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Inwardly rectifying K^+ (Kir) channels have a wide range of functions including the control of neuronal signalling, heart rate, blood flow and insulin release. Because of the physiological importance of these channels, considerable effort has been invested in understanding the structural basis of their physiology. In this review, we use two recent, high-resolution structures as foundations for examining our current understanding of the fundamental functions that are shared by all K^+ channels, such as K^+ selectivity and channel gating, as well as characteristic features of Kir channel family members, such as inward rectification and their regulation by intracellular factors.

POLYAMINES
Long-chain aliphatic compounds that contain more than one amine group. Putrescine, spermine and spermidine are prime examples. Because of the positive charges on these molecules, polyamines bind electrostatically to proteins, DNA and RNA.

RCK DOMAINS
(Regulator of K^+ conductance domains). A ligand-binding domain found in many ligand-gated K^+ channels; in MthK the ligand is thought to be nicotinamide adenine dinucleotide.

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Potassium channels are expressed in many cell types and contribute to a wide range of physiological processes. Consequently, K^+ channel mutations cause diseases of the brain (epilepsy, episodic ataxia), ear (deafness), heart (arrhythmia), muscle (myokymia, periodic paralysis), kidney (hypertension) and pancreas (hyperinsulinaemic hypoglycaemia), and developmental abnormalities of neural crest-derived tissues (Andersen syndrome)[1].

Inwardly rectifying K^+ (Kir) channels are an important class of K^+ channels that regulate membrane excitability, heart rate, vascular tone, insulin release and salt flow across epithelia. Inward rectification refers to the ability of an ion channel to allow greater influx than efflux of ions. In the case of Kir channels, inward rectification is caused by cytoplasmic ions such as POLYAMINES and Mg^{2+}, which plug the conduction pathway on depolarization and thereby impede the outward flow of K^+. There are seven Kir subfamilies (Kir1–7), which can be distinguished by their strength of rectification and their responses to cellular signals. For example, Kir3 (GIRK) channels are strongly rectifying and activated by Gβγ, and Kir6 (K_{ATP}) channels are weakly rectifying and influenced by the ATP/ADP ratio.

Kir channels are tetramers[2,3], with each subunit having two transmembrane (TM) segments called M1 and M2, a pore loop (P), and amino (N)- and carboxy (C)-terminal cytoplasmic domains (FIG. 1a,b). Recently, the high-resolution structure of KirBac1.1, a bacterial ion channel that is closely related to eukaryotic Kir channels, has been reported in its closed conformation[4]. It is the latest in a series of K^+ channel X-ray crystal structures, namely the bacterial 2TM KcsA channel[5–7], the archaebacterial 2TM K^+ channel MthK including its C-terminal RCK (REGULATOR OF K^+ CONDUCTANCE) DOMAINS[8], an archaebacterial 6TM voltage-gated K^+ channel (KvAP)[9] (for review see REF. 10) and the structure of the mammalian Kir3.1 cytoplasmic domain[11].

This review will highlight the convergence of structural studies and functional analyses of Kir channels. Ion selectivity and inward rectification will be discussed in the light of the KirBac structure and extensive MUTAGENESIS studies. With respect to Kir channel gating, we will summarize functional studies on the regulation of gating, and will map the sites of regulatory interactions onto the cytoplasmic domain of KirBac (REF. 4) and Kir3.1 (REF. 11), to gain insight into the complex and synergistic mechanisms of Kir channel modulation and the molecular events that lead to channel opening.

Architecture of Kir channels

The transmembrane domain. The crystal structures of KirBac, KcsA and MthK give insight into the architecture of Kir channels, because they all have two membrane-spanning helices per subunit. In the KirBac and KcsA

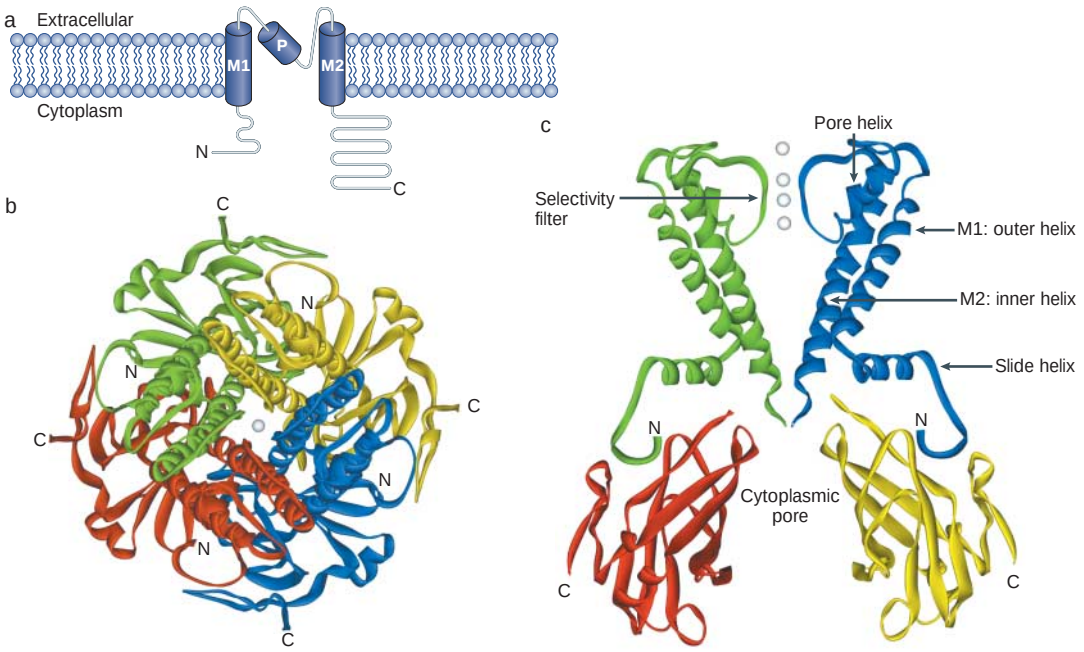


Figure 1 | **Overall architecture of inwardly rectifying K^+ (Kir) channels. a** | Schematic drawing of a Kir channel subunit. Each subunit comprises two transmembrane helices (M1 and M2), a pore-forming region containing the pore-helix (P), and a cytoplasmic domain formed by the amino (N) and carboxy (C) termini. **b** | View of the tetrameric structure of the KirBac1.1 channel[4] (PDB ID: 1P7B) from the extracellular side. Monomers are individually coloured red, green, yellow and blue. A K^+ ion (white) indicates the conduction pathway. **c** | Side view of the KirBac1.1 structure showing the transmembrane domain of two subunits (green and blue) and the C-terminal domains of their neighbouring subunits (red and yellow). White spheres represent K^+ ions in the selectivity filter. Molecular drawings were prepared using WebLab ViewerPro 3.5.

structures, M1 (the outer helix) makes contact with M2 (the inner helix), which lines the pore of the channel and forms an 'inverted tepee' in a closed channel conformation (FIG. 1c). The pore loop, located between the M1 and M2 helices, contains the descending pore helix and the ascending K^+ channel signature sequence. The KirBac structure includes an additional helix, the 'slide helix', which runs parallel to the cytoplasmic face of the membrane.

