Kir4.1 K⁺ channels are regulated by external cations

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Abbreviations: Kir, inwardly rectifying potassium channel; NMDG, N-Methyl-D-Glucamine; K⁺, extracellular K⁺

The inwardly rectifying potassium channel (Kir), Kir4.1 mediates spatial K⁺-buffering in the CNS. In this process the channel is potentially exposed to a large range of extracellular K⁺ concentrations ([K+]o). We found that Kir4.1 is regulated by K⁺. Increased [K+]o leads to a slow (mins) increase in the whole-cell currents of Xenopus oocytes expressing Kir4.1. Conversely, removing K⁺ from the bath solution results in a slow decrease of the currents. This regulation is not coupled to the pH-sensitive gate of the channel, nor does it require the presence of K67, a residue necessary for K⁺-dependent regulation of Kir1.1. The voltage-dependent blockers Cs⁺ and Ba²⁺ substitute for K⁺ and prevent deactivation of the channel in the absence of K⁺. Cs⁺ blocks and regulates the channel with similar affinity, consistent with the regulatory sites being in the selectivity-filter of the channel. Although both Rb⁺ and NH₄⁺ permeate Kir4.1, only Rb⁺ is able to regulate the channel. We conclude that Kir4.1 is regulated by ions interacting with specific sites in the selectivity filter. Using a kinetic model of the permeation process we show the plausibility of the channel’s sensing the extracellular ionic environment through changes in the selectivity occupancy pattern, and that it is feasible for an ion with the selectivity properties of NH₄⁺ to permeate the channel without inducing these changes.

Introduction

The inwardly rectifying potassium channels (Kir) are a family of channels mainly involved in K⁺-vectoral transport and control of cell excitability. These channels are controlled by several factors, some that are general for the family (pH, lipids), and some that are specific for submembers (nucleotides, G-protein, intracellular Na⁺ and extracellular K⁺).1

Although all Kir-channels close in response to a decrease in intracellular pH, only a few do so in a physiologically relevant pH range (Kir1.1, Kir4.1, Kir4.2 and Kir4.x/5.1). Although the mechanism of pH-gating is not fully understood, it is known to involve closure of a region of the protein known as the bundle-crossing through large conformational changes in the channel protein.2-5

In addition to being gated by pH, Kir1.1 is regulated by K⁺. This regulation, which has also been shown to occur in Kir4.2,6 is manifested by a slow increase in whole-cell conductance when [K+]o is elevated and conversely a slow decrease when [K+]o is lowered. This phenomenon is different from the dependence of the open-channel conductance on the [K+]o that is characteristic of Kir-channels and that happens immediately as the external solution is changed. Other ions that interact with the selectivity-filter of the channel, either blocking (Cs⁺) or permeating (Rb⁺) the channel are able to mimic the effect of K⁺.7,8 This finding, in conjunction with mutational studies that have located residues in the selectivity-filter region necessary for this regulation to occur, have lead to the proposal that the selectivity filter acts as the sensor for the extracellular ionic milieu and possibly even as the gate.8-10

Structural and computational evidence has shown that the K⁺-channel selectivity filter consists of five binding sites (S0–S4) with 2–3 sites occupied at any given time.11-14 Under physiological conditions there is abundant K⁺ available from the cytoplasm regardless of the [K+]o, hence if the selectivity filter senses changes in the ionic environment the mechanism must be more complex than a simple ligand binding reaction. In line with this it has been proposed that in similar K⁺-dependent processes, such as C-type inactivation of Shaker channels and slow inactivation of KcsA channels, ions are sensed by the channel through changes in occupancy of a specific site or sites within the selectivity filter.15,16 It is unclear if this hypothesis is consistent with the selectivity of the effect for different permeant and blocking ions. Although pH and K⁺ may control different gates, there is a coupling between pH-gating and K⁺-regulation of Kir1.1 that is apparent either as an increase of the pKₘ when the [K+]o is lowered or an inability to recover from a pH-closed state in the absence of K⁺.7,10,17,18 Although the mechanism of this interaction is not understood, a similar coupling exists between the state of
the bundle-crossing gate and the slow inactivation gate in KcsA and Shaker channels. Furthermore, a lysine in the first transmembrane domain (K61 in Kir1.1b) is a main determinant of pH-sensitive Kir-channel regulation of Kir1.1. However, unlike in the case of pH-gating, where mutating this residue merely shifts the pK to lower values, K-dependent regulation is completely abolished if this residue is removed even if the pK is restored to normal values by introducing an additional mutation.

