Commentary

Spermine Is Fit to Block Inward Rectifier (Kir) Channels

Peter R. Stanfield[1] and Michael J. Sutcliffe[2]

[1]Molecular Physiology Group, Department of Biological Sciences, University of Warwick, Coventry CV4 7AL, UK
[2]Departments of Biochemistry and Chemistry, University of Leicester, University Road, Leicester LE1 7RH, UK

Molecular biology has shown there to be a great diversity of potassium channel isoforms. Among the simplest structurally are the inward rectifier potassium channel, *KCNJ* gene family. These channels are open in the region of the resting potential, a potential that they therefore help to determine. Some 14 genes are known for this family, classified into 7 subfamilies in terms of sequence homology or identity. These subfamilies are identified as Kir1.0 through Kir7.0 (for review see Stanfield et al., 2002).

Inward rectifier channels are tetramers of pore-forming subunits with two transmembrane domains (M1 and M2) separated by a P-region that forms the most selective part of the pore (Fig. 1). Significantly more than half the molecular mass is made up by the intracellular NH_2 and particularly the COOH terminus. The X-ray structure of a bacterial homologue (KirBac1.1) shows that the NH_2 and the COOH terminus form an extension of the pore beyond the inner surface of the membrane into the cytoplasm (Kuo et al., 2003). This extension roughly doubles the pore length. This structure holds for mammalian channels as well (Nishida and MacKinnon, 2002). Additional subunits are required for some members of the family to make channels—e.g., K_{ATP} is formed from Kir6.2 and the sulphonylurea receptor SUR. But the majority appear not to require accessory subunits.

Why are these potassium channels called inward rectifiers? When Katz first discovered the phenomenon of inward rectification (Katz, 1949), he showed in skeletal muscle that if the extracellular solution contained a high $[K^+]$, hyperpolarization gave rise to a high K^+ permeability, while depolarization gave rise to a low permeability. Thus, K^+ moved into the cell more easily than it moved out. This behavior was entirely unexpected, particularly so at a time when the mechanism of the nervous impulse was being elucidated, where K^+ permeability in nerve increased with depolarization. The behavior was later shown to be a characteristic of the resting potassium conductance of skeletal muscle whatever the initial level $[K^+]_o$ (Hodgkin and Horowicz, 1959; Leech and Stanfield, 1981). Thus, even at physiological $[K^+]_o$, K^+ moves in more easily under hyperpolarization than it moves out. Two factors, then, regulate the K^+ permeability underlying inward rectification. It is increased at more negative membrane potentials. And, at a given membrane potential, it is increased with increasing $[K^+]_o$. This latter is perhaps the clue to the physiological importance of inward rectification: essentially these potassium channels are activated by extracellular K^+.

Consider how K^+ acts as a vasodilator in certain parts of the vasculature. It is well established that K^+ induces vasodilatation in coronary, cerebral, and skeletal muscle vascular beds. To do this, an increase in $[K^+]_o$ must hyperpolarize the membrane of vascular smooth muscle cells to reduce Ca^{2+} entry from the extracellular milieu and its release from intracellular stores (Edwards et al., 1988). This hyperpolarizing effect of K^+ is not expected, because the resting membrane potential is normally approximately proportional to *log* $[K^+]_o$. However, as increasing $[K^+]_o$ opens K^+ channels, it is able to increase K^+ efflux at potentials positive to E_K. This increased efflux moves the membrane potential in a negative or hyperpolarizing direction, causing relaxation. The increase in $[K^+]_o$ is due, in part, to electrical activity in neurons or muscle and may also be due to K^+ flux through K_{ATP}. Some have argued that K^+ is released by endothelium as endothelium derived hyperpolarizing factor (EDHF) (Edwards et al., 1998). The channel isoform Kir2.1 (or IRK1) is the inward rectifier that is important; in Kir2.1 knock-out mice, the vasodilator response to K^+ is lost (Zaritsky et al., 2000).