Although the structure of KcsA revealed for the first time the architecture of the transmembrane region of a K^+ channel, it was unclear whether information from KcsA could be generalized to Kir channels given the limited sequence homology. In addition, it was suggested that the helix packing (BOX 1) and dimensions of the inner pore of Kir2.1, as deduced from mutagenesis and functional studies, were distinct from those of KcsA[12–14]. The higher sequence homology between KirBac and eukaryotic Kir channels[4,15] makes it easier to evaluate interactions deduced from functional assays in the context of the relevant channel structure (for further information see online figure).

The cytoplasmic pore. Two-thirds of the Kir channel amino-acid sequence constitutes a large intracellular domain, which forms the cytoplasmic pore (FIG. 1c). This region of the channel is crucial for channel modulation by intracellular regulators and for establishing the strong voltage dependence of inward rectification. The high-resolution structure of a construct containing the N and C termini of Kir3.1 provided the first view of the cytoplasmic pore[11]. A wall of β-sheets containing many polar and charged residues surrounds a large pore ~30 Å in length and 7–15 Å in diameter. At the C-terminal end of the structure, an α-helix projects into the cytoplasm. The KirBac structure contains a cytoplasmic domain that is similar to the Kir3.1 structure in the context of the whole channel[4] (FIG. 2a). An interesting feature of both structures is that the N and C termini interact through two parallel β-strands (FIG. 2b).

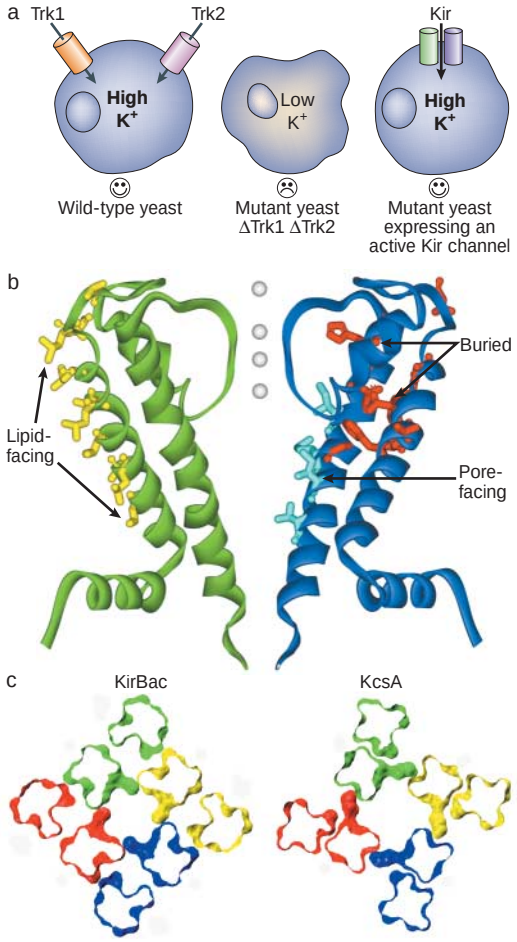
Ion selectivity and permeation

All K^+ channels can discriminate between K^+ and Na^+, the most abundant alkali metal ions in nature. Without such ion selectivity, channels would not be able to generate electrical signals. Selectivity for K^+ is conferred by the selectivity filter, defined as the narrowest part of the conduction pathway in the open channel (FIG. 1c). In all K^+ channel crystal structures solved so far, the main-chain carbonyl oxygens of the conserved K^+ channel signature sequence (TXGYG or TXGFG), which is located in the P-loop, form the selectivity filter (FIG. 3). Point mutations in the K^+ channel signature sequence abolish K^+ selectivity[16]. At the phenotypic level, this loss of K^+ selectivity is linked to the movement disorder that is observed in *weaver* mice[17–20].

A detailed understanding of ion selectivity and permeation was derived from crystal structures of KcsA at varying K^+ concentrations, modelling of the energy landscape of the selectivity filter and other biophysical

MUTAGENESIS
Technique in which an alteration is made either at a specific site or randomly in a DNA molecule. Mutated DNA is then reintroduced into a cell and analysed with various techniques to determine which parts of a protein or nucleotide sequence are crucial for its function.

Box 1 | Using yeast to study inwardly rectifying K^+ (Kir) channel structure–function relationships



A mutant yeast strain lacking the K^+ transporters Trk1 and Trk2 does not grow on a low-K^+ medium (a)[129,130]. This lethal phenotype can be rescued by transformation of the yeast with active Kir channels[131], presenting the opportunity to identify functional Kir channels in libraries containing hundreds of thousands of mutated channels[12,28,55]. This assay has been used to determine the pattern of permissible substitutions in the transmembrane helices of Kir2.1 and to predict which residues face lipid, protein or water. Although it was difficult to map the residues identified in Kir2.1 onto the KcsA structure[12,15], the KirBac structure is in good agreement with the pattern of tolerance that is seen in the yeast study[4] (b). Predicted lipid-facing residues (yellow) localize to the external face of M1, residues that are predicted to face water (cyan) are on the internal part of M2, which lines the pore, and substitution-intolerant residues (red) are buried in the KirBac channel.

Minor *et al.* (REF. 12) proposed that in the open state of Kir2.1 channels, each M1 helix makes contact with M2 from the same subunit as well as M2 from an adjacent subunit. This arrangement differs from that seen in the closed KcsA structure, where each M1 contacts only M2 in the same subunit (c). This difference could mean that KcsA and Kir channels form two structurally distinct classes[12] or that the arrangement of the helices changes between the closed and the open state[28]. Interestingly, the closed KirBac structure shows an arrangement of helices that differs from that seen in KcsA, but is similar to the helix packing that is proposed for the open state of Kir2.1 (c). The similarity between KirBac and the model for Kir2.1 might indicate that Kir channels form a structurally distinct class whose gating transitions are associated with only minor rearrangements of M1 with respect to M2. However, the KcsA and KirBac structures both represent closed states, which might indicate that there are several closed states that differ in the arrangement of M1 with respect to M2.

The amino acids of Kir2.1 (corresponding residues are in parenthesis for KirBac1.1) shown in (b) are I87(S66), L90(A69), A91(L70), L94(V73), L97(T76), F98(L77), C101(L80), W104(Q83), L105(L84) and L108(A87) for lipid-facing, S165(I131), C169(M135), D172(I138) and I176(T142) for pore-lining, and F92(F71), S95(N74), W96(N75), F99(F78), G100(A79), A107(D86), A157(A123), V158(H124), V161(A127), Q164(E130) and G168(G134) for buried residues. In (c), the positioning of the transmembrane helices in KcsA and KirBac is shown at the level of a conserved glycine (G99 in KcsA and G134 in KirBac) from the extracellular side. Molecular drawings were prepared using WebLab ViewerPro 3.5 for panel b and VMD[137] for panel c.

experiments[6,7,21–24]. This work has been reviewed previously[25] (FIG. 3). As the K^+ channel signature sequence is conserved between all K^+ channels, and the KcsA pore can substitute for the pore of other K^+ channels[26], it is assumed that the mechanism of ion selectivity and permeation at the selectivity filter is similar in all K^+ channels.

