – CFTR) in that CFTR decreases Kir1 activity. This effect can be reversed by PKA phosphorylation and may underlie the actions of the antidiuretic hormone arginine vasopressin that increases Kir1 activity, limiting K^+ secretion and urinary K^+ loss (Field et al. 1984).

5.1.1 Subfamily Members and Tissue Distribution

In early 1993 the first two Kir channel cDNAs were reported, Kir1.1 and Kir2.1, followed by Kir3.1 later the same year (Dascal et al. 1993; Ho et al. 1993; Kubo et al. 1993a; Kubo et al. 1993b). Six alternatively spliced isoforms of Kir1.1(a-f) have been identified but only two of these (a, c) give rise to distinct proteins from the other four isoforms (b, d-f) (Boim et al. 1995; Ho et al. 1993; Kondo et al. 1996; Shuck et al. 1994; Zhou et al. 1994). The splice isoforms differ in their N-termini with Kir1.1b being the shortest, Kir1.1a 19 aa and Kir1.1c 26 aa longer than Kir1.1b (the human isoform shown in Fig. 9 is the Kir1.1a isoform with 391 aa). No Kir1.1 heteromers with members of other Kir subfamilies have been reported. As mentioned above, Kir1.1 is expressed in the kidney (TAL, distal convoluted tubule, and

cortical collecting duct) and, as shown by in situ hybridization, in cortical and hippocampal neurons (Kenna et al. 1994).

5.1.2 Physiology/Pathophysiology

Kir1.1 function is regulated by internal pH, phosphorylation by a number of different kinases, miRNAs, and ubiquitination. Acidification closes Kir1.1 channels with a pK_a of ~6.5 (Choe et al. 1997; Doi et al. 1996; Fakler et al. 1996; McNicholas et al. 1998; Tsai et al. 1995). Multiple studies have implicated the involvement of several residues in pH sensitivity but structural insights are still lacking (Hibino et al. 2010). Kir1.1 has ER retention signals (368R-X/A-R370 and K370-R371) (Ma et al. 2001; Yoo et al. 2005). It is regulated by phosphorylation of S44 (by both PKA, SGK – Ser/Thr protein kinases), increasing trafficking to the plasma membrane (McNicholas et al. 1994; Xu et al. 1996; Yoo et al. 2005). Additionally, PKA-mediated phosphorylation of S219 and S313 increases the channel open probability, likely by enhancing channel–PIP_2 interactions (Liou et al. 1999; MacGregor et al. 1998). Another class of Ser/Thr kinases, WNKs (With-no-K – K = Lys) decreases Kir1.1 cell surface expression by unique mechanisms (Hibino et al. 2010). A number of microRNAs have been shown to be upregulated in high-K^+ diet and, in turn, they regulate Kir1.1: miR-802 increased Kir1.1 surface expression and channel activity by reducing caveolin-1 that limits Kir1.1 expression at the plasma membrane (Lin et al. 2014); miR-194 also enhanced Kir1.1 surface expression by counteracting the opposite regulation by WNK and intersectin 1 (He et al. 2007). Finally, monoubiquitination of K22 decreased cell surface Kir1.1 serving as a signal to process the channel for lysosomal degradation (Lin et al. 2005). The Kir1.1 knockout mouse resulted in mice afflicted with Barter's syndrome (BS) (Lorenz et al. 2002; Lu et al. 2002). BS is an autosomal recessive renal tubulopathy characterized by hypokalemic metabolic alkalosis, renal salt wasting, hyperreninemia, and hyperaldosteronism (Asteria 1997; Guay-Woodford 1995; Hebert 2003; Karolyi et al. 1998; Peters et al. 2002; Rodriguez-Soriano 1998). Loss-of-function mutations of Kir1.1 (type II BS) affect many aspects of the functional integrity of the channel and its regulation, including interactions with PIP_2 (Hibino et al. 2010; Lopes et al. 2002). BS patients and Kir1.1 mice are characterized by hypokalemia and excess K^+ in the urine, which is contrary to the role of Kir1.1 as providing the main secretory pathway of K^+ in renal tubules. Bailey and colleagues addressed the conundrum showing that in the absence of Kir1.1 in the TAL cells K^+ secretion is sustained in the late distal tubule by maxi-K^+ channels (Bailey et al. 2006).

5.2 Kir4/Kir5: Historical Perspective and Tissue Distribution

Kir4 are expressed as homotetramers as well as heterotetramers with Kir5.1, while when Kir5.1 is expressed alone in a heterologous expression system it shows no function.

