Molecular Basis of Decreased Kir4.1 Function in SeSAME/EAST Syndrome

David M. Williams,* Coeli M.B. Lopes,† Avia Rosenhouse-Dantsker,‡ Heather L. Connelly,* Alessandra Matavel,* Jin O-Uchi,† Elena McBeath,† and Daniel A. Gray*

*Department of Medicine, Nephrology Division, and †Aab Cardiovascular Research Institute, University of Rochester, Rochester, New York; and ‡Department of Medicine, Section of Pulmonary, Critical Care and Sleep Medicine, University of Illinois at Chicago, Chicago, Illinois

ABSTRACT
SeSAME/EAST syndrome is a channelopathy consisting of a hypokalemic, hypomagnesemic, metabolic alkalosis associated with seizures, sensorineural deafness, ataxia, and developmental abnormalities. This disease links to autosomal recessive mutations in \( KCNJ10 \), which encodes the Kir4.1 potassium channel, but the functional consequences of these mutations are not well understood. In \textit{Xenopus} oocytes, all of the disease-associated mutant channels (R65P, R65P/R199X, G77R, C140R, T164I, and A167V/R297C) had decreased \( K^+ \) current (0 to 23% of wild-type levels). Immunofluorescence demonstrated decreased surface expression of G77R, C140R, and A167V expressed in HEK293 cells. When we coexpressed mutant and wild-type subunits to mimic the heterozygous state, R199X, C140R, and G77R currents decreased to 55, 40, and 20% of wild-type levels, respectively, suggesting that carriers of these mutations may present with an abnormal phenotype. Because Kir4.1 subunits can form heteromeric channels with Kir5.1, we coexpressed the aforementioned mutants with Kir5.1 and found that currents were reduced at least as much as observed when we expressed mutants alone. Reduction of \( pH \) from approximately 7.4 to 6.8 significantly decreased currents of all mutants except R199X but did not affect wild-type channels. In conclusion, perturbed \( pH \) gating may underlie the loss of channel function for the disease-associated mutant Kir4.1 channels and may have important physiologic consequences.


Linkage analysis recently identified, in two separate reports, mutations in the \( KCNJ10 \) gene that were associated with seizures, sensorineural deafness, ataxia, mental retardation, and electrolyte abnormalities, termed SeSAME,\(^1\) or equivalently EAST\(^2\) (for epilepsy, ataxia, sensorineural deafness, and \( tubulopathy \) syndrome). \( KCNJ10 \) codes for Kir4.1, a member of the family of inwardly rectifying \( K^+ \) channels,\(^3,4\) which is present in the brain, inner ear, retina, and kidney.\(^5,5–13\) The electrolyte abnormalities are akin to Gitelman’s syndrome\(^14\) and consist of hypokalemia, hypomagnesemia, metabolic alkalosis, hypocalciuria, and secondary hyperaldosteronism associated with excess urinary loss of \( K^+ \), \( Na^+ \), and \( Mg^{2+} \). The disease has an autosomal recessive pattern of inheritance. Seven mutations in six families were identified with affected individuals either homozygous for R65P, G77R, C140R, or T164I or having the compound heterozygous mutations R65P/R199X or A167V/R297C (Figure 1A).

The Kir4.1 knock-out mouse suffers from seizures, movement disorders, hearing loss, vision ab-
Figure 1. Mutant Kir4.1 channels show markedly decreased function. (A) Structure of Kir4.1 with SeSAME/EAST mutations indicated. (B) I-V curves for Kir4.1 expressed in Xenopus oocytes as bath [K⁺] was lowered from 110 to 4 mM followed by the addition of 5 mM Ba²⁺. Steady-state currents at each stage were measured in response to a series of voltage steps from −140 to +80 mV from a holding potential of −80 mV. I-V curves for each batch of oocytes were normalized by the mean current at +40 mV with 110 mM K⁺ in the bath. The error bars represent SEM for at least 12 oocytes from at least two batches. (C) In the upper panel, representative current traces for WT and SeSAME/EAST syndrome mutants expressed in oocytes. The two compound heterozygotes, R65P/R199X and A167V/R297C, were formed by 1:1 coexpression of the component mutant subunits. The currents were measured as in B with 4 mM K⁺ in the bath. In the lower panel, normalized I-V curves were generated from the current traces above. The currents were leak-subtracted and normalized by the WT current at +40 mV for that day. R65P, R65P/R199X, and A167V/R297C had residual currents of 23, 17, and 13%, respectively, of WT current (at +40 mV), whereas the other mutants had currents similar to H2O-injected oocytes. The error bars indicate SEM for at least 14 oocytes from at least two batches. (D) Representative immunoblot (upper panel) of oocyte lysates probed with a polyclonal Kir4.1 antibody showing a doublet/triplet band at approximately 42 kD as well as a higher molecular mass singlet/
normalities, and urinary Na\textsuperscript{+} wasting.\textsuperscript{2,6–8,10,13} Astrocyte-glial Kir4.1 appears to be involved in reuptake of extracellular K\textsuperscript{+} after an action potential, a process termed “spatial buffering.”\textsuperscript{7,12} Failure of which is thought to result in membrane depolarization and lowered seizure threshold. Kir4.1 also participates in oligodendrocyte development/myelinization and endolymph formation.\textsuperscript{7,13}