It is presumably also lost in humans with Andersen's syndrome, caused by mutation of the Kir2.1 gene (Plaster et al., 2001). The syndrome however is characterized clinically by periodic paralysis of skeletal muscle, by an increase in the QT interval in the ECG often leading to serious cardiac dysrhythmia including *torsades de pointes* and ventricular fibrillation, and by developmental abnormality, with facial dysmorphia. These symptoms speak also to the importance of these channels in the excitability of skeletal muscle and its K^+ homeostasis; in the excitability of heart muscle and in repolarization of its action potential; and in developmental phenomena. Kir2.1 expression, generating a negative resting potential, is thought important in events leading to the fusion of myocytes to form myotubes (Fischer-Lougheed et al., 2001) and is presumably important in

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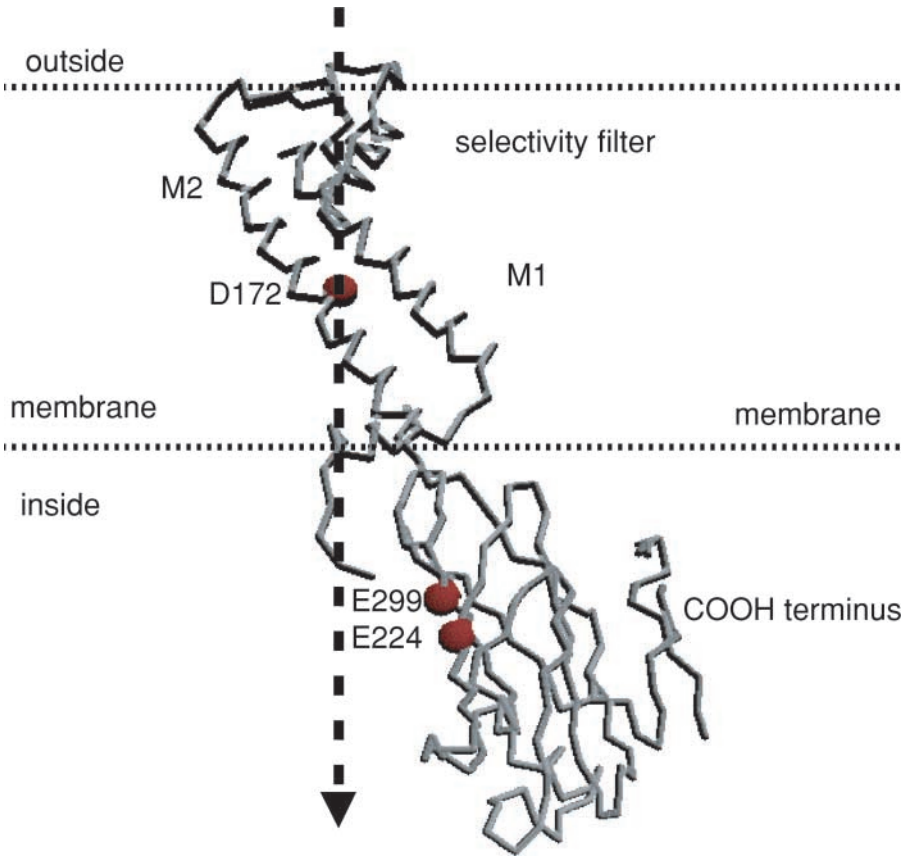

FIGURE 1. Structural model of Kir2.1 (IRK1). The model was produced using homology modeling (program MODELLER) by aligning the sequence of Kir2.1 with that of KirBac1.1 using ClustalX, adjusting manually where necessary the correlation between predicted secondary structural elements and those present in the structure of KirBac1.1, and using KirBac1.1 (Kuo et al., 2003; PDB accession code 1P7B) as the structural template. A single subunit is shown, with the vertical arrow indicating the path taken by a K^+ ion moving inwards across the membrane. Three residues (indicated) are crucial to the gating of channels by polyamines: D172 in M2, and E224 and E299 in the COOH terminus. The residues are pore lining and interact electrostatically with the positively charged amine groups on the polyamines. (This figure was generated using the program Protein Explorer.)

cells involved in the modeling of bone (see Karschin and Karschin, 1997).

What is the mechanism by which K^+ opens these ion channels? For some years it has been understood that channel closure is caused by blockage by intracellular cations. The likelihood that gating might occur in this way came from early experiments of Armstrong (1969) studying internal TEA^+ blockage of potassium channels in squid axon. Mg^{2+} was the first candidate for making such blockage; later, polyamine molecules—putrescine, spermidine, and spermine, which are derivatives of ornithine—were shown to be more important physiologically (Lopatin et al., 1994; Table I). Many have supposed that some change of channel conformation is associated, but Guo and Lu (2002) have shown that this is unlikely to be correct. The channels may show gating changes, but these are likely to be in response to other regulators, such as H^+ in Kir2.3 (for review see Stanfield et al., 2002). However, the inward rectification—the capacity of the channels to be activated by K^+_o—is generated entirely by the blocking and unblocking of the channels by polyamines from the cytoplasm and by the opposing of polyamine occupancy by K^+ from the extracellular fluid (Guo and Lu, 2002, 2003; Guo et al., 2003).

