

# Controlling potassium channel activities: Interplay between the membrane and intracellular factors

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Neural signaling is based on the regulated timing and extent of channel opening; therefore, it is important to understand how ion channels open and close in response to neurotransmitters and intracellular messengers. Here, we examine this question for potassium channels, an extraordinarily diverse group of ion channels. Voltage-gated potassium (Kv) channels control action-potential waveforms and neuronal firing patterns by opening and closing in response to membrane-potential changes. These effects can be strongly modulated by cytoplasmic factors such as kinases, phosphatases, and small GTPases. A Kv  $\alpha$  subunit contains six transmembrane segments, including an intrinsic voltage sensor. In contrast, inwardly rectifying potassium (Kir) channels have just two transmembrane segments in each of its four pore-lining  $\alpha$  subunits. A variety of intracellular second messengers mediate transmitter and metabolic regulation of Kir channels. For example, Kir3 (GIRK) channels open on binding to the G protein  $\beta\gamma$  subunits, thereby mediating slow inhibitory postsynaptic potentials in the brain. Our structure-based functional analysis on the cytoplasmic N-terminal tetramerization domain T1 of the voltage-gated channel, Kv1.2, uncovered a new function for this domain, modulation of voltage gating, and suggested a possible means of communication between second messenger pathways and Kv channels. A yeast screen for active Kir3.2 channels subjected to random mutagenesis has identified residues in the transmembrane segments that are crucial for controlling the opening of Kir3.2 channels. The identification of structural elements involved in potassium channel gating in these systems highlights principles that may be important in the regulation of other types of channels.

Potassium channels decide whether and when to open by integrating signals from multiple directions. Incoming neurotransmitters can affect potassium channel gating by acting on ionotropic receptors, ligand-gated ion channels that alter the membrane potential. Alternatively, neurotransmitters can act on metabotropic receptors that mobilize G proteins and downstream second messengers that interact with cytoplasmic domains of potassium channels to modify gating. Apart from the rapid (millisecond) responses of potassium channels to changes in the membrane potential, second messengers and other cytosolic factors that modulate potassium channels usually exert slower and longer-lasting effects important for fine-tuning neural signaling (1).

Potassium channels are not only extremely low in abundance on the cell membrane, but also extraordinarily heterogeneous *in vivo*. Cloning of potassium channel genes is one approach to studying the function and regulation of individual channel types. We first cloned the *Shaker* voltage-gated potassium channel gene in *Drosophila* (2), and then cloned its mammalian homolog, Kv1.1 (3), thanks to the strong sequence conservation between vertebrate and invertebrate potassium channels. Kv1.1 turns out to be encoded by the first potassium channel gene associated with a disease; mutations of the Kv1.1 gene have been found to cause episodic ataxia type 1 (EA-1) (4). When induced by startle or sudden movements, EA-1 patients exhibit jerking movements

and shaking limbs that bear an uncanny similarity to the *Shaker* phenotype. In the past decade the voltage-gated potassium (Kv) family of potassium channels has grown considerably in number and type. The physiological importance of these potassium channels is evident from the diseases due to mutations of Kv channels, ranging from epilepsy and deafness, to cardiac arrhythmia (5–7).

The large number of Kv family members and their ability to coassemble to form heteromultimeric channels (8), however, cannot fully account for the diversity of potassium channels. For example, the muscarinic potassium channels that mediate the calming effect of acetylcholine on the heartbeat (9), and the ATP-sensitive potassium channels that control insulin release from the pancreas (5), could not be isolated based on their sequence similarity to Kv channels. These potassium channels resemble the inward rectifier potassium channels in neurons, muscles, and other nonexcitable cell types. Steve Hebert's group and our group, therefore, resorted to expression cloning to isolate the first inwardly rectifying potassium (Kir) channels Kir1.1 and Kir2.1 (10, 11). Now the Kir family has approached the Kv family in size and complexity (12) and includes known disease genes responsible for hypertension (Bartter's Syndrome) and unregulated insulin release (Persistent Hyperinsulinemic Hypoglycemia of Infancy) (5).

Mechanistic studies of potassium channel function and regulation will contribute to our understanding of how the myriad of potassium channels *in vivo* might respond to physiological inputs in neural signaling. Moreover, potassium channel blockers and openers have been developed for the purpose of combating convulsion, arrhythmia, or diabetes (5, 13–18). A better understanding of the potassium channel domains that mediate channel modulation by second messengers, as well as the conformational changes that accompany channel opening and closing, may facilitate future development of use-dependent drugs that affect potassium channels according to their recent and imminent activities.

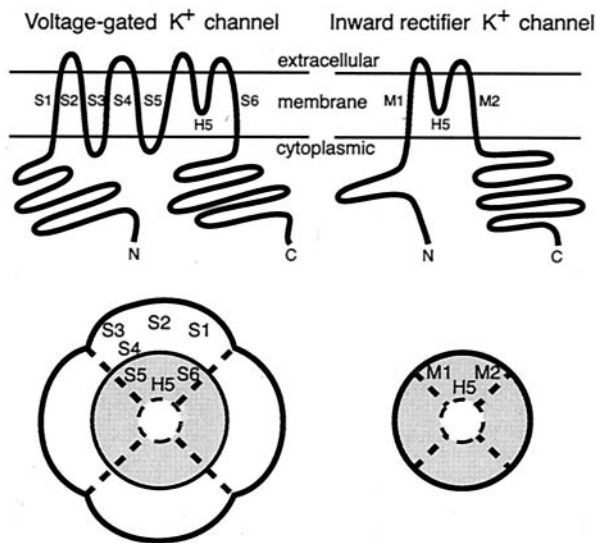
Before considering the issues of channel regulation, a brief review of the basic channel structure is in order. Site-directed mutagenesis and heterologous channel expression have been used extensively to identify structural elements involved in specific channel functions. These studies have unveiled the general blueprint for basic channel design (Fig. 1; ref. 12). As predicted by classical biophysical studies, Kv channels are intrinsically sensitive to membrane potential because of the presence of voltage sensors built into the protein (19, 20). By

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Abbreviations: Kv, voltage-gated potassium; Kir, inwardly rectifying potassium; KcsA, bacterial potassium channel from *Streptomyces lividans*.