In Kir channels, structural features other than the K^+ channel signature sequence are also important for K^+ selectivity. Differences in the residues that surround the selectivity filter account for the reduced K^+ selectivity of Kir3.1 compared with other Kir3 family members[27]. Certain mutations in the pore helix or in M2 of Kir3.2 cause a loss of K^+ selectivity[28]. In most Kir channels, a SALT BRIDGE between conserved charged residues in adjacent subunits has been suggested to anchor and stabilize the K^+ channel signature sequence[29–32]. The salt bridge cannot be formed in KirBac owing to the absence of one of the charged residues. Interestingly, the closest eukaryotic relative of KirBac, Kir7.1, also lacks the salt bridge and its selectivity differs from that of other Kir channels[33].

SALT BRIDGE
Electrostatic interaction between oppositely charged amino-acid side chains in close proximity in a protein.

RESTING POTENTIAL
The separation of positive and negative charges across the cell membrane results in the membrane potential. The resting potential is the membrane potential at which there is no net current flow across the cell membrane.

Inward rectification

Notwithstanding their ability to pass K^+ ions selectively in both directions, Kir channels characteristically interact with cytoplasmic Mg^{2+} and polyamines, which block K^+ efflux at membrane potentials that are more positive than the RESTING POTENTIAL[34–36] (FIG. 4a,b). Among Kir family members, there are strong (Kir2 and Kir3) and weak (Kir1 and Kir6) rectifiers. The degree of rectification is correlated with the binding affinity of the channel for blocking cations. Given the essential role of rectification in the control of the resting membrane potential and

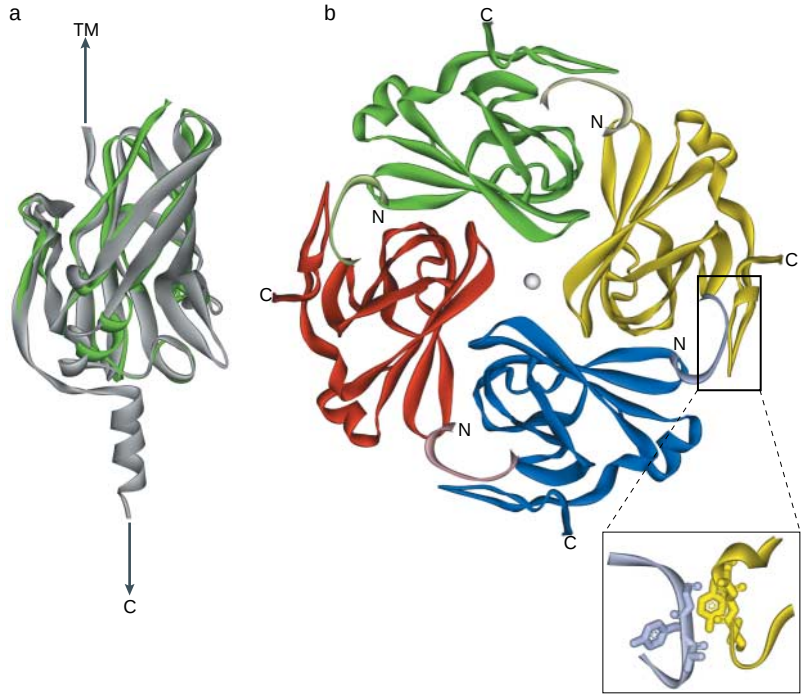


Figure 2 | **Structures of the cytoplasmic domains of KirBac1.1 and Kir3.1. a** | Side view of a superposition of the carboxy (C)-terminal domains of Kir3.1 (REF. 11) (grey, 1N9P) and KirBac1.1 (REF. 4) (green) showing that the two structures have a similar fold. A single subunit of each channel structure is represented from amino acids 151 to 309 for KirBac1.1 and 190 to 370 for Kir3.1. The subunit is oriented such that the pore would be to the right. TM, transmembrane domain. **b** | Top view of the KirBac1.1 cytoplasmic domain after removal of the transmembrane helices. Inset: three residues from the amino (N) terminus (amino acids 40 to 42) interact with three residues of the C-terminal domain (amino acids 298 to 300) of an adjacent subunit through two parallel β-strands. In the Kir3.1 structure a similar interaction takes place within the same subunit, which is probably the result of the direct fusion of the N to the C terminus in this structure. Molecular drawings were prepared using WebLab ViewerPro 3.5.

consequently in setting the excitation threshold, different levels of rectification will allow cells to generate specific responses to stimuli.

Electrophysiological studies on the kinetics of the Mg^{2+} and polyamine block, as well as mutational analyses, indicate that there is more than one binding site for blocking ions in Kir channels. The first to be identified was a residue in the second transmembrane helix of Kir channels. The strongly rectifying Kir2.1 contains a negatively charged residue (D172) at this position, whereas the weakly rectifying Kir1.1 has an uncharged residue (N171). The mutation N171D in Kir1.1 was shown to increase the affinity for Mg^{2+}, thereby increasing rectification[37–40]. Another M2 residue, S165 in Kir2.1, has been shown to be important for the Mg^{2+} block but not the polyamine block[41]. Mutations in the cytoplasmic domain that affect the binding of intracellular blocking ions were found at two negatively charged amino acids (E) and a TITRATABLE RESIDUE (H)[40,42–45]. Support for a direct interaction of these residues with Mg^{2+} or polyamines comes from mutagenesis and SUBSTITUTED CYSTEINE ACCESSIBILITY METHOD (SCAM) experiments, which provide evidence that the residues face the pore[12,13]. The crystal structures of KirBac and Kir3.1 further support the pore-lining location of these residues (FIG. 4c). The side chains of the conserved cytoplasmic glutamates point to the centre of the conduction pathway, forming rings of negatively charged residues that create a complimentary electrostatic match for the binding of a long, positively charged polyamine[4,11]. Although the suggested binding residues for blockers in the transmembrane domain face the pore in the KirBac structure, they are not polar or negatively charged. Future functional studies of KirBac will reveal whether this reflects an important difference in its rectification properties.

What is the importance of having residues that bind blocking molecules in both the transmembrane and the cytoplasmic domain? A two-site model of inward rectification could account for the speed of the polyamine block. It has been proposed that polyamines plug the pore in the transmembrane domain (plugging site), whereas the cytoplasmic region serves as an intermediate binding site (non-plugging site) that increases the local concentration of polyamines around the plugging site[45–48]. Another characteristic feature of inward rectification is its strong voltage dependence, which is consistent with the movement of several ions through the transmembrane electric field. Two factors have been proposed to contribute to the charge movement. First, the blocking sites can accommodate several Mg^{2+} and polyamine molecules[13,49]. Second, the pore of Kir channels is long and provides room for several K^+ ions, which will be forced out of the pore in front of entering blocking ions[11,50–52].

Intrinsic gating

In addition to blockage of the conduction pathway by extrinsic molecules, Kir channels can change their conformation and thereby reduce ion flow through them. Two locations for the intrinsic gate have been proposed: the bundle crossing and the selectivity filter.