These channels are the molecular identities of neuro- and retinal glial cell K^+ channels. Their cloning provided a boost to their long-recognized importance (Kuffler and Nicholls 1966; Newman 1984). Kir4channel cDNAs were first identified in the mid-90s and Kir4.1 has been referred to under a variety of names: BIR10 (Bond et al. 1994), K_{AB}-2 (Takumi et al. 1995), BIRK-1 (Bredt et al. 1995), and Kir1.2 (Shuck et al. 1997). In situ hybridization showed Kir4.1 predominantly expressed in glial cells in the CNS (brain and spinal cord) and retina (Müller cells) (Hibino et al. 2010; Takumi et al. 1995). Kir4.1 has also been reported to be expressed in the kidney (reviewed in Manis et al. 2020), stomach (Fujita et al. 2002), and the cochlea of the inner ear (Hibino and Kurachi 2006). Kir4.2 was first isolated from a kidney library, where it is highly expressed, along with liver, embryonic fibrocytes, and microvascular endothelial cells (Lachheb et al. 2008; Pearson et al. 1999; Shuck et al. 1997). A single member of the Kir5 subfamily, Kir5.1, was identified in 1994 (Bond et al. 1994). It is highly expressed in kidney, spleen, adrenal glands, liver, in several brain regions and in spermatozoa and spermatogenic tissue (Bond et al. 1994; Salvatore et al. 1999). Kir5.1 is only functional as a heteromer with Kir4.1 and Kir4.2. The nephron serves as a great example of where expression and heteromerization with one, the other, or both of the Kir4 isoforms best serves overall renal salt handling (see below in Sect. 5.2.1). Its distribution and co-expression with the Kir4 isoforms vary along the nephron to fulfill specific functional characteristics of each tubule segment (full validation of the expression and activity of Kir4.2 is still pending). Thus, in the proximal convoluted tubule Kir5.1 is co-expressed with Kir4.2, in the cortical collecting duct and also in the connecting tubule (connecting the distal convoluted tubule to the cortical collecting duct) it is co-expressed with Kir4.1, while in the thick ascending limb of Henle it is not expressed at all (only Kir4.1 is expressed), and in the distal convoluted tubule it is co-expressed with both Kir4 isoforms (Manis et al. 2020).

5.2.1 Physiology/Pathophysiology

Kir5.1 confers several unique functional characteristics to Kir4 isoforms: (1) greater pH sensitivity (Tucker et al. 2000) (2) maintenance of pH homeostasis (Puissant et al. 2019), (3) inhibition of the dependence of channel conductance on $[K^+]_o$ (Edvinsson et al. 2011a; Edvinsson et al. 2011b) (shown only for Kir4.2 thus far), and (4) activation by $[Na^+]_i$ (Rosenhouse-Dantsker et al. 2008). Kir4.1/Kir5.1 channels have been shown to act as K^+ sensors in the distal nephron (Cuevas et al. 2017) regulating expression of apical Na^+ transporting proteins and renal salt handling in response to dietary salt intake (Manis et al. 2020). Dysfunction of these channels has been proposed to contribute to the pathogenesis of salt-sensitive hypertension (Palygin et al. 2017; Palygin et al. 2018). Knockouts of either Kir4.1 (Cuevas et al. 2017) or Kir5.1 (Wu et al. 2019) compromise $[K^+]_o$ sensing and K^+ excretion. A unifying link for action of various Kir4/5 modulators could be the allosteric changes in channel–PIP_2 interactions. The modulators proposed to work in this way include $[Na^+]_i$ (entering through the apical side; Rosenhouse-Dantsker et al. 2008)), $[K^+]_o$ at the basolateral side (Harraz et al. 2018), changes in intracellular pH or phosphorylation by kinases, such as WNK-4, (Du et al. 2004). Differences in

Kir4.1 versus Kir4.2 and their relative heteromers with Kir5.1, along with their characteristic expression may underlie their distinct physiological roles. Thus, Kir4.2 is more pH sensitive than Kir4.1. Heteromerization of Kir4.2 with Kir5.1, unlike Kir4.1, converts Kir4.2 from a strong to a weak rectifier, rendering it more sensitive to intracellular pH. Knockout mice have shown that Kir4.2 plays a distinct role in acid-base homeostasis compared to Kir4.1, resulting in metabolic acidosis with intracellular alkanization and membrane depolarization in the proximal convoluted tubules of the kidney (Manis et al. 2020). In the CNS, the Kir4 isoforms and their regulatory Kir5.1 subunits exhibit very interesting physiology by allowing for control of $[K^+]_o$ in nearby neurons to regulate neuronal excitability (Hibino et al. 2010).

5.3 Kir7.1: Historical Perspective

Kir7.1 is the only member identified in this subfamily and the most distant relative of all other Kir channels, showing the highest homology/identity to the Kir4 isoforms (~50% similarity and 40% identity; Fig. 10). Three groups reported cloning of Kir7.1 from CNS neurons in 1998 (Doring et al. 1998; Krapivinsky et al. 1998; Partiseti et al. 1998). A splice variant in native human retinal pigment epithelium has been described to be missing a region from exon 2 (amino acids 244–479), yielding a non-functional truncated Kir7.1 that is expressed at fourfold lower levels than the wild-type protein and does not interfere with the activity of the full-length protein (Yang et al. 2007). Another two splice variants of Kir7.1 were described recently in several mouse tissues. These lacked most of the C-terminal domain, Kir7.1-R166X (in the B-loop, Fig. 9) and Kir7.1-Q219X (in βE, Fig. 9). These truncation variants would be comparable to mutations associated with Leber congenital amaurosis, a rare recessive hereditary retinal disease that results in vision loss at early age. Simultaneous expression with the full-length Kir7.1, however, led to a reduction in activity of the wild-type channel (possibly due to partial proteasome degradation of WT-mutant channel heteromers) (Vera et al. 2019).