In most physiological situations [K] does not vary greatly. However, as the apical K-channel of principal cells in the cortical collecting duct of the nephron, Kir1.1 is exposed to large range of [K]. Thus this regulation may be physiologically relevant. Another case where sensitivity of a Kir-family member to changes in [K] might be important involves K-spatial buffering in the CNS and Kir4.1.

In the CNS the K-concentration is controlled at around 3 mM. However, during bursts of activity the extracellular K-concentration can locally increase to about 10 mM and under pathological conditions even higher. One of the mechanisms to clear elevations of [K] and prevent spontaneous neuronal firing or spreading depression is K-spatial buffering. This process takes advantage of the electrical coupling between glial cells to effectively transport K through the glial syncytium, from areas of high to low concentration. An increasing body of evidence suggests that the glial channel involved in this process is Kir4.1. As this channel is closely related to Kir1.1 both by sequence homology and function, it is a likely candidate for K-regulation. In the present study we demonstrate and characterize K-dependent regulation of Kir4.1. Additionally, using a kinetic model of permeation we show that the changes in occupancy in the selectivity filter associated with changes in the ionic environment is consistent with the selectivity-filter’s being the K-sensor.

Results

Kir4.1 is regulated by K-pH-sensitivity. To test if K-regulation occurs in Kir4.1 we incubated oocytes in solutions containing 1 mM K for 1 h prior to the start of the TEVC recording. After obtaining baseline whole-cell measurements in the 1 mM K solution, the bath solution was replaced with one containing 10 mM K and whole-cell currents were measured periodically. After 40 min, the bath solution was returned to 1 mM K. To control for time-dependent effects not attributable to the solution changes during these long recordings some oocytes were kept in 1 mM K for the duration of the recording. As seen in Figure 1C, showing outward currents measured at 60 mV, there was a slow increase in the currents upon increasing [K]. The increase occurs uniformly throughout the voltage range tested (-150 mV to 100 mV) as seen in the representative IV curves in Figure 1A. We chose to plot outward currents as they have a smaller acute dependence on the extracellular ionic composition. This increase persists after the oocytes are returned to the 1 mM K solution. At this point (t = 50 min) the whole-cell currents, measured in the same solution, had increased 2.62 ± 0.33 (n = 6) fold compared to baseline (t = 5 min), whereas the time control did not change significantly; 0.86 ± 0.10 (n = 3). Executing the reverse protocol (Fig. 1B and D), i.e., incubating in 10 mM K and exposing the oocytes to 1 mM K, resulted in a slow decrease (t = 50 min: 0.66 ± 0.06, n = 7) of the whole-cell currents, whereas the time control increased slightly (1.27 ± 0.10, n = 3).

Since we substituted the K in the bath solutions with Na it is possible that it was the increase in [Na] rather than the decrease in [K] that affected the whole-cell currents. To test this we compared the effects of the Na-containing solution to that of one containing 109 mM N-Methyl-D-Glucamine (NMDG) (Fig. 2). NMDG is a large cation that is unlikely to affect the channel in a similar manner to the smaller Na or K. We used 110 mM KCl as our baseline condition so that it would be identical for both groups. There is no significant difference in the fractional decrease in currents between the groups. Hence increased [Na] is not downregulating the channel. Taken together these results demonstrate that Kir4.1 is regulated by K per se.

There is no coupling between Kir4.1 K-regulation and pH-sensitivity. The findings above indicate that Kir4.1 is regulated by external cations, similar to what has previously been found in Kir1.1. We next investigated if there exists a coupling between K-regulation and pH-sensitivity in Kir4.1 as has been found in Kir1.1. A permeant acetate buffer was used to acidify the cytoplasm of intact oocytes expressing Kir4.1 with either 10 mM or 110 mM K in the bath. In Kir1.1, decreasing [K] causes an increase of the pK. As evident in Figure 3A, this is not the case in Kir4.1 where we found a small but statistically significant increase in the pH-sensitivity in the 110 K-solution. To investigate this lack of coupling further, we introduced a mutation, K67M, into the N-terminal of Kir4.1. A homologous mutation in Kir1.1, K80M, dramatically lowers the pK and completely abolishes the K-sensitivity of the channel. As shown in Figure 3A, the K67M mutation had a similar effect on the pK of Kir4.1. It did not, however, decrease the K-sensitivity. The ratio of outward currents before and after exposure to 1 mM K for 45 min was 0.29 ± 0.08 for K67M (Fig. 3B and compared to 0.52 ± 0.09 for WT, Fig. 2A). Both these findings suggest that K-regulation in Kir4.1 does not depend on the state of the pH-sensitive gate.