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**Fig. 1.** Voltage-gated potassium (Kv) channels and inwardly rectifying potassium (Kir) channels belong to two distantly related families. Common to both potassium channel families is the pore-forming structure comprised of two transmembrane segments and the H5/P loop in between, in each of the four  $\alpha$  subunits. Kv but not Kir channels contain intrinsic voltage sensors, which correspond primarily to the S4 segment with basic residues at every third position. [Reproduced with permission from ref. 11 (Copyright 1993, MacMillan Magazines, Ltd., www.nature.com).]

contrast, Kir channels display voltage dependence, although they lack voltage sensors because these channels are blocked from the inside by cations like magnesium and polyamines that are normally present in all cells (21). Thus, potassium ions flow through Kir channels more readily into the cell than out of the cell, also known as “inward rectification.” Both Kv and Kir channels are tetramers (22, 23). Between the last two transmembrane segments of Kv channels and the two transmembrane segments of Kir channels is a highly conserved P region that has been implicated along with the last transmembrane segment in forming the potassium-selective pore. Crystallographic determination of the bacterial potassium channel, KcsA, revealed that the P region consists of a pore-helix and a pore-loop that contributes to the narrow potassium selectivity filter of the channel pore (24).

The intrinsic voltage sensor of Kv channels corresponds primarily to the fourth transmembrane segment (S4), which contains basic residues at every third position in an otherwise hydrophobic segment that spans the membrane. In recent studies, fluorescent probes were attached to the S4 segment of voltage-gated sodium channels and potassium channels to detect the movements of S4 relative to its surroundings as the channel undergoes voltage-induced conformational changes (25–29). Remarkably, several residues on S4 that face the cytoplasm in the closed channel become buried in the membrane or exposed to the outside of the cell in the open channel. This change in accessibility is commonly interpreted as an outward movement of the S4 segment, but the actual motions may also involve rotation and tilting of S4 when the electrical potential on the cytoplasmic side of the membrane becomes more positive (depolarization). How this motion of S4 causes Kv channels to open is not known, although it presumably prompts the movements of structures that form the pathway for ions to flow through the channel. The extent to which Kv and Kir channels share common mechanisms in channel regulation is unknown; however, both types are amenable to modulation by cytoplasmic factors (1), and the gross design of their channel pores appears to be similar (11, 30). Detailed electron paramagnetic resonance studies of the

activation of KcsA (31) and cysteine scan mutagenesis studies of Kv channels (32) suggest that some sort of conformational change occurs in the intracellular end of the pores of both of these channels as the gates open. Despite the tantalizingly rapid progress of this field, several aspects of channel gating remain mysterious. Given the relatively hidden location of the pore-lining structures, how do various intracellular signals influence channel activity? And how does a channel move as it opens?

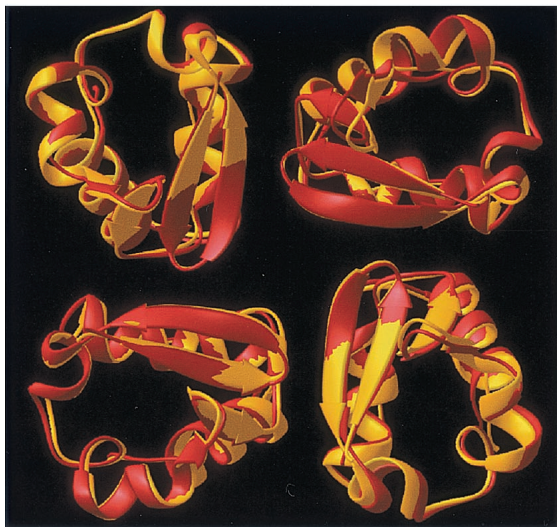
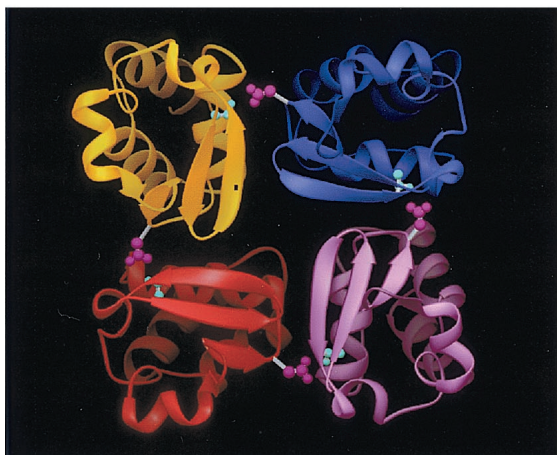
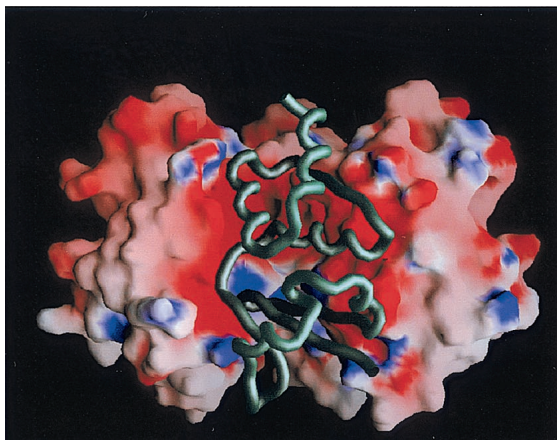
We explored these issues by examining the role of a highly conserved cytoplasmic domain in modulating the voltage gating of Kv channels (33). We also used yeast mutant screens to probe at the different conformations of the open and closed channel of a class of Kir channels modulated by G protein (34). Our studies indicate that Kir channel gating involves the transmembrane domain near the inner end of the pore as well as the P region near the selectivity filter. If the conformational changes during channel opening include cytoplasmic domains, such as the T1 tetramerization domain of Kv channels, then channel modulation may be mediated by interactions between cytoplasmic domains and second messengers that shift the relative stability of the open and the closed conformation of the channel.