Many groups have contributed to the polyamine story, including that of Guo and Lu (2003), whose latest contribution in this issue shows us how exquisitely well the channel and spermine match each other. Channels have acidic residues that bind the polyamines. These residues are an aspartate in the second transmembrane domain (D172 in M2; Stanfield et al., 1994) and two glutamate residues (E224 and E299) in the COOH terminus (Yang et al., 1995; Kubo and Murata, 2001). Current ideas about Kir channel structure (Kuo et al., 2003) are entirely consistent with these residues, giving rise to two negatively charged rings in the pore—one just below the selectivity filter and a double ring in the cytoplasmic mouth of the pore (Fig. 1). It is the negative charge of the residues that is important—replacement of D172 by glutamate gives wild-type behavior; replacement by asparagine or by glutamine weakens polyamine blockage. Further, the exact position of D172 is not vital to polyamine blockage; it can be moved by molecular engineering one turn of the α-helix up or down the pore and still offer reasonably high-affinity binding (Guo et al., 2003).

In their latest paper, Guo and Lu (2003) have carefully examined blockage by the polyamines putrescine, spermidine, and spermine. They have compared blockage by these agents with blockage by alkyl bis-amines, and have studied the affinity, rate of blockage, and, from the voltage dependence, the observed valence in wild type channels and in channels where one or more of the residues conferring blockage have been substi-

TABLE I

Structures of Polyamines and of bis-C9, the Alkyl bis-amine That Most Closely Mimics the Binding of Spermidine and Spermine

Putrescine (C4)	$H_2N.CH_2.CH_2.CH_2.NH_2$
Bis-C9	$H_2N.CH_2.CH_2.CH_2.CH_2.CH_2.CH_2.CH_2.CH_2.NH_2$
Spermidine	$H_2N.CH_2.CH_2.CH_2.NH.CH_2.CH_2.NH_2$
Spermine	$H_2N.CH_2.CH_2.CH_2.HN.CH_2.CH_2.CH_2.NH.CH_2.CH_2.CH_2.NH_2$

Note that spermidine is, however, shorter than C9, and its two leading amine groups (to the right) are thought to interact electrostatically with D172. Spermine has the highest affinity: its two leading and two trailing amines groups enhance its electrostatic interaction with both D172 and E224/E299, respectively.

tuted (D172N, E224G, E299S). Their results address a number of issues.

Observed Valence

Inward rectification in Kir2.1 is steep, so the blocking cations must block with a high observed valence—spermine blocks with a valence of ~5. It might be expected that the high charge on spermine is partly responsible for this high observed valence, but this is not the case. The length of the blocking amine, not its charge, determines valence. Since longer molecules displace more K^+ ions, this number gives the high observed valence of spermine.

The observed valence of the bis-amines increases effectively stepwise with increasing chain length. For bis-C2–bis-C4, the valence is approximately two. It increases to three for bis-C5, to four for bis-C7, and to five for alkyl bis-amines of longer chain length. A bis-amine, with the same length as spermine has the same observed valence. It displaces the same number of K^+ ions.

Observed valence is reduced by all the mutants, which remove negative charges from the wall of the pore. This is consistent with a reduced K^+ occupancy of the inner part of the pore.

Rate of Blockage

If blockage is treated as a first order process, the transition rate constant k_{on} is reduced from $\sim 10^9$ M^{-1} s^{-1} in wild type to $\sim 10^5$ M^{-1} s^{-1} in E224G and E299S. The mutation D172N does not reduce blocking rate. Similar effects are seen with the bis-amines. This result implies that polyamine docks first with E224/E299. The voltage dependence of the blockage is low, with an observed valence of less than one. This is consistent with the channel being blocked immediately the polyamine first docks with the channel, because only about one K^+ ion is displaced in this first interaction. The polyamine then moves into the channel, where it interacts with D172 as well as with E224/E299. Thus, there are two blocked states, one immediately the polyamine docks with the channel at E224/E299 and subsequently one where it moves to lie deeper in the channel, interacting also with D172.

Affinity

The affinity of polyamines for wild-type channels increases from putrescine to spermidine to spermine. These affinities are reduced by the mutants D172N, E224G, and E299S. The reduction in affinity is greatest for D172N. D172N also reduces the affinity for the longer chain bis-amines. The reduction is greatest when the two amine groups are separated by nine $-CH_2$ groups (bis-C9).