Other pore-interacting ions can regulate Kir4.1. In the case of Kir1.1, other cations that interact with the channel pore, both permeable ions and voltage-dependent blockers, are able to mimic the effect on the whole-cell currents of K. Before addressing the specificity of the effect of K on Kir4.1, we measured the selectivity of the channel to two ions that permeate most K-channels, Rb and NH4+. The results from the ion substitution experiments are shown in Figure 4. Both ions had reduced conductance and permeability compared to K. For Rb, the conductance ratio was smaller than the permeability ratio, while the opposite was found for NH4+.

Next, we investigated the specificity of the effect of K on Kir4.1. Using the protocol established above, but varying the cation in the low K-bath solution. If the cation replacing K is able to mimic the effect of K, the decrease of currents will be less than that observed when the K is replaced with Na or NMDG. Figure 5 shows examples of this protocol using 110 Rb (A) and 110 NH4 (B) in the bath. Rb was able to substitute for K and the
Both the pore blocking ions, Cs⁺ and Ba²⁺, are able to substitute for the removed K⁺ and prevent the decrease seen with 1 mM K⁺ in the bath. In contrast to the permeant ions, the interaction between the channel and a blocking ion, in terms of pore occupancy, is more straightforward; when the channel is blocked, the blocking ion is occupying the pore. This allows us to correlate occupancy of the pore and regulation of the channel by Cs⁺. First we measured Cs⁺-block with 1 mM K⁺ in the bath, a concentration that led to a decrease in the currents in the regulation experiments (Fig. 6A and B).

To measure regulation by Cs⁺, we preincubated oocytes in 110 mM K⁺ before adding 1 mM K⁺ and varying Cs⁺ concentrations to the bath. After 40 min the bath solution was returned to 110 mM K⁺, whole-cell currents were measured and normalized to the initial currents in 110 mM K⁺. Since occupancy of

currents were stable or even increased slightly. NH₄⁺, on the other hand, was not able to substitute for K⁺ and the currents decreased. Since both these ions permeate the channel it follows that they must occupy the pore, but only Rb⁺ is able to substitute for K⁺ in keeping the channels in the open state. This suggests that the specific interaction between the sites in the selectivity filter and the given ions are important in order to maintain channel activity. We will discuss this issue further in the simulation section of the paper.

The solutions we tested in this manner are summarized in Figure 5C where we plot the currents after the bath solution was changed back to 110 mM K⁺ (t = 50 min) relative to the baseline current (t = 5 min) under the same conditions. In this way we control for the acute effects that both blocking and permeant ions may have on the currents independent of the slow regulation studied in this paper.

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**Figure 1.** Kir4.1 is regulated by external K⁺. Representative I-V relationships for switches from 1 K⁺ to 10 K⁺ (A) and 10 K⁺ to 1 K⁺ (B). Time points refer to (C and D) for (A and B) respectively. (C and D) Time course of measurements from oocytes preincubated in 1 mM (C) or 10 mM (D) K⁺ for 1 h prior to the start of the experiment. The bath solution was switched to 10 mM (C) or 1 mM (D) K⁺ after 5 min and then returned to the original solution after 45 min. The currents measured at 60 mV were normalized to the value at 5 min. Control oocytes were kept in the original solution for the duration of the recording period. Boxed in values are 2.62 ± 0.33 (n = 6) vs. 0.86 ± 0.10 (n = 3) for the control (p < 0.01) in (C) and 0.66 ± 0.06 (n = 7) vs. 1.27 ± 0.10 (n = 3) for the control (p < 0.001) in (D).
the pore, like block, is a voltage dependent process the oocytes were clamped at -120 mV during the 40 min exposure period. The time course for the activation experiments are plotted in Figure 6C. The concentration dependencies of block and regulation by Cs+ at -120 mV are plotted in Figure 6D. There is a reasonable agreement between the two measures, consistent with the idea that Cs+ blocks and regulates the channel at the same site, presumably within the selectivity filter.