### T1: A Multifunctional Domain of Kv Channels?

The N-terminal T1 domain is best known for its role in sorting different Kv channel subunits and initiating their assembly (35–37). Biogenesis of Kv channels proceeds from the N terminus with the tetramerization of the T1 domains followed by the packing of the transmembrane segments and finally the folding of the C-terminal cytoplasmic domains around the N-terminal domains (38). With the exception of hyperexpression, Kv channel  $\alpha$  subunits that lack the T1 domain cannot achieve a local concentration high enough for channel assembly (39). Numerous subfamilies of Kv channels have been characterized in vertebrates and invertebrates. Members of the same subfamily can coassemble and form heteromeric channels (40). The heterogeneity of potassium channels *in vivo* is greatly enhanced by the mix and match of different Kv subunits with different channel properties. The x-ray crystal structure of the T1 domain provides a physical explanation for why only members of the same subfamily are able to coassemble: the T1 interface contains structural determinants that make it compatible only with other members of the same subfamily (35).

The T1 tetramer most likely remains in mature Kv channels on the cell membrane (41). Does the T1 domain merely provide a physical platform for channel assembly or could it also be a receptor for regulatory molecules and be somehow involved in channel gating? Extensive studies by Peralta’s group of one Shaker family member, Kv1.2, reveal that channel inhibition by the m1 muscarinic acetylcholine receptor is due to tyrosine phosphorylation of the channel (42). Moreover, Kv1.2 channel is modulated by a small GTPase and tyrosine phosphatase that physically interact with its N-terminal T1 domain (43, 44), a domain also known for its role in initiating Kv channel assembly and determining the compatibility of subunit interactions (35–38).

An unusual feature of the T1 tetramer is the highly polar interface between T1 monomers (refs. 33 and 45; Fig. 2). The stability of most protein complexes derives largely from the burial of exposed hydrophobic residues in the interface between proteins (46, 47). Is there an evolutionary advantage to having the T1 interface occupied by mostly polar residues that are highly conserved among Kv channel family members? To explore this issue, we replaced Kv1.2 residues at the T1 interface, one at a time, with alanine or with more conservative amino acids where alanine substitution resulted in nonfunctional channels (33). Some of these substitutions had effects on voltage-dependent channel gating, whereas others did not. Many of the residues that did affect gating were situated across complementary surfaces of



**Fig. 2.** Mutation of the polar interface between the Kv1.2 tetramerization domain T1 stabilizes the closed state relative to the open state without altering the appearance of the T1 tetramer. (*Top*) Molecular surface and electrostatic potentials for the T1 tetramer: cutaway view from the side with one of the four subunits depicted in RIBBONS. (*Middle*) RIBBONS (75) depiction of the T1 tetramer viewed from the N-terminal side showing the buried threonine 46 (T46) and its hydrogen bond partner aspartate 79 (D79). (*Bottom*) RIBBONS depiction of the superpositions for the wild-type (yellow) and T46V mutant (red) T1 tetramer, both crystal structures solved to a resolution of 1.6 Å, based on a comparison of  $C_{\alpha}$  atoms. [Adapted with permission from ref. 33 (Copyright 2000, Elsevier Science).]

the T1 interface forming “hot spots” on neighboring T1 monomers. Surprisingly, almost all of these substitutions stabilized the closed state relative to the open state of the channel indicating that the polar interaction between T1 monomers is not designed for maximal stability of the closed channel. Instead, the polar interface of the T1 domain of Kv channels may be balanced to accommodate some sort of conformational change that happens in the whole channel at membrane potentials most suitable for neuronal signaling.

#### Valine Substitution for a Buried Threonine at the T1 Interface Stabilizes the Closed Channel Without Altering the Surface of the T1 Tetramer

A detailed analysis of one mutation at the T1 interface, T46V, of Kv1.2 suggests how the T1 domain might regulate gating. The isosteric T46V mutation disrupts two hydrogen bonds that span the T1 interface between T46 on one monomer and D79 on the neighboring monomer. Aside from minor displacements of the residue at position 46 and its immediate neighbors buried at the interface, crystallographic studies revealed identical structures for the wild-type and the T46V mutant T1 tetramers (ref. 33; Fig. 2). However, Kv1.2 T46V channels exhibit a slower rate of activation and a + 24.3 mV shift in the midpoint of channel activation, indicating that T46V stabilizes the closed state of the channel. Moreover, isolated tetramers of the T46V mutant are significantly more stable than the wild-type T1 domain (33). If the T1 domains were retained in the same conformation in the open and the closed Kv channel, altering the interactions between the T1 domains that stabilize or destabilize the tetramer should not have affected channel function. The stabilization of the closed state by the T46V mutation suggests that channel opening may be accompanied by conformational changes at the interface between T1 monomers and possibly between the T1 tetramer and the rest of the channel itself. Exactly when these conformational changes at the T1 interface take place—during the rotation and translation of S4 or opening of the activation gates in the pore—and the extent of the conformational changes in this part of the channel remain to be determined.