5.3.1 Tissue Distribution

The Kir7.1 protein has been reported to be expressed along with the Na^+/K^+-ATPase for "K^+ recycling" either in the apical membranes of epithelial cells of the choroid plexus (Doring et al. 1998; Hasselblatt et al. 2006; Nakamura et al. 1999) and retinal pigment epithelium (Kusaka et al. 2001; Shimura et al. 2001) or in basolateral membranes of thyroid follicular cells (Nakamura et al. 1999), in the distal convoluted tubule, proximal tubule, and collecting duct of renal epithelia (Derst et al. 2001; Ookata et al. 2000) as well as in the small intestine and stomach (Partiseti et al. 1998). Kir7.1 has also been reported to be expressed in hypothalamic arcuate neurons involved in the appetite control circuit, where they are coupled to the MC4R G Protein Coupled Receptor in a G protein-independent manner (Ghamari-Langroudi et al. 2015) as well as in the myometrial smooth muscle, where it controls uterine excitability throughout pregnancy (McCloskey et al. 2014).

5.3.2 Physiology/Pathophysiology

A hallmark of Kir channels is a dependence of their conductance on $[K^+]_o$ (Fig. 1a). Kir7.1 is also characterized by very weak rectification and a very low unitary conductance (Krapivinsky et al. 1998). The crucial difference appears to lie in the structure of the outer mouth of the selectivity filter, where Kir7.1 has a Met residue (M125), instead of Arg that is found in every other Kir channel (Fig. 9). M125R point mutation in Kir7.1 not only increased its unitary conductance by nearly 20-fold, strengthened its inward rectification and sensitivity to Ba^{2+} but also reinstated strong dependence on $[K^+]_o$ (Doring et al. 1998; Krapivinsky et al. 1998). The corresponding residue R148 in Kir2.1 had been shown to have the opposite effect, namely the Kir2.1(R148Y) mutant showed a much reduced $[K^+]_o$ dependence (Kubo 1996) while the R148H mutation increased sensitivity to pH_o (but not pH_i) (Shieh et al. 1999). Taken together, this evidence suggests that a positively charged residue is needed at the outer mouth of the selectivity filter to confer the dependence on $(V\text{-}E_K)$ shown by Kir channels. Several questions remain for a better understanding of this property. How do extracellular Na^+ ions serve as blocking particles to regulate this dependence (Ishihara 2018; Soe et al. 2009) and how such interactions couple allosterically to control channel–PIP_2 interactions (Harraz et al. 2018)? Dynamic computational studies will be instrumental in answering these questions. We will next focus discussion of the role of Kir7.1 channels in health and disease in the best studied system, the retinal pigment epithelium (RPE).

Retinal pigment epithelium: The RPE is a hexagonally packed, tight-junction connected monolayer of post-mitotic, pigmented cells between the neuroretina and the choroids. The apical membrane of the polarized RPE cells faces the photoreceptor outer segment (POS), while the basolateral membrane faces the fenestrated endothelium of the choriocapillaris. Interactions between the RPE and the POS are essential for visual function. The RPE's primary functions are: a) to transport nutrients, ions, and water between the blood supply and the POS, b) to absorb light and protect against photo-oxidation of proteins and phospholipids of the POS, c) the isomerization of retinal which serves as the agonist of the rhodopsin GPCR to signal to the phosphodiesterase to cleave cGMP to 5'-GMP, d) phagocytosis of shed photoreceptor membranes, and e) secretion of essential elements for the morphological integrity of the retina (Kumar and Pattnaik 2014). In the dark, the cGMP levels are maintained to signal to the cGMP-gated (CNG) cation channels in the POS and allow the influx of Na^+ and Ca^{2+} to the photoreceptor cell (rod or cone). This is countered by a net K^+ efflux in the photoreceptor inner segment (PIS) mediated by various K^+ channels (e.g., KCNQ, and 8 Kir currents, with Kir7.1 being the predominant one) giving rise to the so-called dark current. The efflux at the PIS pulls the resting V_m toward E_K but the cationic influx at the POS counters the K^+ efflux, maintaining a rather depolarized level in the dark. Upon light illumination, the resulting 5'-GMP no longer keeps the CNG channels open, causing the K^+ efflux to dominate and an ensuing hyperpolarization of the photoreceptor cell. This signal will decrease glutamate release that once integrated to the retinal ganglion cells, it will be communicated to the brain through the optic nerve and be interpreted as "light." The new balance of Na^+ and K^+ ions decreases the PIS Na^+/K^+ ATPase

activity and the $[K^+]_o$ falls from 5 mM in the dark to 2 mM in the light, making E_K more negative increasing Kir currents toward re-establishing $[K^+]_o$ to the 5 mM levels. The predominant Kir7.1 resting conductance, unlike other Kir currents, does not decrease its conductance with the drop of $[K^+]_o$ as we discussed above, carrying the weight of re-establishing the "normal" higher $[K^+]_o$ levels. This suggests another case of K^+ recycling in conjunction with the Na^+/K^+ ATPase (Kumar and Pattnaik 2014). Loss of function mutations in Kir7.1 result in the autosomal-dominant disease called snowflake vitreoretinal degeneration, which progressively causes fibrillar degeneration of the vitreous humor, early-onset cataract, minute crystalline deposits, and retinal detachment. The R162W mutation, characteristic of this disease has been suggested to affect channel–PIP_2 interactions (Pattnaik et al. 2013; Zhang et al. 2013).