Gating at the bundle crossing. In the closed channel structures of KcsA[5] and KirBac[4], the M2 transmembrane helices cross one another at a point that is close to the intracellular surface of the membrane. This bundle crossing seems to be narrow enough to restrict the passage of hydrated K^+ ions[53]. In addition, hydrophobic residues that line the pore in this region, especially the large phenylalanine at position 146 in KirBac, create an unfavourable environment for K^+ ions[4]. We will discuss gating at the bundle crossing with regard to three questions.

Does the M2 helix bundle move to open the channel? If so, SCAM might reveal state-dependent accessibility during gating. In Kir6.2, large modifying reagents applied intracellularly react with engineered cysteines in the M2 helices at a slower rate in the closed than in the open state, implying that an intracellular gate regulates access to the pore[54]. Further evidence for an inner gate was obtained by randomly mutating the Kir3 channel sequence and recovering gating mutations in the lower half of the transmembrane domain[28,55] (FIG. 5a). Mutations of N94 in M1 and V188 in M2 of Kir3.2 produce similar gating phenotypes, indicating

TITRATABLE RESIDUE
An amino acid with a side chain that can bond and release protons within a physiological pH range. Seven of the twenty amino acids are titratable (pKa of the free amino acid is given in parenthesis; this can vary in the protein): aspartate (4.4), glutamate (4.4), histidine (6.5), cysteine (8.5), tyrosine (10), lysine (10) and arginine (12).

SUBSTITUTED CYSTEINE ACCESSIBILITY METHOD (SCAM). An approach to the characterization of channel and binding site structures that probes the environment of any residue by mutating it to cysteine and characterizing the reaction of the cysteine with sulphydryl reacting and coordinating reagents.

that these two residues might be part of the same inner gate[28]. Mutation of F181 in Kir3.1/4 to a smaller residue (F181S) also produces constitutively open Kir3 channels[55]. Interestingly, this residue corresponds to F146 in KirBac, whose large side chain was proposed to block ion conduction[4] (FIG. 5b).

How might the M2 helices move? SITE-DIRECTED SPIN LABELLING (SDSL) and ELECTRON PARAMAGNETIC RESONANCE (EPR) studies of KcsA indicate that the M1 and M2 helices rotate and translate relative to the central axis of the channel, thereby widening the pore at the bundle crossing[53,56]. The structure of the open MthK channel indicates that the M2 helices kink during gating so that they splay open at the intracellular side[8,57]. The kink in M2 is thought to occur at a glycine residue that is conserved in many K^+ channels. Mutation of this glycine reduces currents through Kir3 channels[58]. However, sequence alignment of Kir channels shows that this glycine is not conserved in all Kir family members (Kir4 and Kir5). A different glycine residue (G143 in KirBac), which is located beneath the originally proposed hinge, is more conserved in Kir channels[4]. As both glycines are positioned above the bundle crossing, either one or both together could bend the M2 helices. In support of a model in which the M2 helices of Kir channels bend during gating, introduction of a kink in M2 using proline mutations stabilizes the open state of Kir3 (REFS 55,58).

Does the bundle crossing form a physical barrier that prevents K^+ flow? This question was addressed in SCAM studies using Cd^{2+} and Ag^+, which are similar in size to K^+. In the case of voltage-gated K^+ (Kv) channels, the central cavity is accessible to Cd^{2+} and Ag^+ in the open but not the closed state[59,60]. A similar tight physical barrier at the intracellular side of the membrane has been proposed for hyperpolarization-activated cation (HCN) channels[61], but not for cyclic-nucleotide-gated (CNG) channels[62] (see REF. 63 for review). SCAM studies on inward rectifiers have shown that Cd^{2+} accessibility in Kir2.1 and 6.2 channels is state-dependent[64,65]. However, Ag^+ ions can access the central cavity of Kir2.1 channels in both the open and closed states[65]. Overall, it seems likely that the diameter of the bundle crossing in Kir channels is narrow, but future studies using independent techniques will be needed to determine whether it closes tightly enough to prevent K^+ access to the central cavity in the closed state.

Gating at the selectivity filter. Traditionally, the most studied property of the selectivity filter is its ability to discriminate between K^+ and other ions (see above). C-TYPE INACTIVATION of Kv channels provided the first evidence for an additional function of the selectivity filter in gating. During C-type inactivation, Kv channels stop conducting K^+ and transiently acquire permeability for the smaller Na^+ ions before closing completely[66–68]. This implies that the selectivity filter constricts during the inactivation process, and is consistent with fluorometry and crosslinking data showing that engineered cysteines at the extracellular entrance of the channel approach each other during inactivation of Kv channels[69,70].

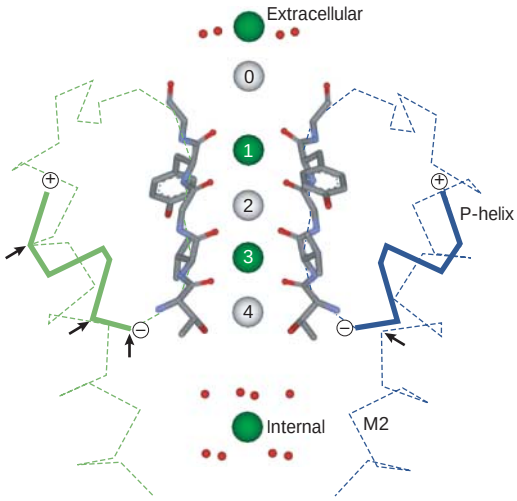


Figure 3 | **Structure of the K^+ selectivity filter supporting a multi-ion pore model.** Close-up view of the selectivity filter of KcsA (1K4C)[7] showing the linear array of K^+ binding sites. The front and back subunits have been removed for clarity and only residues of the K^+ channel signature sequence (TVGYG) are shown in ball-and-stick representation. At the extracellular and internal ends of the filter, K^+ ions are surrounded by water molecules. As K^+ ions enter the filter, their hydration shell is progressively replaced by interactions with the backbone carbonyls of the selectivity filter (positions 0 to 4). In the KcsA structure, the filter contains two K^+ ions simultaneously, either at positions 1 and 3 (green spheres), or at 2 and 4 (white spheres). In Kir channels, several mutations that affect selectivity have been identified in the pore helices (arrows on left) and the M2 helices (arrow on right) that surround the selectivity filter. The mutated amino acids are F137(S69) and A142(T74) in Kir3.1, and E152(A73) and S177(M96) in Kir3.2; corresponding residues are given in parentheses for KcsA. The orientation of the pore helices, with their negative ends pointing towards the central cavity, was proposed to promote ion conduction by stabilizing the K^+ ion at the internal site. P-helix, pore helix. Molecular drawings were prepared using WebLab ViewerPro 3.5.