Our studies of the T1 domain suggests a mechanism by which cytoplasmic factors can modulate channel gating. If conformational changes of cytoplasmic domains accompany the conformational changes of the transmembrane domains, one way for cytoplasmic factors to modulate channel activity would be to stabilize one of the conformations of the cytoplasmic domains, thereby stabilizing either the open or the closed channel. In one sense, this scenario is analogous to current models for cyclic nucleotide channel gating. Instead of supposing that the cyclic nucleotide binds to a receptor and triggers conformational changes that open the channel, it appears likely that cyclic nucleotide-gated channels are capable of opening on their own; ligand-binding may open channels by simply shifting the equilibrium toward the active conformation (48). The cytoplasmic domains of these and many other voltage-gated channel members are sensitive to the binding of molecules that affect channel opening (43, 44, 49, 50). The discovery that the cytoplasmic domains can affect Kv channel gating suggests that Kv channels share this common feature with other voltage-gated channel superfamily members like cyclic nucleotide-gated channels (51), calcium-sensitive potassium channels (52), and hyperpolarization-activated channels (53–55). It would be of interest to explore the possibility of common modes of coupling between the cytoplasmic and transmembrane domains of these different ion channels.

#### Functional Studies of Inwardly Rectifying Potassium Channels in Yeast

The Kir family contains both potassium channels that are active most of the time and potassium channels whose activity are

acutely sensitive to transmitters and the internal metabolic state, thereby altering the membrane potential and the excitability of the cell (1, 5, 12, 21). For example, Kir2.1 (IRK1) channels are constitutively active and exhibit an open probability near one, whereas Kir3 (GIRK) channels activate in the presence of the  $\beta\gamma$  subunits of G proteins mobilized by metabotropic transmitter receptors (9, 56, 57). Cytoplasmic factors may regulate channel activity by modulating their interaction with the phospholipid PIP<sub>2</sub>. Kir channels that are constitutively active tend to exhibit a high affinity for PIP<sub>2</sub>, whereas Kir channels that are modulated by cytoplasmic factors have low intrinsic affinity for PIP<sub>2</sub> (58, 59). Just how these channels alter their conformation as they open and close, however, is not known.

There are precedents for ion channels opening by movements of extracellular, intracellular, or transmembrane domains of the channel. Therefore, a search for mutations in the entire channel sequence that alter the ability of a channel to open or close are preferable to strategies that target a small region, such as site-directed mutagenesis. The ability of Kir channels to rescue potassium transport-deficient yeast for growth in low potassium medium makes it possible to screen hundreds of thousands of randomly mutagenized channels for those that support potassium-selective permeation (60, 61).

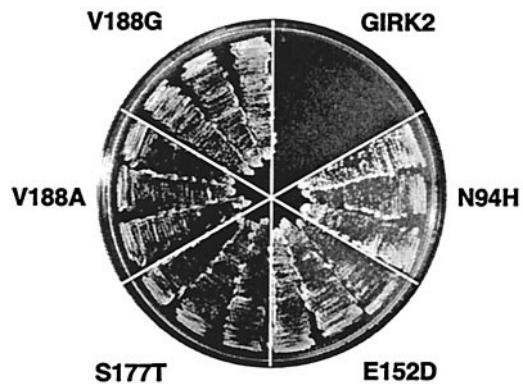
For Kir channels to functionally substitute for yeast potassium transporters, these channels have to be sufficiently active to support potassium uptake at a level necessary for yeast growth. Kir2.1 (IRK1) with an open probability close to one can rescue mutant yeast (60, 61), but Kir3.2 (GIRK2) channels cannot because they open rarely in the absence of mammalian G protein  $\beta\gamma$  subunits (34). Therefore, mutations in Kir3.2 that stabilize the open channel and increase the probability of opening are, in principle, one class of mutations that we expected from this screen. Interestingly, all of the mutations we identified in our screen turned out to affect the gating of Kir 3.2 channels.

#### DNA Shuffling, Yeast Screens, and *in Vitro* Backcrosses to Isolate GIRK2 Gating Mutants

We used the DNA shuffling method of Stemmer (62) to introduce random mutations into Kir3.2. By cutting the Kir3.2 cDNA into pieces of 50–100 base pairs with DNase I and reassembling these pieces with *Taq* polymerase without added primers under low stringency conditions, we were able to generate as many as ten mutations per clone. A unique advantage of the DNA shuffling method is the ability to “backcross” the mutant cDNA with wild-type cDNA *in vitro* (63). Once active clones are found, this procedure permits one to quickly sort functional from spurious mutations when active clones contain multiple changes. After the initial mutagenesis and isolation of mutants that permitted yeast growth under low potassium conditions, we mixed cDNA from these active clones with an excess wild-type Kir3.2 cDNA and then repeated DNA shuffling under high stringency conditions. The relevant mutations for functional complementation could then be isolated via another round of growth selection.

From the first group of single mutants recovered, substitutions of V188 with alanine or glycine each emerged from at least six independent clones, whereas mutations of three other residues, N94, E152, and S177, were each represented by a single clone (ref. 34; Fig. 3). The mutant screen was far from saturation. Nonetheless, it is remarkable that all mutations identified thus far affect residues in the transmembrane domains even though Kir3.2 channel activity is normally regulated by cytoplasmic factors.