5.4 Transport Channel Pharmacology

Kir1, Kir4, and Kir7 channel inhibitors: Dozens of small molecule inhibitors for Kir1, Kir4, and Kir7 channels have been reported (Table 6). VU590 was discovered through HTS of 126,009 small molecules as Kir1.1 modulators. It inhibits Kir1.1 with submicromolar affinity, but has no effect on Kir2.1 or Kir4.1. It also inhibits Kir7.1 at low micromolar concentrations. Electrophysiological studies indicated VU590 is an intracellular pore blocker (Lewis et al. 2009). In an effort to achieve more selective inhibitors for Kir1.1, VU591 was developed based on VU590. VU591 is a potent inhibitor like VU590 for Kir1.1, but it is selective for Kir1.1 over Kir7.1. It blocks the intracellular pore of the channel through interactions with V168 and N171 (Bhave et al. 2011; Swale et al. 2015). VU573 was discovered through a thallium (Tl^+) flux-based high-throughput screen of a Kir1.1 inhibitor library for modulators of Kir3. It inhibits selectively Kir2.3, Kir3.X, and Kir7.1 with similar potency over Kir1.1 and Kir2.1 (Raphemot et al. 2011). ML418 was discovered based on a lead compound VU714, which was identified by a HTS screen as a novel Kir7.1 channel inhibitor. It selectively inhibits Kir7.1 and Kir6.2/SUR1 over Kir1.1, Kir2.1, Kir2.3, Kir3.1/Kir3.2, and Kir4.1 channels (Swale et al. 2016).

VU0134992 was discovered through HTS of 76,575 compounds from a Vanderbilt University library for small molecule modulators of Kir4.1. It inhibits selectively Kir4.1 and Kir3.1/3.2, Kir3.1/3.4 and Kir4.2 with similar potency over (30-fold selective) Kir1.1, Kir2.1, and Kir2.2. It also weakly inhibits Kir2.3, Kir6.2/SUR1, and Kir7.1 (Kharade et al. 2018). Tricyclic antidepressants (TCA), nortriptyline, amitriptyline, desipramine, and imipramine were found to act as inhibitors for Kir4.1 channel expressed in HEK293T cells, using the whole-cell patch-clamp technique. These compounds inhibited Kir4.1 currents in a voltage-dependent fashion, and marginally affected neuronal Kir2.1 currents (Su et al. 2007). Antidepressant, selective serotonin reuptake inhibitor (SSRI), fluoxetine, was found to inhibit the Kir4.1 channel expressed in HEK293T cells using whole-cell patch clamp. It inhibited the Kir4.1 channel in a concentration-dependent and voltage-independent manner. Fluoxetine had little or no effect on Kir1.1 and Kir2.1 channels. Other SSRIs, sertraline and fluvoxamine also inhibited the Kir4.1 channel (Ohno et al.

Table 6 Kir1, Kir4, and Kir7 channel inhibitors

Drug name	Species system	Potency (IC_{50}/EC_{50})	References
Inhibitor			
VU 590	HEK-293 cells	Kir1.1 (IC_{50} = 294 nM) Kir7.1 (IC_{50} = 8 μM)	Lewis et al. (2009), Kharade et al. (2018)
VU 591	HEK-293 cells	Kir1.1 (IC_{50} = 300 nM)	Bhave et al. (2011)
VU 0134992	HEK-293 cells	Kir4.1 (IC_{50} = 0.97 μM)	Kharade et al. (2018)
Pentamidine (aromatic diamine)	HEK-293 cells	Kir4.1 (IC_{50} = 97 nM)	Arechiga-Figueroa et al. (2017)
Quinacrine (cationic amphiphilic drug)	HEK-293 cells	Kir4.1 (IC_{50} = 1.8 μM)	Marmolejo-Murillo et al. (2017a)
Nortriptyline (tricyclic antidepressant)	HEK293T cells	Kir4.1 (IC_{50} = 16 μM)	Su et al. (2007)
Fluoxetine (SSRI)	HEK293T cells	Kir4.1 (IC_{50} = 15.2 μM)	Ohno et al. (2007)
Chloroquine (4-aminoquinoline)	HEK-293 cells	Kir4.1 (IC_{50} = 0.5 μM)	Marmolejo-Murillo et al. (2017b)
Gentamicin (aminoglycoside antibiotic)	HEK293T cells	Kir4.1 (IC_{50} = 6.2 μM)	Moran-Zendejas et al. (2020)
VU573	HEK-293 cells	Kir7.1 (IC_{50} = 4.9 μM)	Raphemot et al. (2011)
ML 418	HEK-293 cells	Kir7.1 (IC_{50} = 0.31 μM)	Swale et al. (2016)