Single-channel currents. To better understand the process of K permeation through Kir4.1, we measured single-channel currents in cell-attached patches under conditions of low, intermediate and high K+ concentrations in the patch-clamp pipette.
Results are shown in Figure 7. With high (110 mM) K\textsuperscript{+}, the inward conductance was 23 pS, similar to previous results.\textsuperscript{38,39} We were unable to detect outward single-channel currents under these conditions. The inward conductance was a saturating function of K\textsuperscript{+} with an estimated g_{max} of 24.4 pS and an apparent K_{m} of 16.7 mM.

Simulations. Our data suggest that the channel directly senses the extracellular ionic composition in the selectivity filter. As mentioned above this cannot simply be a matter of a general occupancy of ions in the selectivity filter, as even under low [K\textsuperscript{+}]_{o} there are abundant K\textsuperscript{+}-ions available from the cytoplasm that has a constant high [K\textsuperscript{+}]. Current kinetic models of K\textsuperscript{+}-channel permeation, based on both structural\textsuperscript{11,14} and computational\textsuperscript{12,13} studies, are often represented with six binding sites (four in the selectivity filter of the channel (S1–S4) one in the extracellular vestibule (S0) and one in the central cavity of the channel (SC)) that are occupied by 2–3 ions at any given time. In a multisite pore such as this, we suggest that rather than the general presence of ions in the pore, a specific site or sites within the pore needs to be occupied to prevent deactivation of the channel.

To investigate if this is a plausible mechanism for K\textsuperscript{+}-dependent regulation in Kir4.1, we constructed a kinetic model using a 6-site model with five states (Fig. 8A) similar to that proposed previously in references 40 and 41. We fit the model to the data by altering the binding energies for the site and the energy barrier between them as this limited the amount parameters, particularly once the model was expanded to accommodate two ionic species. These parameters were then used to calculate rate constants.

We first investigated changes in occupancy induced by [K\textsuperscript{+}]_{o} itself. Fitting the model to single-channel currents from cell-attached patches at three different [K\textsuperscript{+}]_{o} (Fig. 7), we found a family of solutions that fit the data well and also predicted a relatively linear i-V curve in symmetrical K\textsuperscript{+} even outside the voltage region used for the fit. The parameters are listed in Supplemental Table 1. The i-V relationships for [K\textsuperscript{+}]_{o} = 10, 40 and 110 mM at constant [K\textsuperscript{+}]_{in} (110 mM), calculated using the mean parameter values, are shown in Supplemental Figure 1A, together with the measured values.

[K\textsuperscript{+}]_{o} dependent changes in occupancy at the reversal potential are shown in Supplemental Figure 1B and summarized in Figure 8B. The reversal potential was the most relevant voltage domain since in our experiments the oocytes are unclamped and the high K\textsuperscript{+} conductance keeps the voltage close to the equilibrium potential for K\textsuperscript{+}. Increasing [K\textsuperscript{+}]_{o} leads to a decrease in occupancy of S2/S4 and an increase in the occupancy of S1/S3 and S0. This is a robust result that pertains generally to most solutions to the observed i-V relationships. The increase in occupancy of S0 directly reflects the equilibration of this site with external K\textsuperscript{+}. The increases in S1/S3 are largely due to changes in membrane potential that shift the ions from the S2/S4 position at less negative voltages.

Next we investigated the effects of Cs\textsuperscript{+} by expanding the model to accommodate two ionic species. The resulting model has 32-states, since each ion in the pore as shown in Figure 8A can be either K\textsuperscript{+} or Cs\textsuperscript{+}. We assumed that the number of Cs\textsuperscript{+} binding sites and their placement in the electrical field are identical to those of K\textsuperscript{+}. We fit the model to the blocking data shown in Figure 5B over the voltage range -150 to 0 mV, using the parameters for K\textsuperscript{+} from Figure S1A and altering the parameters for Cs\textsuperscript{+}. These data can be fitted in either of two general ways with average and variance for each shown in Supplemental Table 1. Figure S1C shows simulated voltage-dependent blocking curves at different [Cs\textsuperscript{+}]_{o} for one of these cases. As was observed with K\textsuperscript{+} there is an increase in S1/S3 occupancy with increasing [Cs\textsuperscript{+}]_{o}. Another set of parameters shown in Supplemental Table 1 predicts increased
We have demonstrated for the first time direct regulation of Kir4.1 by external cations. Although the physiological significance of this regulation is unknown, in the CNS, where Kir4.1 is expressed, areas of high neuronal activity can experience a local increase of \([K^+]_o\). During periods of high activity the \([K^+]_o\) can rise to around 12 mM and during pathological conditions as high as 60 mM. One of the proposed mechanisms to prevent this build up of \([K^+]_o\) is known as \(K^+\) spatial buffering.