All of the mutations recovered from our yeast screen are gating mutations that increase the activity of Kir3.2 channels. Single-channel analysis of Kir3.2 channels expressed in *Xenopus* oocytes indicated that one pair, E152D and S177T (the “outer pair,” because those residues are closer to the extracellular



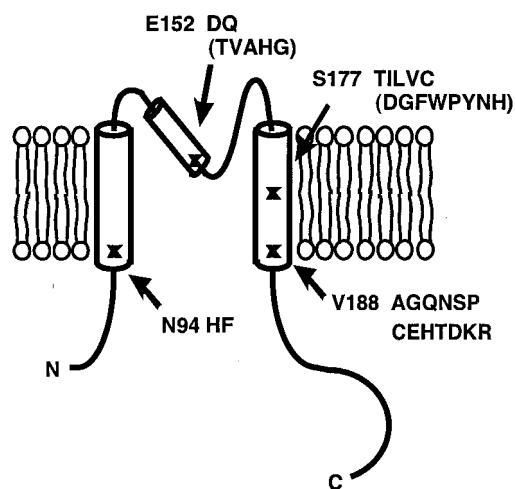
**Fig. 3.** Growth phenotype of yeast expressing wild-type Kir3.2 (GIRK2) or single mutants identified by selection among a randomly mutagenized Kir3.2 library, on a plate supplemented with 0.1 mM KCl. [Reproduced with permission from ref. 34 (Copyright 2001, Elsevier Science).]

surface), yielded similar phenotypes, increasing the channel open time by 3-fold and introducing substate openings. On the other hand, a pair of mutations closer to the cytoplasmic side of the membrane, N94H and V188G (the “inner pair”), caused Kir3.2 channels to exhibit prominent bursts of channel opening in the absence of active G protein subunits. These mutations thus appear to stabilize the channel in a high activity mode that is rarely visited by the wild-type channel unless it is exposed to G protein  $\beta\gamma$  subunits (34).

The N94H and V188G mutations alter residues near the beginning and the end of the transmembrane domain, and yet cause similar stabilization of the high activity mode of Kir3.2 channels. Could it be that they affect a common physical entity in the channel? We explored this possibility in double mutant studies (34). The open probability of double mutants carrying substitution of one residue from the outer pair, E152D, and another gating mutation of one residue from the inner pair, V188G, is the sum of those for the two single mutants, indicating that they affect different gating processes. By contrast, the gating properties of the N94H V188G double mutant of the inner pair were similar to those channels carrying either single mutation alone. Moreover, V188I, a mutation that reduces the basal activity but still allows channel to be activated by G protein  $\beta\gamma$  subunits, suppresses the N94H gating mutation in both the yeast growth assay and single-channel analysis. These strong interactions between the inner pair of N94 and V188 indicate that they are involved in the same aspect of channel gating. Hints for their role in channel gating emerged from structural considerations as well as studies of the ability of each of the 20 amino acids to occupy these positions in the open or the closed channel.

#### Patterns of Tolerance for Amino Acid Substitutions Indicate That Both S177 and V188 of the M2 Helix Face the Water-Filled Pore in the Open but Not the Closed Channel

To learn about the possible roles played by the inner pair and the outer pair in channel gating, we introduced all 20 amino acids into each of the four positions affected by the gating mutations (34). Only a small subset of these amino acids can substitute N94 of M1 and E152 in between M1 and M2, in either the open channel or the closed channel. This pattern indicates that these two residues are buried within the channel protein. Remarkably, all 20 amino acids can replace the two M2 residues, S177 of the outer pair and V188 of the inner pair, and allow the open channel to conduct ions although certain S177 substitutions abolish potassium selectivity (Fig. 4). Very few of these mutations, however, are compatible with the conformation of the closed channel; most of the mutations render the channel constitutively open (ref. 34; Fig. 4). The pattern of tolerance of these two M2



**Fig. 4.** A schematic representation of a Kir3.2 (GIRK2) subunit showing mutations of the inner and the outer pair that result in constitutive channel opening (34). Residues in parenthesis represent substitutions that cause a loss of potassium selectivity.

residues indicates that, in the closed channel, V188 is buried in a hydrophobic pocket that accepts only hydrophobic residues of a certain range of sizes. Likewise, the interactions between S177 and its surroundings are even less tolerant of side chain alterations. As the channel opens, however, both M2 residues must be facing the pore, so that substitution with polar and even charged amino acids is compatible with ion permeation and the integrity of the open channel. It thus appears that the conformation of the open Kir3.2 channel differs substantially from the conformation of the closed channel. What might these two channel conformations be like?

#### Yeast Functional Screens Predict a Model for Packing the Transmembrane Segments of the Open Kir2.1 Channel

The similar gating phenotypes of the outer pair and the strong interaction between the inner pair of residues could provide some clues. Conceivably, gating mutations of these residues may disrupt crucial interactions that hold the channel in the closed conformation, thereby causing constitutive activation. One possible explanation for the similar gating phenotypes of the inner pair and the outer pair, respectively, is that residues of each pair are in physical proximity in the closed channel, so that their mutations affect the same gating process.