2007). Aréchiga-Figueroa and colleagues discovered pentamidine, a drug for treatment of protozoan infections, inhibited Kir4.1 channels. This drug potently inhibited the Kir4.1 channel from the cytoplasmic side in the inside-out patch-clamp configuration. The inhibition was voltage dependent. Molecular modeling predicted the binding of pentamidine to the transmembrane pore region interacting with residues T127, T128, and E158 (Arechiga-Figueroa et al. 2017). Quinacrine, an old antimalarial drug, was discovered as an inhibitor of the Kir4.1 channel expressed on HEK293 cells, using patch clamp. It inhibited Kir4.1 channel with an IC_{50} of 1.8 μM and in a voltage-dependent manner. Molecular modeling and mutagenesis studies suggested quinacrine blocks Kir4.1 through the central cavity interacting with residues E158 and T128 (Marmolejo-Murillo et al. 2017a). Similarly, chloroquine, an aminoquinoline derivative anti-malarial drug, was discovered as an inhibitor for Kir4.1 channel (Marmolejo-Murillo et al. 2017b). More recently, Morán-Zendejas and colleagues discovered that aminoglycoside antibiotics (AGAs), such as gentamicin, neomycin, kanamycin, inhibited the Kir4.1 channel. Using patch-clamp, mutagenesis, and molecular modeling, the AGAs were characterized as pore blockers plugging the central cavity of the channel. The residues T128 and E158 were identified as critical determinants for AGA inhibition activity (Moran-Zendejas

et al. 2020). From these studies, we can conclude that residues E158 and T128 of the central pore in Kir4.1 are critical for the activity of several channel blockers. Insight can be gained by comparing conservation of the key residue determinants of intracellular pore blockers. The residue E158 (Kir4.1) is conserved in Kir4.2 (E157) and in Kir7.1(E149), but not in Kir1.1(N171) (Fig. 9). Kir1.1-specific intracellular pore blockers of Kir1.1, e.g. VU590 or VU591, utilize position 171 to exert their specific Kir1.1 effect. Indeed, VU591 interacts with Kir1.1 through this residue as a hydrogen bond donor with the nitro group acceptor of the compound. While the negatively charged residues, such as Glu and Asp, form unfavorable, repulsive interactions with the nitro group of the compound (see Fig. 11). A double mutation, Kir1.1(N171E/K80M), abolished VU591 activity (Swale et al. 2015). This explains that VU591 selectively inhibits Kir1.1 over Kir7.1, and UV590 has no effect on Kir4.1 and Kir2.1 (D172 is the corresponding residue). On the other hand, antibiotics, chloroquine, quinacrine, pentamidine utilize their amine groups to interact with Kir4.1 through E158 as a hydrogen bond acceptor to block the channel. Although discovery of selective channel blockers is challenging, the structural information of determinant residues could be useful for future structure-based drug design for selective channel inhibitors. In addition, identification of new allosteric binding sites could be valuable for discovery of novel selective modulators.

5.4.1 Transport Channel Structural Determinants

Kir1, 4, and 7 Channel Structure and Dynamics

Linder et al. (2015) conducted MD simulations (200 ns) on wild-type KirBac1.1 and a stimulatory mutant (G143E) to investigate activation gating of KirBac channels. The simulations revealed that a Glu mutation at G143 position caused significant widening at the HBC gate, enabling water flux in the cavity. Both global and local rearrangements were observed. Opening of the HBC gate could trigger twisting of the CTD, which was mediated by electrostatic interactions between the TMD and CTD. In addition, the channel's slide-helix and C-linker interactions with lipids were strengthened during gating the channel open (Linder et al. 2015). Hu and colleagues predicted the TPNQ (Tertiapin-Q) and Kir1.1 channel interactions using homology modeling, protein docking, and MD simulations. The results suggested TPNQ toxin interacts with Kir1.1 through its helical domain as its interacting surface along with residue H12 as a pore-blocking residue. Residues F146 and F148 in Kir1.1 formed dominant nonpolar interaction with the toxin (Hu et al. 2013). To understand the mechanism for the antidepressant–Kir4.1 channel interaction, Furutani and colleagues conducted homology modeling and molecular docking simulations on Kir4.1 channel with fluoxetine and nortriptyline. Chimeric and site-directed mutagenesis studies suggested the residues T128 and E158 on the TM2 were critical for the drug inhibitory activity. In addition, a 3D quantitative structure-activity relationship (3D-QSAR) model of antidepressants was generated, which suggested common features of a hydrogen bond acceptor and positively charged moiety from the drugs interacting with the channel (Furutani et al. 2009). Using similar homology modeling and molecular docking approaches, in combination with mutagenesis and electrophysiology experiments, several Kir4.1 channel inhibitors were

characterized: Quinacrine (Marmolejo-Murillo et al. 2017a), Pentamidine (Arechiga-Figueroa et al. 2017), Chloroquine (Marmolejo-Murillo et al. 2017b), and aminoglycoside antibiotics (Moran-Zendejas et al. 2020; Arechiga-Figueroa et al. 2017; Marmolejo-Murillo et al. 2017a, b; Moran-Zendejas et al. 2020; Swale et al. 2016).

Swale et al. (2016) discovered a novel Kir7.1 channel inhibitor, VU714, through fluorescence-based high-throughput screening. Homology model and docking predicted the binding site of VU714 in Kir7.1 central pore cavity. Site-directed mutagenesis suggested that residues E149 and A150 are essential determinants of VU714 activity. Lead optimization based on VU14 generated ML418, which exhibited high potency ($IC_{50} = 310$ nM) and superior selectivity over other channels (Kir1.1, Kir2.1, Kir2.2, Kir2.3, Kir3.1/3.2, and Kir4.1) except for Kir6.2/SUR1 (Swale et al. 2016).