There is accumulating evidence that the selectivity filter and outer pore of Kir channels undergo conformational changes during gating. First, simulations of Kir channels based on homology models with KcsA have indicated a significant degree of flexibility in the selectivity filter[71–73]. Second, the single-channel kinetics of Kir channels differ depending on whether K^+ or Tl^+ is present as the permeant ion[74]. Indeed, permeant ions such as K^+ can occasionally cause brief channel closures, presumably by inducing a pore conformation that traps the permeant ion[75,76]. Third, mutations in the pore helix and the residues that connect the filter and the inner helix can affect gating rather than K^+ selectivity[72,76–78] (FIG. 5a). Even changes within the K^+ channel signature sequence can affect gating rather than selectivity[79,80]. For example, a subtle amide-to-ester substitution at the GYG motif produced channels with intact selectivity, but with altered kinetics and distinct subconductance states[80]. Finally, there are differences between the selectivity filter of KirBac and the high K^+-concentration KcsA structures. The TEMPERATURE FACTORS in KcsA are the

SITE-DIRECTED SPIN LABELLING (SDSL). In SDSL, a nitroxide side chain is introduced by cysteine substitution mutagenesis followed by modification of the unique sulphydryl group with a specific nitroxide reagent. Measurements of the spectral properties of the paramagnetic nitroxide probe with electron paramagnetic resonance (EPR) spectroscopy provide information on its environment in the protein.

ELECTRON PARAMAGNETIC RESONANCE (EPR). When an atom with an unpaired electron is placed in a magnetic field, the spin of the unpaired electron can align, either in the same direction or in the opposite direction. EPR is used to measure the absorption of microwave radiation that accompanies the transition between those two states.

C-TYPE INACTIVATION Two distinct molecular mechanisms for voltage-gated K^+ channel inactivation have been described: N-type, which involves occlusion of the pore by an intracellular domain of the channel, and C-type, which involves a conformational change in the outer pore.

TEMPERATURE FACTOR (B-factor, Debye-Waller factor). A measure of atomic vibration as described by the spread of the electron density. A low B-factor indicates low atomic mobility.

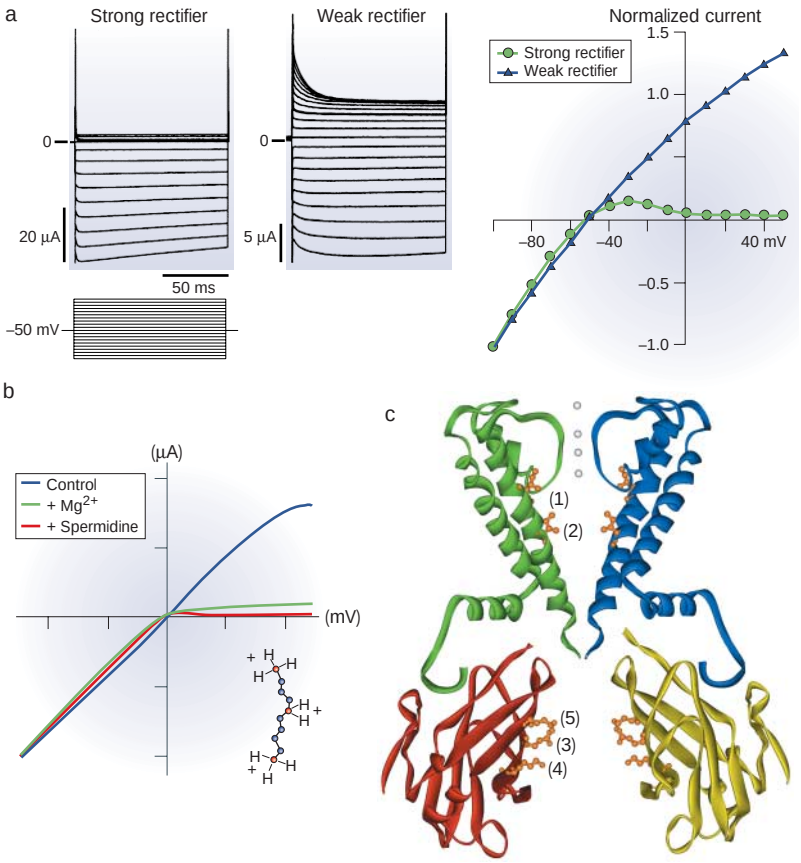


Figure 4 | **Inward rectification. a** | Macroscopic current traces and current–voltage curves of a strong and a weak inward rectifier. The examples shown were obtained under a two-electrode voltage clamp of *Xenopus* oocytes expressing wild-type (left) Kir2.1 and E224G/E299S mutant (right) channels. The protocol of stimulation shown below the current traces consists of voltage steps of 10-mV increments from –140 mV to +50 mV from a holding potential of –50 mV. **b** | Inward rectification is caused by cytoplasmic Mg^{2+} and polyamines blocking the pore. Current–voltage relationships were recorded from inside-out patches of *Xenopus* oocytes expressing Kir2.1. In the absence of Mg^{2+} or polyamines the current increases linearly with voltage. The addition of Mg^{2+} or polyamines decreases outward currents at membrane potentials that were depolarized from the reversal potential. The inset shows the structure of spermidine as an example of a polyamine. **c** | Mapping of the binding sites for polyamines and Mg^{2+} identified in eukaryotic Kir channels onto the KirBac structure. Sites 1 and 2 are in the transmembrane domain, Kir2.1 S165(I131) and Kir2.1 D172/Kir1.1 N171(I138), respectively. Sites 3, 4 and 5 are in the cytoplasmic domain, Kir2.1 E224(E187), Kir6.2 H216(K191) and Kir2.1 E299(E258), respectively. Sites are numbered according to their appearance in the KirBac sequence (numbers in parentheses). C, carboxy; N, amino. Molecular drawings were prepared using WebLab ViewerPro 3.5. Panel **a** modified, with permission, from REF. 45 © (2001) Cambridge University Press; panel **b** modified, with permission, from REF. 40 © (1995) Elsevier Science.

same for all K^+ ions, whereas in the KirBac selectivity filter one ion has a lower temperature factor than the others. This might indicate that KirBac is not as efficient as KcsA at conducting ions[4]. In addition, the pore helices in KcsA and KirBac are oriented differently with respect to the centre of the cavity[4] (FIG. 5c). Both observations indicate that the two structures might correspond to the closed (KirBac) and open (KcsA) selectivity filter and that KirBac might undergo a transition between the two arrangements during gating at the selectivity filter[4]. Future experiments, for example, crystal structures of the same channel in the open and closed state, are needed to confirm this proposal.

Although these observations support the idea that the selectivity filter moves during gating, how it might regulate K^+ flow remains unclear. Constriction of this part of the channel might physically obstruct the permeation pathway or trap a permeant ion in a deep ENERGY WELL. Alternatively, conformational changes in the K^+ channel signature sequence or rearrangement of the pore helices might lead to misalignment of the coordination sites for K^+ ions, thereby impeding K^+ flow.

Fast versus slow gating. Single-channel recordings provide evidence that Kir channels gate at two locations, possibly corresponding to the bundle crossing and the selectivity filter. The single channel behaviour of several Kir family members shows bursts of openings interspersed with long closures (FIG. 5d). This has been described as dual-component gating, composed of fast and slow gating. Slow gating refers to the transition between a burst of activity and interburst closure; it is measured by the burst and interburst durations. Fast gating refers to the brief openings (flickerings) within a burst and is assessed by the intraburst open and closed durations. Do the two functionally observed gating components correspond to the two gates?