For potassium channels, we were confronted with two distinct structural models: the x-ray crystal structure of the bacterial channel KcsA (24) and our model for Kir2.1 (IRK1) based on a mutational analysis of functional Kir2.1 channels selected from yeast (60). In the KcsA structure, the M2 helices from the four subunits make subunit–subunit contacts and line the pore below the selectivity filter formed by the pore loops. The M1 and M2 from the same subunit contact each other and are arranged like an antiparallel coiled-coil. M1 is not engaged in subunit–subunit contacts (24). Structural constraints from our studies of Kir2.1 suggest that M1 and M2 are arranged in a similar way *within* the same subunit—that is, like a pair of antiparallel coils. However, other constraints from our selections also strongly suggest that M1 contacts M2 from the adjacent subunit. Therefore, the Kir2.1 arrangement places the M1 helices in the groove between two M2 helices, thereby suggesting a more compact quaternary structure than KcsA (60).

The Kir2.1 model has been subjected to multiple functional tests. Fourier analysis of the patterns of tolerance for substitutions of M1 and M2 residues indicates that both transmembrane segments are  $\alpha$  helices (60). The nature of the permitted

substitutions suggests helical faces that make lipid–protein, water–protein, and protein–protein contacts. The lipid facing positions were tested by sequence minimization experiments whereby all ten putative lipid facing M1 residues were found to tolerate simultaneous substitution with the hydrophobic residues phenylalanine, leucine, and alanine, but not the polar residue serine. Moreover, replacing all four M2 residues predicted to be lining the pore wholesale with aspartate, asparagine, alanine, or serine yielded functional channels (60).

A number of site-specific second-site suppressors have been isolated to further constrain the Kir2.1 model. Channels bearing a nonconservative mutation of a residue that is intolerant of substitution and predicted to be at a protein–protein interface are nonfunctional and cannot rescue the yeast. In the background of such lethal mutations in one transmembrane segment, a selection with channels having random mutations introduced into the other transmembrane segment identifies allele-specific second-site suppressors (60). The Kir2.1 model is constrained to have each of the allele-specific second suppressors on the face of the transmembrane helix adjacent to their respective lethal mutation (Fig. 5). By contrast, it was not possible to have physical proximity between the lethal mutations and their own second-site suppressors in a model based on the KcsA structure. Given that Kir2.1 channels have an open probability close to 1, and hence can functionally complement for potassium transport functions in yeast, the Kir2.1 model deduced from yeast mutant screens most likely corresponds to the conformation of an open Kir channel.

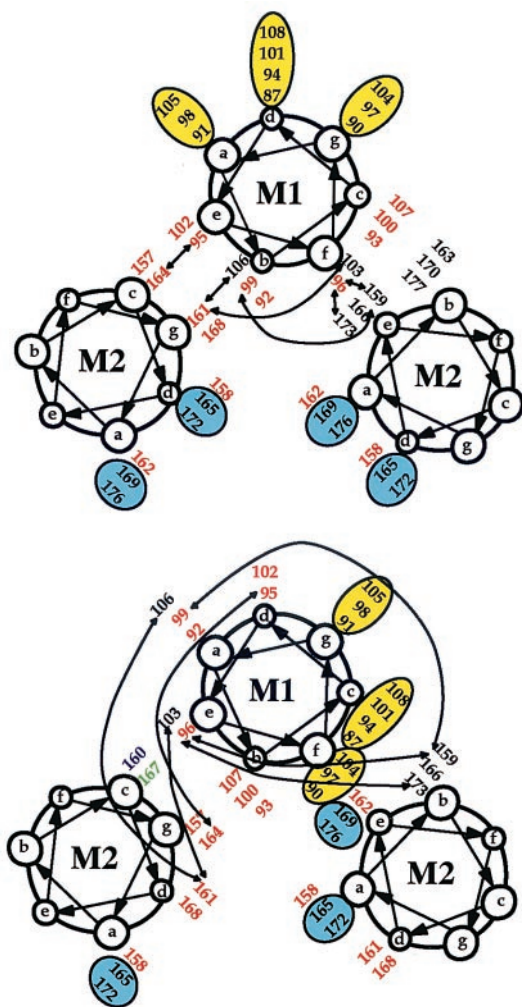
#### The KcsA Crystal Structure as a Model for the Closed Kir3.2 Channel

The reasoning that the channel in the KcsA structure is closed falls mainly along two lines (31, 64, 65). First, the crystals were grown at pH 7.5, which favors the closed conformation of the channel (31, 64). Secondly, site-directed spin labeling studies suggest that there is a significant change from the crystal structure on channel activation (31). Can the KcsA structure approximate the closed conformation of Kir3.2 channels? Relying on our sequence alignment that preserves the contacts between M1 and M2 helices within a subunit (60), we find that the inner pair of mutations and the outer pair each localize to a small region in KcsA. This physical proximity provides a plausible explanation for the similar gating mutant phenotypes of the inner pair and the outer pair, respectively (ref. 34; Fig. 6).

Of the outer pair, E152 of Kir3.2 corresponds to A73 near the end of the pore helix of KcsA, whereas S177 of Kir3.2 corresponds to the KcsA residue G99 in the immediate vicinity of A73. In other words, the outer pair of Kir3.2 represents neighbors in the KcsA model. This placement of the outer pair as immediate neighbors provides one plausible explanation why even the most conservative mutations of the outer pair, namely E152D and S177T, increase channel opening in a similar way. Given the proximity of the outer pair to the narrow passage of the channel pore at the selectivity filter, it is perhaps understandable that amino acid substitution of either residue of the outer pair often results in a loss of potassium selectivity (ref. 34; Fig. 4).

Of the inner pair, V188 of Kir3.2 corresponds to L110 of KcsA, an M2 residue buried at the interface between M2 helices of neighboring subunits, and N94 of Kir3.2 corresponds to H25 of KcsA. Both L110 and H25 contact W113 within the same subunit in the KcsA structure. It thus appears possible that the strong interaction between the Kir3.2 residues of the inner pair reflects direct as well as indirect involvement of these residues in securing the interaction between the subunit containing these two residues and one neighboring subunit.