6 Conclusions

Kir channels are mostly active at negative potentials influencing cells to rest near E_K. They are either constitutively active or opened by external signals (e.g., neurotransmitters and G protein signaling) or closed by internal signals (e.g., ATP/ADP ratio). They are fundamental to the proper functioning of many cells and systems in our body and their malfunction yields a multitude of diseases, making them desirable targets for therapeutic drug discovery and development. Great insights from 3-D atomic resolution structures of these K^+ channels have begun to provide insights for the molecular basis of their fundamental functional properties, such as inward rectification, dependence on $[K^+]_o$, and interactions with PIP_2 to control gating. More specialized functional attributes are also being deciphered, such as Gβγ binding and allosteric control of gating or ATP binding in relation to the gates and the interplay with PIP_2 gating. Computational studies have begun to offer dynamic views of ion permeation and gating by physiological regulators. The pharmacology of these channels has also been expanding providing insights as to how to best synthesize specific and effective drugs. Dynamic structural studies are paving the way toward successful structure-based drug design for this family of K^+ channels. Thus, the physiology and pharmacology of Kir channels in the context of dynamic atomic resolution structures have provided the groundwork for numerous therapeutic opportunities. The next decade promises great advances in Kir pharmacology with therapeutic potential (Figs. 9, 10, and 11).

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Appendix

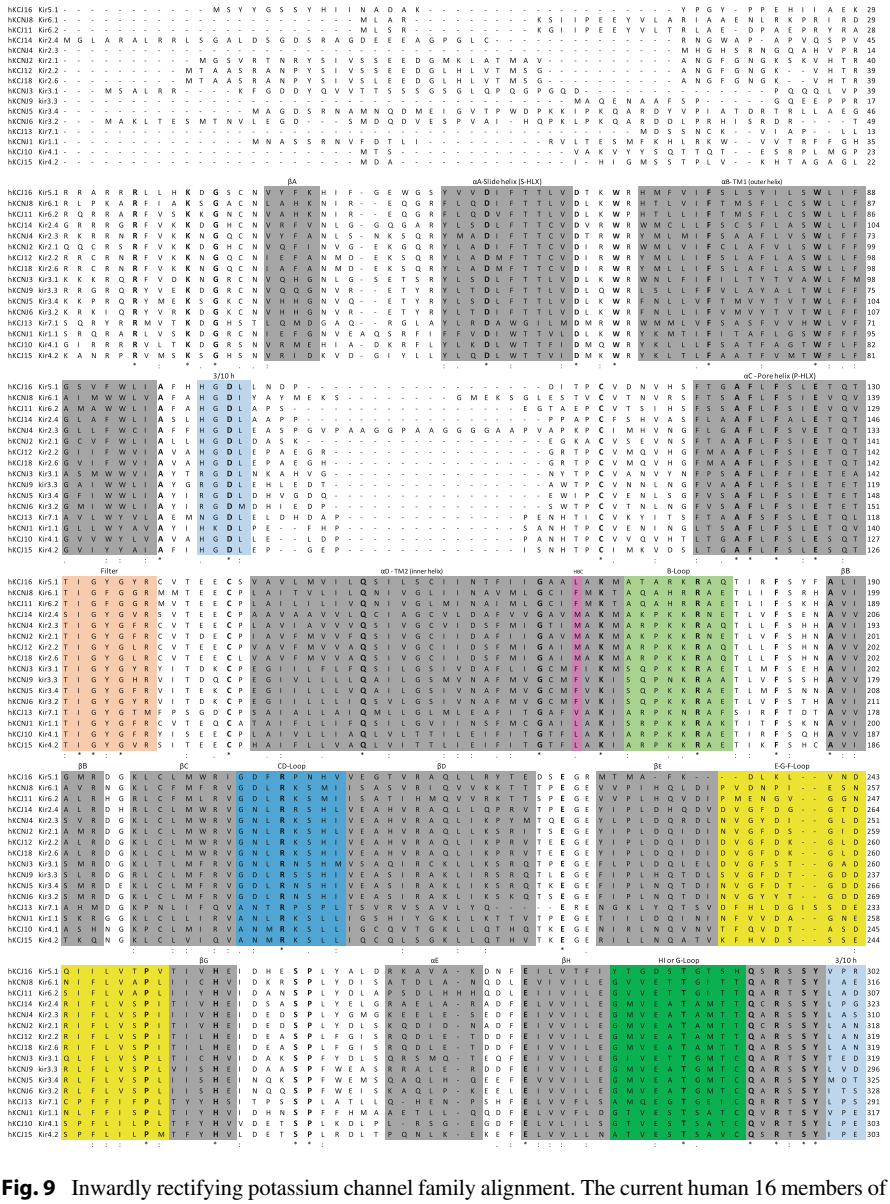

Fig. 9 Inwardly rectifying potassium channel family alignment. The current human 16 members of the Kir family were aligned using the Clustal Omega multiple sequence alignment program, which uses seeded guide trees and HMM profile–profile techniques to generate the alignments for all Kir members. Fully conserved resides are denoted with an "*", residues with strongly similar properties are denoted with a ":", and residues with weakly similar properties are donated with a ".". The important structural and gating features are highlighted and labeled (Madiera et al. 2019)