Mutations within the pore loop of Kir channels affect the intraburst kinetics, indicating that the fast gate might correspond to the gate at the selectivity filter[76,77,81–83]. For example, Kir2.1 channels, which, unlike Kir3 channels, do not exhibit fast gating, can be converted to Kir3-like channels by a point mutation (Q140E) in their pore helix[76]. By contrast, the frequency and duration of bursts are affected by mutations at the intracellular end of M2, indicating that slow gating of K^+ channels might be controlled by the gate at the bundle crossing[64,84,85]. The idea of two gates is further demonstrated by two pairs of mutations in Kir3.2 (REF. 28). Mutations of the outer pair (E152D and S177T) lengthen the mean open time — that is, they affect fast gating — whereas changes of the inner pair (N94H and V188G) prolong bursts — that is, they alter slow gating. Double mutants that combine mutations of the inner and outer pair show additive effects, indicating that there are two independent gates[28]. Consistent with these findings, the residues of each pair are in close proximity in the KirBac structure, and the outer pair localizes to the selectivity filter region whereas the inner pair is close to the bundle crossing (FIG. 5a).

Cytoplasmic regulatory factors

The activity of Kir channels can be modulated by several cytoplasmic factors, including PHOSPHATIDYLINOSITOL-4,5-BISPHOSPHATE ($PtdIns(4,5)P_2$), arachidonic acid, Na^+ and Mg^{2+} ions, pH, heterotrimeric G proteins (specifically Gβγ), ATP, phosphorylation, oxidation/reduction and interactions with PDZ DOMAINS (BOX 2). Although some regions and residues that are involved in modulation have been identified, it has not been easy to define their exact roles, because they might be part of the binding site for the modulator, the transduction mechanism to the gate or the gate itself. The structural information that is available helps to resolve some of these questions with

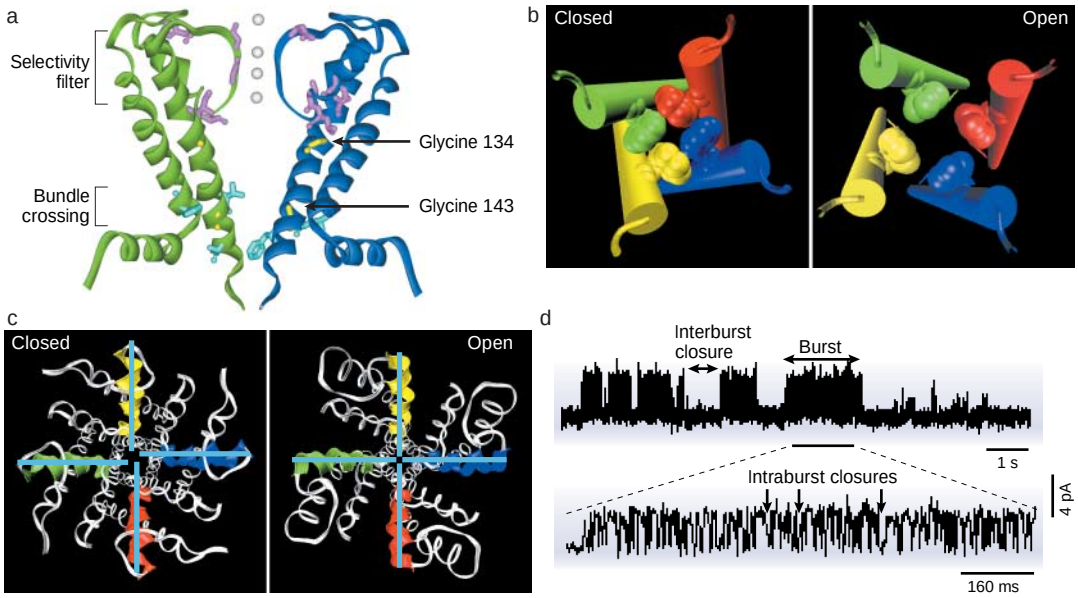


Figure 5 | **Gating of inwardly rectifying K⁺ (Kir) channels. a** | Residues that were found to affect gating fall into two regions of the KirBac structure: the selectivity filter (pink) and the bundle crossing (cyan). In the region containing the selectivity filter, the following residues are shown (numbers for KirBac1.1 in parentheses): Kir3.1 F137/Kir3.4 S143(S104); Kir6.2 V127(T107); Kir2.1 Q140/Kir3.2 E152(L108); Kir2.1 T141/Kir1.1b V121(A109); Kir2.1 G144(G112); Kir2.1 G146(G114); Kir6.2 G135(D115); Kir6.2 M137(H117); Kir3.1 Q165(E130); Kir3.2 S177(I131). In the region of the bundle crossing, residues Kir3.2 N94(F63), Kir3.2 V188(T142), Kir3.1 C179/Kir3.4 C185(L144), Kir3.1 F181(F146) and Kir3.1 I182(A147) are shown. Also depicted are the two glycine residues (yellow: G134 and G143 in KirBac) that are proposed to form hinges at which the M2 helices could bend. **b** | Schematic showing how the bundle crossing could widen during gating. The left panel shows the arrangement of the M2 helices in the closed KirBac structure as seen from the cytoplasm. The right panel depicts a possible open state of the bundle crossing that is generated by tilting the M2 helices of KirBac. However, the movements of the M2 helices during channel opening could also include rotation and/or bending. The phenylalanine (F146), which points into the conduction pathway at the bundle crossing, is shown in van-der-Waals representation. **c** | Comparison of the KirBac[4] and KcsA[5] structures, indicating that gating at the selectivity filter involves movements of the pore helices. A view into the KirBac pore (left) from the extracellular side shows that the pore helices are misaligned. The same view in KcsA (right) shows that all four pore helices point towards the centre of the channel cavity. **d** | Single channel traces showing the dual-component gating that is observed in several Kir channels. Recordings were obtained by cell-attached patch-clamp of *Xenopus* oocytes expressing Kir3.2 V188G mutant channels (holding potential –100 mV). Channel openings are represented as upward deflections. The lower trace shows the underlined part of the upper trace at an expanded timescale. Modified, with permission, from REF. 28 © (2001) Elsevier Science. Panel **a** was prepared using WebLab ViewerPro 3.5, and panels **b** and **c** using VMD[137], with futher modifications.

ENERGY WELLS
Discrete sites along the conduction pore of the channel, which are energetically favourable. These sites arise from a delicate balance between interactions with the channel atoms, water in the channel and other ions. Wells are separated by barriers, which hinder diffusion. When the energy wells are low in energy compared with the barriers, the residence time of ions at these positions is long.

PHOSPHATIDYLINOSITOL-4,5-BISPHOSPHATE
($PtdIns(4,5)P_2$). An anionic phospholipid found at low concentrations in biological membranes. It acts as a membrane-delimited second messenger, regulating the activity of a number of transporters and channels.

PDZ DOMAIN
A peptide-binding domain that is important for the organization of membrane proteins, particularly at cell–cell junctions, including synapses. They can bind to the carboxy termini of proteins, or can form dimers with other PDZ domains. PDZ domains are named after the proteins in which these sequence motifs were originally identified (PSD95, Discs-large, zona occludens-1).

RUNDOWN (OR WASHOUT)
Decrease in channel activity over time. Loss of phosphorylation and decrease of the levels of $PtdIns(4,5)P_2$ and ATP have been suggested to cause rundown, but other processes that are not yet understood might occur.