Guo and Lu (2003) examined the energetics of the interactions of each polyamine with each of the acidic residues that confer binding, comparing these with interactions of each of the alkyl bis-amines. This was achieved by calculating a coupling coefficient from the dissociation constants measured at 0 mV for the polyamine and for the bis-amine for wild-type and mutant channels (see also Hidalgo and MacKinnon, 1995). The coupling coefficient compares the difference in the affinity for a polyamine caused by substitution of a residue (${}^{wt}K_d^{PM}/{}^{mt}K_d^{PM}$) with the difference in affinity for a bis-amine caused by the same substitution (${}^{wt}K_d^{bis-Cn}/{}^{mt}K_d^{bis-Cn}$). The coupling coefficient, Ω, will be given by $({}^{wt}K_d^{PM} \times {}^{mt}K_d^{bis-Cn})/({}^{mt}K_d^{PM} \times {}^{wt}K_d^{bis-Cn})$, where *wt* and *mt* represent wild-type and mutant, respectively, and where *PM* and *bis-Cn* represent polyamine and alkyl bis-amine, respectively. Because of the relationship between equilibrium constants and the standard free energy change of reactions, the interaction energy with a particular residue, relative to that of the bis-amine, will be given by $RT.\ln\Omega$. This quantity will be negative if the interaction of the polyamine is the stronger and positive if it is the weaker.

There is little difference in interaction energy for the interactions with E224 and E299 in the COOH terminus with spermidine, putrescine, or any of the other alkyl bis-amines. Each of these interacts with E224/E299 in essentially the same way and presumably sits at essentially the same distance from these residues. The interaction with spermine is, however, markedly tighter with an interaction energy difference of about -1.5 kcal.mol^{-1}. Evidently, the two trailing amine groups of spermine interact with E224/E299 in its final position in the pore.

Much more marked differences are seen with mutations of D172. Putrescine interacts more weakly with D172 than any of the longer bis-amines. The energy difference is greatest with bis-C9, whose leading amine group is therefore likely to come closest to the aspartate residue. With longer bis-amines, the leading charge may move further into the channel than D172,

or the bis-amine may become folded. Spermidine shows a stronger interaction than all bis-amines except bis-C9. Spermidine is, however, only as long as C8, so it interacts more strongly than is expected from its length. Thus, its two leading amine groups are presumed to interact with D172.

Spermine interacts much more strongly than does any bis-amine. The difference is least with C9, but even here it is -1.3 kcal.mol^{-1}. It is greatest with putrescine. Thus, spermine contributes its leading two positive charges to the interaction with D172 and is extraordinarily well fit to the structure of the channel and to act as blocker. Spermine blocks with high affinity and high valence.

The hypothesis is highly attractive, particularly for its simplicity. It reflects and extends classic ideas about ionic blockage of channels that began to be developed when intracellular TEA^+ blockage of squid axon was first studied by Armstrong (1969): The transition rate for the onset of blockage depended principally on the concentration of TEA^+ in axoplasm and the transition rate constant for the reversal of blockage depended on voltage and on $[K^+]_o$. One surprise for spermine blockage of Kir2.1 comes from what is known so far about structure; the distance between D172 and E299 is thought to be ~35Å, spermine is only 16Å long. The structure, however, is for a closed form of the channel, but this distance is unlikely to be substantially different in the open state. If the hypothesis is correct, then the trailing charge(s) must interact with E224/E299 at a distance. This interaction at a distance is consistent with the modest energetic effect (~0.7 kcal.mol^{-1}) of replacing one of these residues with a neutral one. However, the electrostatic effect of D172 is likely to be more localized.

Polyamines have long been implicated in stabilizing DNA molecules. But they appear to play roles in the regulation of proteins also. As well as inward rectifier K^+ channels, polyamines have been shown to affect glutamate receptors, Ca^{2+} channels, other classes of K^+ channels, cyclic nucleotide–gated channels, and Na channels (for review see Stanfield et al., 2002). They may play other roles in regulation of proteins: recently they have been implicated in the folding and aggregation of α-synuclein, whose aggregation leads to cellular degeneration in Parkinson's and Alzheimer's diseases (Antony et al., 2003).

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REFERENCES

Antony, T., W. Hoyer, D. Cherny, G. Heim, T.M. Jovin, and V. Subramaniam. 2003. Cellular polyamines promote the aggregation of α-synuclein. *J. Biol. Chem.* 278:3235–3240.

Armstrong, C.M. 1969. Inactivation of potassium conductance and related phenomena caused by quaternary ammonium ion injected into squid axon. *J. Gen. Physiol.* 54:553–575.