Lastly, we examined \(NH_4^+\), an ion that permeates the channel but is not able to prevent its inactivation. We again assume that the number of binding sites and their placement in the electrical field are identical to those of \(K^+\), and that the macroscopic conductance ratio (Fig. 5) reflects changes in the single-channel conductance. The model was fit to the average \(NH_4^+\) currents from the selectivity experiments scaled to the \(K^+\) single-channel data. Parameter values are given in Supplemental Table 1. Figure S1F shows the i-V relationship for the 95 fits that converged to a solution. The change in the occupancies of the binding sites with addition of \(NH_4^+\) to the extracellular side is shown in Supplemental Figure 1F and summarized for the case of 110 mM \(NH_4^+\) in Figure 8B. S0 occupancy increased similar to that calculated for \(K^+\), but S1/S3 occupancy did not.

**Discussion**

We have demonstrated for the first time direct regulation of Kir4.1 by external cations. Although the physiological significance of this regulation is unknown, in the CNS, where Kir4.1 is expressed, areas of high neuronal activity can experience a local increase of \([K^+]_o\). During periods of high activity the \([K^+]_o\) can rise to around 12 mM and during pathological conditions as high as 60 mM. One of the proposed mechanisms to prevent this build up of \([K^+]_o\) is known as \(K^+\) spatial buffering.
Influx of K\(^+\) into glial cells exposed to the elevated [K\(^+\)]\(_o\) will depolarize both that cell and the entire electrically coupled synctium. This will create a driving force for efflux of K\(^+\) in the areas not exposed to the elevated [K\(^+\)]\(_o\), clearing K\(^+\) from the areas of K\(^+\) accumulation. Evidence points to Kir4.1 as the K\(^+\)-channel involved in this clearance.\(^{35-36}\) The K\(^+\)_o-dependent regulation that we have demonstrated offers a means to selectively upregulate the channels exposed to the elevated [K\(^+\)]\(_o\).

Our data suggest that the selectivity filter of the channel directly senses the changes in the ionic composition of the extracellular solution. This is based on two observations: (i) only ions that interact with the selectivity-filter can substitute for K\(^+\); (ii) both permeant and voltage dependent blockers can substitute for K\(^+\). Since voltage-dependent blockers bind inside the electric field, i.e., the selectivity-filter,\(^{42}\) and do not permeate the channel at a significant rate, it is unlikely that this process requires actual movement of ions across the membrane. Our data comparing Cs\(^+\)-block and Cs\(^+\)-regulation of Kir4.1 are consistent with the idea that these processes require occupancy of the same site or sites.

The idea that occupancy of a specific site within the selectivity filter controls the activity of the channel has been proposed for KcsA and Shaker channels.\(^{16,19,43,44}\) We propose here that a similar mechanism occurs in Kir4.1. In our kinetic model, we found that increasing [K\(^+\)]\(_o\) increases occupancy of the S0, and the S1/S3 sites. At a first glance this seems to contradict the results of MD-simulations of KcsA, where S2 occupancy appears to keep the selectivity filter in the conducting state.\(^{16}\) However a recent computational study based on the KirBac1.1 structure suggests that even though S2 is the site where the closure of the filter occurs, this process is initiated from the S2–S4 configuration by flickering behavior from the ion in S2 into either S1 or S3.\(^{35}\) S1/S3 occupancy could prevent this process, which could
also be inhibited by an ion present at the S0 site by repelling any ion entering S1. Effectively, this would mean that in our model (Fig. 6A and B) states (or groups of states) B, C and E would prevent deactivation of the channel. In fact, this combination of states shows the strongest dependency on the [K+]o (Sup. Fig. 1B).