PHOSDUCIN
A phosphoprotein that modulates the phototransduction cascade by interacting with the βγ-subunits of the retinal G-protein transducin.

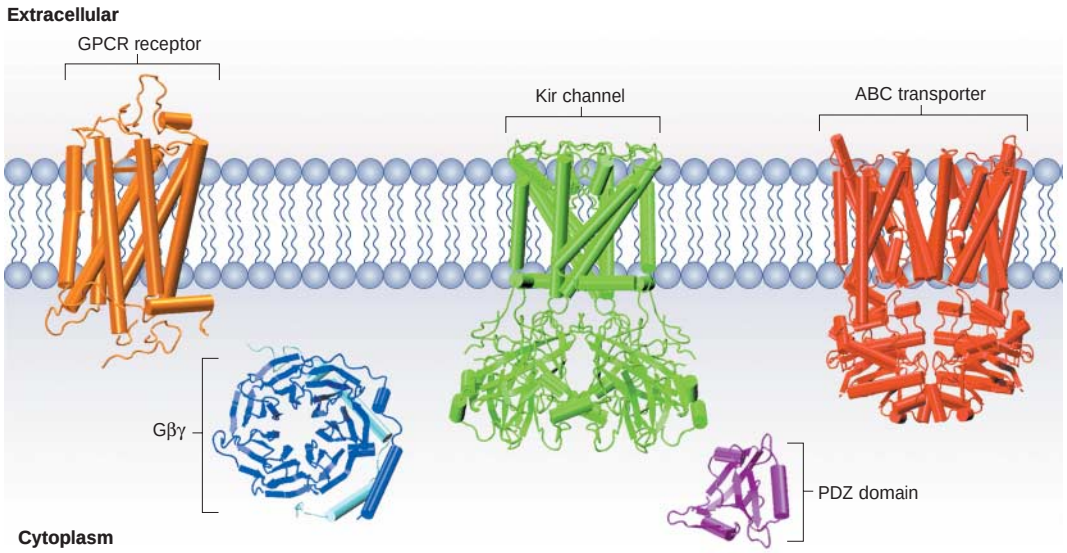
regard to $PtdIns(4,5)P_2$, Gβγ, Na^+, proton and ATP binding to Kir channels.

$PtdIns(4,5)P_2$ enhances the currents of Kir channels; conversely, its depletion from excised patches is thought to underlie channel RUNDOWN[86–90]. The binding of $PtdIns(4,5)P_2$ seems to depend on basic residues in the N- and C-terminal domains. The first to be identified was a conserved arginine in a stretch of positively charged residues (KKR) just beyond M2 (REFS 86,91–93). A second $PtdIns(4,5)P_2$-interacting domain in the proximal C terminus was identified using chimaeras of Kir2.1 and 3.4, which show different sensitivities to $PtdIns(4,5)P_2$ (REF. 94, see also REFS 91,93). These residues can functionally replace the putative $PtdIns(4,5)P_2$-binding domain of the transient receptor potential channel TRPV1[95]. Mutations of two N-terminal residues also reduce $PtdIns(4,5)P_2$ binding and therefore channel activity[93,96]. Mapping the residues that are implicated in $PtdIns(4,5)P_2$ binding onto the KirBac structure shows that a pocket that lies just below the membrane is the most obvious location for a $PtdIns(4,5)P_2$-binding site (FIG. 6).

Kir3 channels are gated by Gβγ, which is released on activation of G-protein-coupled receptors[97,98]. Gβγ binds directly to a region of the C terminus (residues 273–462) and the N terminus (1–85) of Kir3.1 (REF. 99). Specifying the binding site for Gβγ proved difficult and many regions in the intracellular domain have been implicated in Gβγ binding[100–105]. On the basis of the structure of the intracellular domain of Kir3.1, Nishida and MacKinnon[11] suggested that an α-helix towards the end of the C terminus mediates the interaction with Gβγ in a manner similar to that found for Gβγ and PHOSDUCIN. As Gβγ also contains several distinct domains that interact with Kir3 channels[106], the two probably interact through several contact points.

Several Kir channels are inhibited by intracellular acidification[107,108]. Kir1.1 and Kir2.3 are protonated at a number of C-terminal histidines[109,110] (FIG. 6). A lysine in M1 (K80 in Kir1.1) was also proposed as a protonation site[111], but its location in the membrane makes it more likely that K80 relays the effects of protonation to the gate[107] (FIG. 6). The finding that pH regulates the binding between the N and C termini of Kir2.3 might indicate

Box 2 | Crystal structures of inwardly rectifying K^+ (Kir) channel subunits and modulators



To the left of the KirBac1.1 structure (green, 1P7B[4]), Gβγ (blue and cyan, 2TRC[132]) and rhodopsin (orange, 1F88 (REF. 133)), an example of a G-protein coupled receptor (GPCR) are shown. To illustrate the interaction between some Kir channels and PDZ domains, the third PDZ domain of PSD-95 (purple, 1BFE[134]) is depicted below KirBac. Functional K_{ATP} channels are composed of four Kir6 subunits and four sulphonylurea receptors[135], which share sequence homology with the *Escherichia coli* ABC transporter BtuC (red, 1L7V[136]). The structures are drawn to scale to allow size comparison. The approximate thickness of the membrane is indicated. Molecular drawings were prepared using VMD[137].

that this interaction could translate the effect of protonation into gating movements of the transmembrane domains[110].

Binding of intracellular ATP closes Kir6.2 channels[112]. Three C-terminal residues and one N-terminal amino acid have been implicated in ATP binding[85,91,112–116] (FIG. 6). A model of Kir6.2 that is based on the Kir3.1 structure shows that the C-terminal residues form an ATP-binding pocket[116]. The N-terminal residue R50 is not resolved in the Kir3.1 structure. Interestingly, the KirBac structure shows this residue as part of the β-strand that interacts with the C terminus (FIG. 6). This observation, in combination with the model proposed for pH gating, raises the question of whether modulation of the interaction between the N and C terminus is a general mechanism for gating of Kir channels by intracellular modulators.

In the physiological setting, several cytoplasmic regulators modulate channel activity at the same time. Studies that explored the combined effects of cytoplasmic regulators found that they can act in an additive, synergistic or competitive manner. In some cases the interplay might be based on binding of modulators to neighbouring sites (FIG. 6). For example, Na^+, which activates Kir3.2 channels[87], binds to an aspartate in close proximity to the PtdIns(4,5)P_2-binding site, indicating that shielding of the negatively charged aspartate by Na^+ might increase the affinity of the channel for PtdIns(4,5)P_2 (REFS 117,118). A similar mechanism might be at work in the case of activation of the Kir6 channel by protons, which add a positive charge to a histidine next to the PtdIns(4,5)P_2-binding site[119]. Arachidonic acid inhibits Kir3.2 and Kir3.4 channels by binding in the proximity of the Na^+-binding site, which might lead to competition between PtdIns(4,5)P_2 and arachidonic acid for binding in the same region[120]. Another example of competition between PtdIns(4,5)P_2 and other intracellular modulators is seen in the case of ATP binding to Kir6.2 channels[121,122]. Kir3 channel activity is modulated by an interplay between Gβγ, PtdIns(4,5)P_2, Na^+ and Mg^{2+} (REFS 86–88,123). Future work defining exactly where Gβγ binds will help to build a model for the activation of Kir3 channels by Gβγ and its relationship to other modulators.