In the KcsA model, all four Kir3.2 residues affected by gating mutations would be buried within the channel protein and interact



**Fig. 5.** Allele-specific second-site suppressors and sequence minimization of Kir2.1 (IRK1). Arrows indicate the positions of the suppressor mutations relative to the position of the nonfunctional mutant they suppress. The mutation and its suppressors were as follows: W96A was suppressed by F159I/A173P or F159V/A173P; F99A was suppressed by F159I; Q164S was suppressed by S95E; and V161W was suppressed by F103Y/I106N. Positions of the lipid-facing residues are highlighted in yellow and tolerate wholesale substitution with hydrophobic residues. Positions of the pore-lining residues are highlighted in blue and tolerate wholesale substitution with polar or charged residues. Restricted positions from the yeast selection experiments are indicated in red. For simplicity, only the relevant residue numbers are displayed. (Upper) Model for the open Kir2.1 channel. Intrasubunit M1/M2 interactions are conserved between this model for Kir2.1 and the KcsA structure, based on the sequence alignment shown in Fig. 6. (Lower) A different sequence alignment for KcsA and Kir channels (24) cannot account for the second-site suppression and sequence minimization results obtained for Kir2.1. [Adapted with permission from ref. 60 (Copyright 1999, Elsevier Science).]

with neighboring residues. Consistent with this prediction, only highly conservative substitutions of a subset of these four residues are compatible with the closed conformation of the channel. Thus, no substitution for N94 is compatible with the closed channel. And V188 can only be replaced with isoleucine, leucine, or phenylalanine without substantially increasing the basal current, suggesting that the residue at position 188 must fit into a hydrophobic pocket and secure the channel in the closed conformation (34).

### Clockwise Rotation of the Pore-Lining Transmembrane Helices to Open the Channel

Mutagenesis of S177 and V188, located on the same face of the M2 helix, indicates that both residues face the water-filled pore

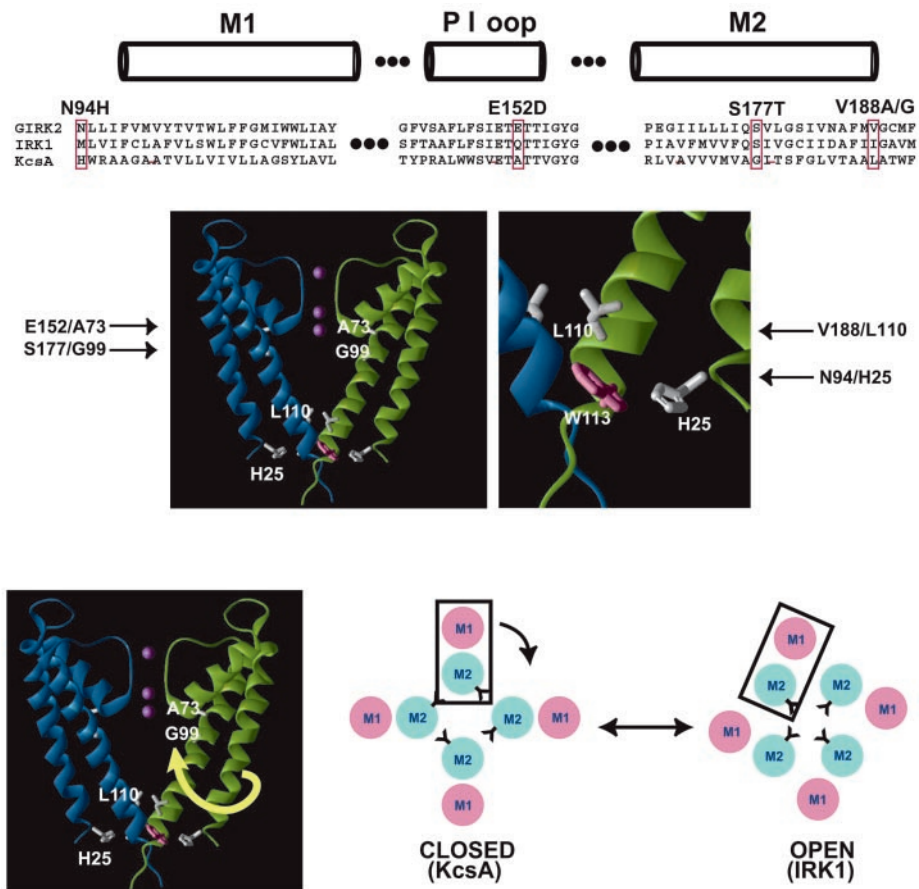
in the open channel. All 20 amino acids can occupy position 188 and support inward rectification gating as well as potassium-selective permeation. Among them, polar residues and hydrophobic residues that differ substantially in size from valine cause the channel to be constitutively active (Fig. 4). Likewise, residues of different sizes and polarity can replace S177 in an open channel, although potassium selectivity is compromised by several mutations at this location near the narrow opening of the channel pore. Tolerance of these M2 residues for substitution with charged or bulky residues suggests that they face the pore in the open channel. The placement of S177 and V188 in pore-lining positions in Kir3.2 agrees with the Kir2.1 model (60) where their equivalent positions (amino acids 165 and 176) face the pore (Fig. 5), further corroborating the notion that the Kir2.1 model resembles the open Kir3.2 channel.