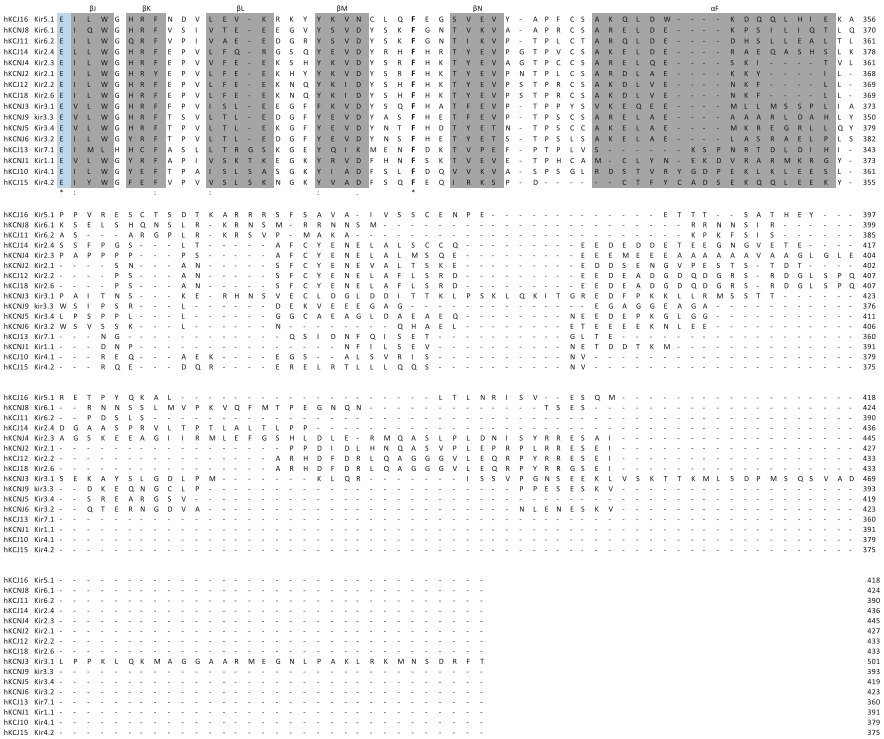

Fig. 9 (continued)

	Kir Family - % Similarity/ % Identity															
Kir1.1	100/100															
Kir2.1	54/40	100/100														
Kir2.2	54/42	80/71	100/100													
Kir2.3	53/41	70/61	71/64	100/100												
Kir2.4	49/37	67/58	64/57	62/54	100/100											
Kir2.6	54/42	79/70	99/99	70/63	64/56	100/100										
Kir3.1	51/40	57/45	55/43	51/40	50/40	54/43	100/100									
Kir3.2	52/40	57/45	55/43	56/44	55/41	54/43	62/53	100/100								
Kir3.3	49/38	60/49	60/51	62/50	57/49	59/50	64/55	76/68	100/100							
Kir3.4	51/41	57/44	55/45	57/45	55/44	55/45	62/54	76/72	71/65	100/100						
Kir4.1	56/45	50/37	50/38	49/36	47/36	50/38	49/36	48/35	48/36	48/36	100/100					
Kir4.2	57/45	49/36	49/37	47/36	47/35	49/37	46/33	47/33	45/31	48/35	71/61	100/100				
Kir5.1	48/34	52/42	51/41	51/40	50/40	51/40	47/34	46/33	49/36	47/32	47/34	46/32	100/100			
Kir6.1	49/37	53/42	53/41	51/40	50/36	53/41	52/39	52/41	55/42	50/39	49/35	46/32	47/35	100/100		
Kir6.2	49/37	56/43	55/44	54/43	54/41	55/44	53/40	56/42	54/43	53/42	50/35	47/33	49/36	79/69	100/100	
Kir7.1	46/35	44/32	44/32	42/30	44/33	43/32	41/31	41/30	40/31	41/30	51/40	50/37	41/28	41/28	39/27	100/100
	Kir1.1	Kir2.1	Kir2.2	Kir2.3	Kir2.4	Kir2.6	Kir3.1	Kir3.2	Kir3.3	Kir3.4	Kir4.1	Kir4.2	Kir5.1	Kir6.1	Kir6.2	Kir7.1

Fig. 10 Inwardly rectifying potassium channel family similarity and identity. The 16 members of the Kir family were analyzed for percent similarity and percent identity using the Sequence Identity And Similarity (SIAS) tool which uses a pairwise analyses utilizing several methods. Amino acid similarity and sequence length is used to calculate the values (% = 100*(identicall similar residues/sequence length)). Parameters were set to the standard settings relative to amino acid similarities. Sequences were input from the Clustal Omega alignment. The values are listed as percent similarity/% identity