Coupling channel activation to opening

K^+ channels could open and close at the bundle crossing and/or the selectivity filter. Which gate responds to intracellular modulators? Several studies have tested whether gating mutations in the pore loop or at the bundle crossing can mimic the effect of a modulator on channel activity. The effects of ATP and Gβγ on Kir6.2 and Kir3 channels, respectively, are occluded by mutations in the lower M2 helices[28,81,84]. By contrast, Kir3 channels with gating mutations in the pore helix, though already more active than wild-type channels, can be further activated by Gβγ application[124]. These experiments indicate that modulators of Kir channels might affect primarily the slow gate that is located at the bundle crossing. However, other experiments indicate that the selectivity filter, rather than the bundle crossing, forms the gate that is regulated by cytoplasmic modulators[65,125,126]. Some of the differences in the interpretation of data concerning the location of the gate that is affected by modulators might arise from different definitions of

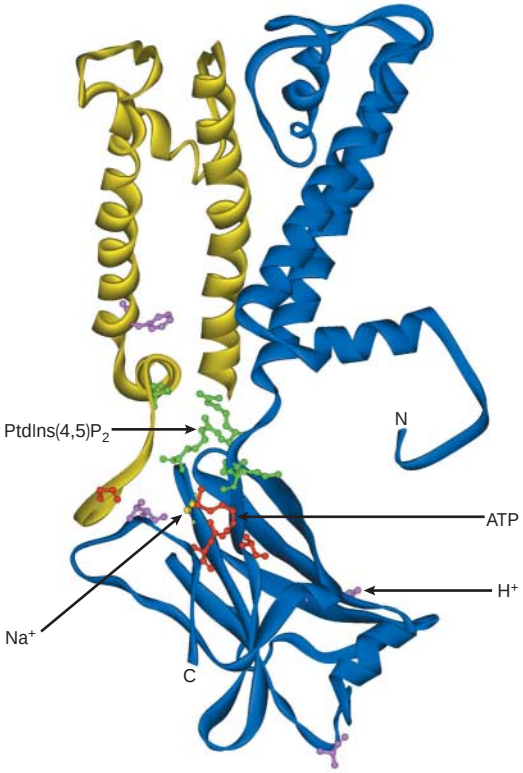


Figure 6 | **Modulation of inwardly rectifying K^+ (Kir) channel activity by intracellular regulators.** Residues of mammalian Kir channels that are implicated in the binding of intracellular regulators, mapped onto the KirBac1.1 structure. A complete subunit (blue) of KirBac1.1 and the amino (N) terminus and transmembrane domain of the adjacent subunit (yellow) are shown from the side. In green are residues that have a role in phosphatidylinositol-4,5-bisphosphate (PtdIns(4,5)P_2) binding (Kir1.1 I63/Kir2.1 R67/Kir6.2 R64(S46), Kir1.1 K186/Kir2.1 K187(R153), Kir2.1 K188/Kir6.2 R176 (A154), Kir1.1 R188/Kir2.1 R189/Kir6.2 R177(K155) and Kir1.1 R217/Kir2.1 R218/Kir6.2 R206(R181)), in red are residues that are involved in ATP binding to Kir6.2 (R50(G42), I182(M157), K185(R160) and G334(N304)), the pink residues are involved in pH gating of Kir1.1 (K80(F63), H225(R189), H274(E234) and H342(T301)), and the yellow residue (Kir3.2 D228/Kir3.4 D223(N179)) forms the binding site for sodium. C, carboxy. Molecular drawings were prepared using WebLab ViewerPro 3.5.

the term 'gate'. It can encompass any structural determinants that are involved in opening and closing the channel, or it can refer strictly to the physical barrier to the flow of K^+.

Based on the available data that indicate movements at both the bundle crossing and the selectivity filter, gating in response to modulator binding could involve one or two physical gates. In the one-gate models, either the selectivity filter or the bundle crossing obstructs K^+ flow. If the bundle crossing is the gate, activation of the channel would correspond to a widening of the conduction pathway at the bundle crossing, which then unmasks the ongoing movement of the selectivity filter. If the selectivity filter is the physical gate, modulator binding would induce bursts of opening at the selectivity filter, possibly transduced by a movement at the bundle crossing. The two-gate model proposes that the gates at the selectivity filter and the bundle crossing both open during channel activation by modulators. This could happen as a concerted movement of the whole channel or in a wave that successively opens one and then the other gate.

How is the activation signal transmitted from the cytoplasmic domain to the transmembrane domain? The answer to this question remains mostly speculative. There is some evidence that modulator binding causes rearrangements of the cytoplasmic domains. For example, two cysteines in the N and C termini of Kir1.1 are accessible to thiol reagents only in the closed state, indicating that the cytoplasmic domain undergoes a conformational change during pH gating[127]. Moreover, the distance between FLUORESCENCE RESONANCE ENERGY TRANSFER (FRET) probes attached to the N and C termini of Kir3.1/4 changes during stimulation by $G\beta\gamma$[128]. This movement was interpreted as a rotation and expansion of the N and C termini, reminiscent of the gating motions that have been proposed for the intracellular RCK domains of MthK[8]. The missing link is how these movements translate into a conformational change in the transmembrane domain of the channel. The motion could be transduced from the C terminus to M2 and/or from the N terminus to M1. The KirBac structure encourages the view that transduction occurs through M1 because it has an amphipathic helix, the slide helix, at the junction between the N terminus and M1, which seems to be well placed to transduce the force[4]. In addition, the linker between the C terminus and M2 was suggested to be flexible[4], which would be unexpected if this region propagates the force from the C terminus to M2. Although the structure forms a starting point in the search for the missing link, future functional and structural studies are needed to resolve this issue.

Conclusion

The cloning of the first Kir channels, ten years ago, opened the opportunity to study these channels in isolation in heterologous systems. We have learned a great deal about their function and regulation from studies of a large number of mutant and wild-type channels under defined experimental conditions. Recently, high-resolution structures of several K^+ channels have become available. These snapshots facilitate both the design and the interpretation of mutagenesis studies of channel function. The next challenge will be to illuminate the dynamic processes that underlie the inner workings of these fascinating miniature machines. The power of the multi-disciplinary approach, combining experimental manipulations, high-resolution structures of ion channels in various physiologically important conformations, and computational studies, will provide answers to these exciting questions. In parallel, the information that is gained from the study of ion channels at the single molecule level, when placed into the cellular context of signalling complexes, will provide valuable insight into how ion flow is regulated in response to hormones, transmitters and second messengers, as well as the metabolic state of the cell.

FLUORESCENCE RESONANCE ENERGY TRANSFER (FRET). A spectroscopic technique that is based on the transfer of energy from the excited state of a donor moiety to an acceptor. The transfer efficiency depends on the distance between the donor and the acceptor. FRET is often used to estimate distances between macromolecular sites in the 20–100-Å range or to study interactions between macromolecules *in vivo*.

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Competing interests statement

The authors declare that they have no competing financial interests.

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