A clockwise rotation of the M2 helix, when viewed from outside the cell, would allow the Kir3.2 channel to resemble the KcsA channel structure when it is closed, but to take on the Kir2.1 conformation when it opens (Fig. 6). This motion would bring M2 residues such as V188 from buried locations within the interior of a closed channel to face the pore of the open channel. If the motion for channel opening occurs without altering the contacts between M1 and M2 helices of the same subunit, the M1 helix would be brought into contact with the M2 helix of a neighboring subunit, in addition to the M2 helix of the same subunit. Clockwise rotation of the M1 and M2 helices as a unit would transform a KcsA-like conformation of the closed channel into a Kir2.1-like conformation of the open channel. How would a rotation of the transmembrane helices affect the pore-helix and pore-loop structure seen in KcsA? One possibility is that the pore-loops and pore-helices could be stabilized by ions in the pore, resulting in relative movements between the P region and the transmembrane helices as the channel opens and closes. A second possibility is that conformational changes also happen in the pore region, possibly providing a mechanistic connection between permeation and channel gating.

### Prospectus

Different functions of potassium channels are tied to the movements of various channel parts. The future challenge is to develop a better picture of what the potassium channel parts look like and concurrently refine models of how they move. In the voltage-sensing step, the outward movement of S4 relative to the electric field across the membrane accounts for the gating charge movement (25–29). The movement of S4 then triggers further conformational changes that open the channel. There are, however, more questions that need to be answered to better understand this step. How well conserved is the basic pore design of Kv and Kir channels? Will opening of Kv channels also involve a clockwise rotation of the S5 and S6 segments perhaps similar to the proposed rotation of M1 and M2 segments in Kir channels? Given that some models propose a rotation of S4 (26, 27), might the S4 segment rotate in the same direction as S5 and S6? What might be the relative motions between different transmembrane segments within a Kv subunit?

The primary function of ion channels, allowing charged ions to pass through the hydrophobic membrane, is regulated by a wide range of soluble factors on either side of the membrane (1). As a recurrent theme, these membrane proteins adopt multiple conformations that permit or deny the passage of ions; these different conformations provide substrates for channel modulation. Regulatory molecules inside or outside the cell may interact with and confer stability to one of these conformations, thereby favoring channel opening or closing. One Kir channel, the ATP-sensitive potassium channel ( $K_{ATP}$ ), exhibits abundant examples of channel regulation by soluble factors.  $K_{ATP}$  channels are comprised of the pore-forming Kir6.2 subunit and the transporter-like  $\beta$  subunit, sulfonylurea receptor (SUR) of the



**Fig. 6.** Gating model derived from an open-state model of Kir2.1 and the KcsA structure. (Top) Sequence alignment of M1 (amino acids 94–118), the P loop (amino acids 140–158), and M2 (amino acids 167–192) of Kir3.2 (GIRK2) with Kir2.1 (IRK1) and KcsA. This alignment is based on structural analysis of KcsA and mutational analysis of Kir2.1. The residues altered by gating mutations isolated from the yeast screen are indicated. (Middle Left) A side view of the KcsA potassium channel showing the physical proximity of the corresponding outer pair of residues. (Middle Right) A close-up view showing that the corresponding inner pair of residues contact the same residue, W113, in the same subunit. (Bottom) A model for the gating motion as the Kir3.2 channel opens. In this model, each subunit rotates clockwise when viewed from outside the cell; V188 (drawn in the diagram on the right) is buried and involved in M2-M2 interactions between subunits in the closed state, but faces the pore in the open state. [Adapted with permission from ref. 34 (Copyright 2001, Elsevier Science).]

ATP-binding cassette (ABC) family (5, 66). SUR interacts primarily with the M1 transmembrane segment of Kir6.2 (67). It is known that SUR conveys metabolic signals such as ADP binding and ATP hydrolysis, as well as pharmacological signals such as potassium channel blockers and openers, to Kir6.2, causing the channel to open and close (5, 66, 68). Could SUR in different physiological or pharmacological states promote rotations of Kir6.2 transmembrane segments in a way similar to the proposed mechanism for Kir3.2 (GIRK2) channel gating? It should be noted that these sorts of camera iris-like rotations between channel subunits have been a prevailing model for the opening and closing of ion channels for some time (69). It is gratifying to see the development and refinement of models of channel opening that match this very basic theme.

Just how universal might be the mechanisms for transducing signals from cytoplasmic factors to the channel pore in the membrane? In Kv channels, movements at the interface of the T1 tetramer are likely to accompany conformational changes that open the channel (33). Conceivably, interaction between T1 and Kvβ subunits, as well as active small GTPases and other regulatory molecules, may affect the stability of the T1 tetramer and hence modulate channel activity. The superfamily to which Kv channels belong also includes channels that respond to membrane potential in different ways, such as the hyperpolarization-activated cation channel (I<sub>h</sub>) and plant potassium channels that activate on hyperpolarization (70, 71). Other family members are hardly voltage-

sensitive, such as the cyclic nucleotide-gated channels and certain calcium-activated potassium channels. Cytoplasmic factors that control channel activities include cyclic nucleotides, calcium, calmodulin, kinases, and phosphatases (42–44, 51, 52, 72–74). In one hypothetical scheme that could apply to Kv, Kir, and other channels, channel interaction with cytoplasmic factors would alter the energetics of interaction between cytoplasmic domains of neighboring subunits. Shifting movements at the interface between cytoplasmic domains of neighboring subunits may then be coupled to movements around the pore, such as clockwise rotation of transmembrane segments in each of the four α subunits.

The modulation of channel activities by neurotransmitters and cytoplasmic factors is important for the transmission of signals between neurons. An ever-expanding collection of experimental tools has enabled steady progress in the study of channel regulation. Much remains to be done, however, to address the questions raised here and to determine the general themes for channel regulation.

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