The Cs+ data could be explained by increases in either S1/S3 or S2/S4 occupancy (Sup. Table 1). However, structural information using ion replacement in KcsA show minimal occupancy of S2 by Cs+ indicating that the fit with favorable S1/S3 binding sites, and [Cs+] dependent increase in S1/S3 occupancy, might be more accurate.59 In line with this, MD simulations have also shown that S2 is the most selective site.13,46 Zhou and MacKinnon also showed that Rb+ does not bind in the S2 site either.45 Both Cs+ and Rb+ are, however, able to upregulate Kir4.1 (Fig. 4) and Kir1.1.7 Rb+ is also able to prevent inactivation of KcsA47 and Shaker.19

Lack of structural information on NH4+ in the selectivity filter makes it unclear how this ion fits into this scheme. In our model substituting K+ with NH4+ in the bath solution lead to a decrease of S1/S3 occupancy, consistent with the idea that occupancy at these sites is important for channel activity. Substituting NH4+ did however also lead to an increase in S0 occupancy, which argues that S0 occupancy is insufficient to maintain the channel in an active state. It is possible that NH4+ interacts very differently with the selectivity filter than does K+, invalidating our modeling process. However, our results make it clear that the peculiar properties of NH4+ can be explained if NH4+ experiences a different energetic landscape inside the pore rather than a fundamentally different permeation process.

In Kir1.1 it has been proposed that K+-dependent regulation occurs due to closure of the selectivity-filter similar to C-type inactivation of KcsA.10,28 C-type inactivation, or slow inactivation, was originally observed and studied in Shaker,19,43,48 and later it was found that a similar process occurs in KcsA.49 The rate of inactivation is on the order of seconds, and slows down in the presence of permeant extracellular cations.19,47 Detailed structural studies of KcsA11,14,45 and the computational studies that this information enabled,16,21,22 provide good evidence that C-type

**Figure 7.** Effect of [K+]o on Kir4.1 single channel conductance. (A) Representative current traces in cell-attached patches with 110 or 40 mM K+ in the pipette as indicated. (B) i-V relationships at the indicated [K+]o. (C) Single-channel conductance as a function of [K+]o. The curve represents the best fit to the equation: g = gmax/(1 + ([K+]/K1/2)n) where: K1/2 = 16.7 mM, gmax = 24.4 pS and n = 1.4.
Oocyte preparation. Oocytes were harvested from *Xenopus laevis* with approval of and in accordance with the guidelines of the IACUC of Weill Cornell Medical College. The animals were anesthetized through immersion into 1 L of tap water containing 1.5 gL⁻¹ tricaine methanesulphonate and HEPES (adjusted to pH 7.4). A small incision was made in the abdomen and part of the ovary removed. Oocytes were dissociated by incubation in OR2 solution (in mM: NaCl 82.5, KCl 2.5, CaCl₂ 1, MgCl₂ 1, Na₂HPO₄ 1, HEPES 5 pH 7.4) containing 2 mgml⁻¹ collagenase type II (Worthington) and 2 mgml⁻¹ hyaluronidase type II (Sigma-Aldrich) and incubated with gentle shaking for 60 min. Oocytes were then injected with 10 ng of RNA and incubated in L-15 solution supplemented with HEPES (pH adjusted to 7.45), penicillin 63 mgL⁻¹ and streptomycin 145 mgL⁻¹ in 16°C overnight. All chemicals were from Sigma-Aldrich unless otherwise noted.

Mutagenesis. Site-directed mutagenesis was performed using the HotStar High Fidelity Polymerase kit (Qiagen, Hilden, Germany) according to the manufacturer’s instructions. Primers were synthesized by Operon Technologies, Inc., (Huntsville, AL). The cDNA were sequenced by the Cornell University Bio Resource Center (Ithaca, NY, USA).

Two-electrode voltage clamp. For all experiments involving changes in [K⁺], the oocytes were incubated in the initial recording solution for 1 h prior to being placed in TEVC recording chamber. All recording solutions contained (in mM): 2 CaCl₂, 1 MgCl₂, 5 HEPES. Solutions with variable [K⁺] (in mM) 1 K, 4 K, 10 K, 40 K, 110 K were osmotically balanced by NaCl so that KCl + NaCl = 110. Other solutions contained (in mM): NMDG: 1 KCl, 109 NMDG; Rb: 110 Rb; Cs: 1 KCl 109 Na 10 CsCl; Ba: 1 KCl, 109 NaCl, 5 BaCl₂; Na: 1 KCl, 109 NaCl; NH₄: 110 NH₄Cl. The pH was adjusted to 7.4 for these solutions.

In conclusion, we have found K⁺-regulation in Kir-family member that is important in both neural and renal function. The recently described SESAME syndrome involves seizures and electrolyte imbalances due to loss-of-function mutations in Kir4.1. We suggest that activation by K⁺ may play a role in the regulation by glial cells of extracellular K⁺ in the brain.