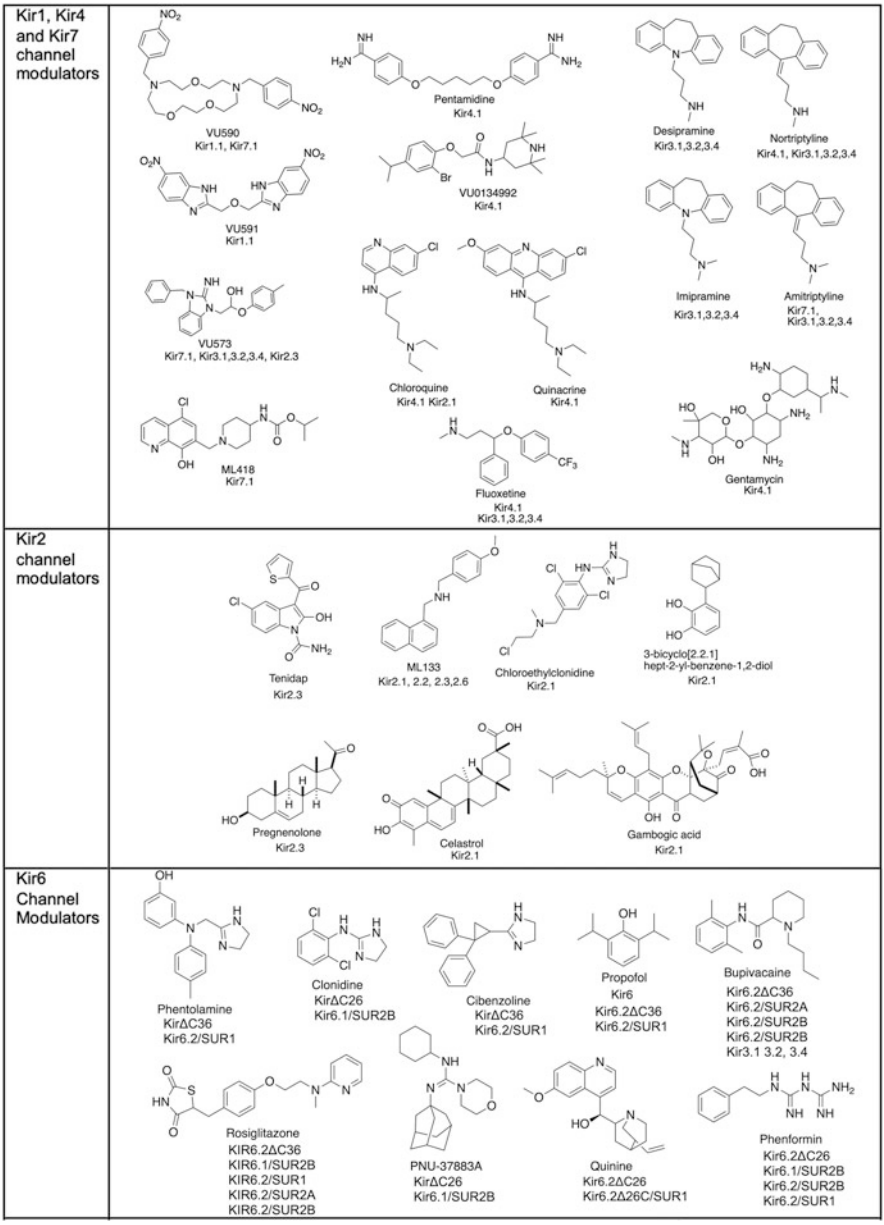

Fig. 11 Chemical structures of drugs targeting Kir channels

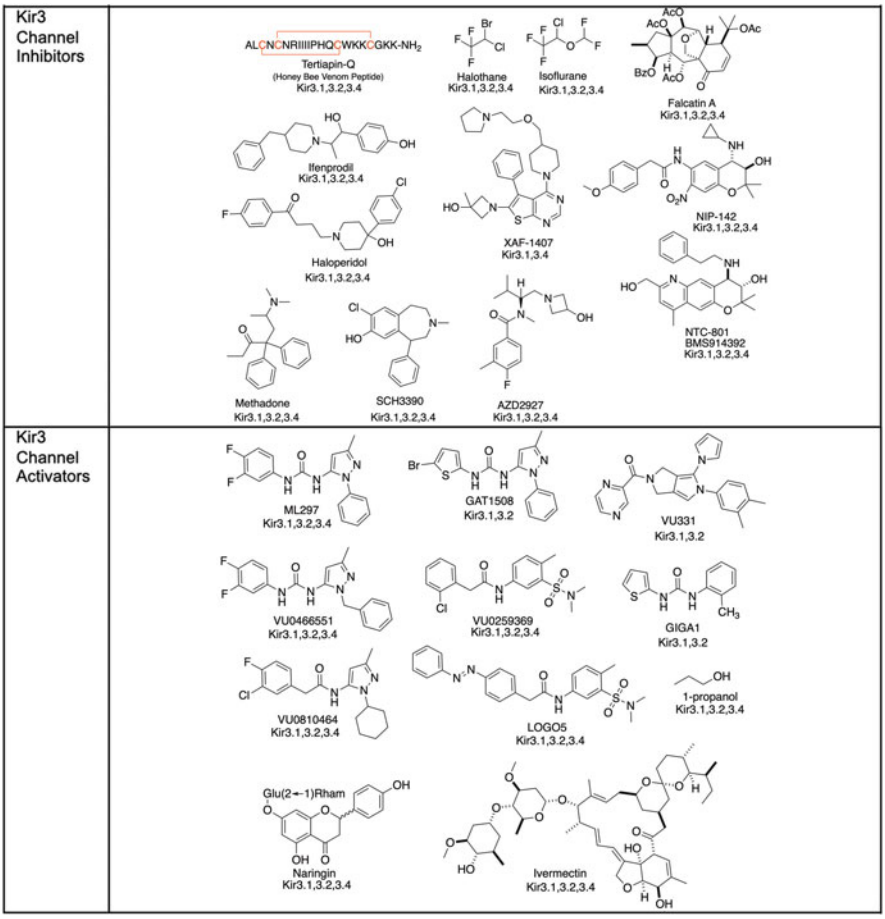

Fig. 11 (continued)

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