Edwards, F.R., G.D.S. Hirst, and G.D. Silverberg. 1988. Inward rectification in rat cerebral arterioles; involvement of potassium ions in autoregulation. *J. Physiol.* 404:455–456.

Edwards, G., K.A. Dora, M.J. Gardener, C.J. Garland, and A.H. Weston. 1998. K^+ is an endothelium-derived hyperpolarising factor in rat arteries. *Nature.* 396:269–272.

Fischer-Lougheed, J., J.-H. Liu, E. Espinos, D. Mordasini, C.R. Bader, D. Belin, and L. Bernheim. 2001. Human myoblast fusion requires expression of functional inward rectifier Kir2.1 channels. *J. Cell Biol.* 153:677–685.

Guo, D., and Z. Lu. 2002. IRK1 inward rectifier K^+ channels exhibit no intrinsic rectification. *J. Gen. Physiol.* 120:539–551.

Guo, D., and Z. Lu. 2003. Interaction mechanisms between polyamines and IRK1 inward rectifier K^+ channels. *J Gen. Physiol.* 122:485–500.

Guo, D., Y. Ramu, A.M. Klem, and Z. Lu. 2003. Mechanism of inward rectification in inward rectifier K^+ channels. *J. Gen. Physiol.* 121:261–275.

Hidalgo, P., and R. MacKinnon. 1995. Revealing the architecture of a K^+ channel through mutant cycles with a peptide inhibitor. *Science.* 268:307–310.

Hodgkin, A.L., and P. Horowicz. 1959. The influence of potassium and chloride ions on the membrane potential of single muscle fibres. *J. Physiol.* 148:127–160.

Karschin, C., and A. Karschin. 1997. Ontogeny of gene expression of Kir channel subunits in the rat. *Mol. Cell. Neurosci.* 10:131–148.

Katz, B. 1949. Les constants électriques de la membrane du muscle. *Arch. Sci. Physiol. (Paris).* 3:285–299.

Kubo, Y., and Y. Murata. 2001. Control of rectification and permeation by two distinct sites after the second transmembrane region in Kir2.1 channel. *J. Physiol.* 531:645–650.

Kuo, A., J.M. Gulbis, J.F. Antcliff, T. Rahman, E.D. Lowe, J. Zimmer, J. Cuthbertson, F.M. Ashcroft, T. Ezaki, and D.A. Doyle. 2003. Crystal structure of the potassium channel KirBac1.1 in the closed state. *Science.* 300:1922–1926.

Leech, C.A., and P.R. Stanfield. 1981. Inward rectification in frog skeletal muscle fibres and its dependence on membrane potential and external potassium. *J. Physiol.* 319:295–309.

Lopatin, A.N., E.N. Makhina, and C.G. Nichols. 1994. Potassium channel blackages by cytoplasmic polyamines as the mechanismof intrinsic rectification. *Nature.* 372:366–369.

Nishida, M., and R. MacKinnon. 2002. Structural basis of inward rectification: cytoplasmic pore of the G-protein gated inward rectifier GIRK at 1.8Å resolution. *Cell.* 111:957–965.

Plaster, N.M., R. Tawil, M. Tristani-Firouzi, S. Canun, S. Bendahhou, A. Tsunoda, M.R. Donaldson, S.T. Iannaccone, E. Brunt, R. Barohn, et al. 2001. Mutations in Kir2.1 cause the developmental and episodic electrical phenotypes of Andersen's syndrome. *Cell.* 105:511–519.

Stanfield, P.R., N.W. Davies, P.A. Shelton, M.J. Sutcliffe, I.A. Khan, W.J. Brammar, and E.C. Conley. 1994. A single aspartate residue is involved in both intrinsic gating and blackage by Mg^{2+} of the inward rectifier, IRK1. *J. Physiol.* 478:1–6.

Stanfield, P.R., S. Nakajima, and Y. Nakajima. 2002. Constitutively active and G-protein coupled inward rectifier K^+ channels: Kir2.0 and Kir3.0. *Rev. Physiol. Biochem. Pharmacol.* 145:47–179.

Yang, J., Y.N. Jan, and L.Y. Jan. 1995. Control of rectification and permeation by residues in two distinct domains in an inward rectifier K^+ channel. *Neuron.* 14:1047–1054.

Zaritsky, J.J., D.M. Eckmann, G.C. Wellman, M.T. Nelson, and T.L. Schwarz. 2000. Targeted disruption of Kir2.1 and Kir2.2 genes reveals the essential role of the inwardly rectifying K^+ current in K^+ mediated vasodilation. *Circ. Res.* 87:160–166.