**Methods**

Channel expression. Human Kir4.1 cDNA cloned into the pGEMHE-vector (a kind gift from Dr. Diomedes Logothetis) was linearized with NheI restriction enzyme (New England Biolabs, Ipswish, MA) and cRNA was made using mMESSAGE mMACHINE T7 kit (Ambion, Austin, TX).
55 KAcetate, or 10 KCl, 45 NaCl and 55 NaAcetate. The pH, was calculated from the pHi, as previously described, using the formula pHi = 0.595 x pHi + 2.4. Whole-cell currents were measured in intact oocytes using a two-electrode voltage clamp (OC-725, Warner Instrument Corp.,) with ITC-16 interface (Instrutech) running Pulse software (Heka Elektronik). Pipettes were made from haematocrit capillary tubes (Fisher Scientific) with a three-step vertical pipette puller. Pipette resistances were 0.5–1 MΩ, when filled with 3 M KCl. Steady-state current-voltage curves were generated from a step-voltage protocol consisting of 26 pulses lasting 50 ms from -150 to +100 mV, from a holding potential set to the resting membrane potential.

**Patch clamp.** For single-channel measurements the vitelline membrane was mechanically removed from the oocytes in a hypertonic 110 mM K+ bath solution containing 200 mM sucrose. The bath solution contained (in mM) 110 KCl, 2 CaCl2, 1 MgCl2, 5 HEPES, pH 7.4. The pipet solution contained (in mM): 1 MgCl2 and 5 HEPES, at pH 7.4. Patch-clamp pipets were prepared from hematocrit capillary glass (Fisher Scientific) using a vertical puller and fire polished with a microforge to yield resistances of 1–5 MΩ. Currents were recorded using an EPC-7/patt-clamp amplifier (Heka Elektronik) and digitized with a Digidata 1332A interface (Axon Instruments). Data were filtered at 0.5 kHz and analyzed with pCLAMP9 software (Axon Instruments). For analysis, data were filtered at 1 kHz, and analyzed using Pulsefit software (HEKA Elektronik).

**Kinetic modeling.** The model shown in Figure 8 was evaluated using Matlab 7.8.0. The rate constants were calculated based on the values set for binding energies, energy barriers and electrical distances. For example, for kDE, the transition between D and E:

\[
k_{DE} = Q \cdot \exp(-\Delta G_{DE} + \delta_D + \delta_E)
\]

**Eqn. 1**

where \(\Delta G_{XY}\) is the height of the energy barrier between sites X and Y, \(E_D\) and \(E_E\) is the binding energy for site \(S_X\) and Q is the frequency factor arbitrarily set to 1 x 10\(^7\). The reverse rate constant was then calculated:

\[
k_{ED} = k_{DE} \cdot \exp((E_D + E_E) - (E_D + E_E))
\]

**Eqn. 2**

A repulsion energy between the ion in the cavity and S4, \(E'\) (2 R-T), was added to state A. A voltage dependence was added to each of the rate constants. For example:

\[
k_{DE} = k_{DE} \cdot \exp(\frac{\delta_D - \delta_E}{RT})
\]

**Eqn. 3**

where \(\delta\) indicates the fraction of the electric field that needs to be crossed to reach the site from the neighboring site (Fig. 9C). The values for \(\delta_1, \delta_2, \delta_3\) and \(\delta_4\) were 0.07, 0.113, 0.233, respectively, as used in a previous study of KcsA channels.\(^{46}\) \(V_m\) is the membrane potential in volts, F is Faraday’s constant, R is the ideal gas constant, and T is temperature in degrees Kelvin. In the above example, the transition involves movement of two ions the electrical distance, \(\delta\). The voltage dependence was partitioned equally between the forward and reverse rate constants. The rates were then entered into a rate matrix and solved for the steady-state occupancy.

The model with two ionic species was solved using the same approach with a separate set of parameters for binding energy and energy barriers used for Cs+ or NH\(_4\)+. The model was fitted to the appropriate experimental data using MatLab’s built in lscurvefit function. To minimize the effect of the initial values provided to the fitting routine, each fit was repeated 10–100 times, with the initial values randomized each time. The numbers for the fit in **Supplemental Table 1** are given as mean ± SD. The \(K^+\) single-channel data points used to fit the model were corrected for offsets from the predicted reversal potential.

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**Note**

Supplemental materials can be found at: www.landesbioscience.com/journals/channels/article/15827
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