The presence of hypomagnesemia suggests that Kir4.1 plays an important functional role in the distal convoluted tubule (DCT), the site of active Mg\textsuperscript{2+} reabsorption.\textsuperscript{15,16} Indeed, immunolocalization and patch clamp studies have demonstrated Kir4.1, perhaps associated with Kir5.1, on the basolateral surface of K\textsuperscript{+} secreting cells of the distal nephron.\textsuperscript{2,9,17–22} Loss of Kir4.1 function in the DCT would be expected to depolarize the basolateral membrane and reduce recycling of K\textsuperscript{+} across this surface. The renal phenotype implies that this is coupled to decreased apical reabsorption of Mg\textsuperscript{2+} and Na\textsuperscript{+}, leading to increased Na\textsuperscript{+}-associated K\textsuperscript{+} secretion in the connecting tubule and collecting ducts.\textsuperscript{1}

Kir4.1 function is modulated by pH\textsubscript{i}, phosphatidylinositol 4,5-bisphosphate (PIP\textsubscript{2}), and extracellular potassium.\textsuperscript{23} It is relatively insensitive to pH\textsubscript{i} within the physiologic range, with a pK\textsubscript{a} of approximately 6.0 in excised patches, but it becomes much more sensitive when coexpressed with Kir3.1, with pK\textsubscript{a} increasing to 7.35,\textsuperscript{24} Despite extensive study, the pH gating mechanism within the Kir family remains incompletely understood. Recently, a K67–T164 gating mechanism has been proposed in which H\textsuperscript{+}-bonding between these residues at the helix-bundle crossing stabilizes the closed state of the channel.\textsuperscript{25} Mutations that modulate the interaction between these residues would, according to this model, alter pH sensitivity.

To better understand the mechanisms underlying the decreased Kir4.1 function in the SeSAME/EAST syndrome, we have studied all of the reported Kir4.1 mutations linked to disease in Xenopus oocytes. All of the mutants were found to have decreased function when expressed alone and when coexpressed with Kir3.1. Decreased surface expression was seen for G77R, C140R, and A167V in transfected HEK293 cells. Coexpression with WT (in oocytes), to mimic the heterozygous state, revealed a partial dominant-negative effect of R297C on A167V. These decreases in current were more substantial than those seen for G77R, C140R, and A167V/R297C, perturbed pH gating may underlie the increased pH sensitivity and loss of channel function. The physiologic implications of these results in the affected patients, their parents, and the general population is discussed.

RESULTS

Mutant Channels Demonstrate Substantially Decreased Function

To assess the effect of the mutations on channel function, we expressed wild-type (WT) and mutant Kir4.1 subunits in Xenopus oocytes. Using two-electrode voltage clamp, we measured Ba\textsuperscript{2+}-sensitive K\textsuperscript{+} currents in response to a series of voltage steps (Figure 1B). Mildly rectified currents, typical of Kir4.1, are seen in high K\textsuperscript{+} bath solution with less rectified currents observed in low K\textsuperscript{+} solution. Henceforth, low K\textsuperscript{+} bath solution was used to reproduce the extracellular milieu of this channel. All six disease-associated mutations showed decreased current ranging from 0 to 23% of WT (Figure 1C). Channels formed by R65P, R65P/R199X, and A167V/R297C had small residual currents with increased rectification, and the rest had negligible currents. Immunoblots of oocyte lysates showed a doublet/triplet band near the expected molecular mass of Kir4.1 (42 kD) as well as a higher molecular mass band of approximately 200 kD (Figure 1D), neither of which was seen in water-injected oocytes. Total protein expression of most mutants was similar to WT. G77R had approximately 75% of WT expression, but this small reduction cannot account for the nearly complete lack of current observed (Figure 1C). Expression for R65P/R199X also appeared reduced, but much of this may be accounted for by the fact that the R199X subunit is not detected by the C-terminal antibody. The higher molecular mass band, which may correspond to a tetrameric form of the channel, was minimally present in G77R and present to a greater extent in T164I. To examine the contribution of each allele to compound heterozygote mutant function, currents from each component mutation were measured separately (Figure 1E). For R65P/R199X, both mutations strongly decreased channel current individually, with R199X having essentially no detectable current when expressed alone. Coexpression of these subunits yielded currents intermediate between the two mutants expressed separately. For A167V/R297C, channels formed by A167V had only mildly reduced current (approximately 60% of WT), whereas those formed by R297C had essentially no current. The resultant current with coexpression was also significantly reduced, suggesting a partial dominant-negative effect of R297C on A167V. These decreases in current cannot be explained by reduced protein expression (Figure 1F).

G77R, C140R, and A167V Show Decreased Surface Expression in HEK293 Cells

HEK293 cells, transfected with mutant subunits, were fixed and probed with a Kir4.1 antibody. Confocal immunofluorescence microscopy showed mild surface expression of the WT channel (Figure 2A). Significantly decreased surface expres-
sion was seen for G77R, C140R, and A167V, which was typically associated with a reticular intracellular distribution, particularly for the former two (Figure 2, A and B). Increased surface expression was seen with most of the other mutants. Coexpressing Mutants with WT Subunits Restores WT Function for Several Mutants, But for G77R and C140R, a Partial Dominant Negative Effect Is Seen

To test for a potential phenotype in carriers of SeSAME/EAST mutations, WT cRNA was injected either alone (WT/WT) or in a 0.5:0.5 molar ratio with each mutant (WT/mutant) or H2O (WT/H2O) to mimic the homozygous, carrier, and haplo-insufficient states, respectively. WT/R199X, WT/C140R, and WT/G77R had reduced currents compared with WT/WT of 55, 40, and 20%, respectively (Figure 3, A and B), raising the possibility of a phenotype in carriers of these mutations. Note that although R297C exerted a dominant-negative effect on A167V (Figure 1E), this was not the case when it was coexpressed with WT. WT/R199X had current indistinguishable from WT/H2O, suggesting that R199X, with its large C-terminal deletion, does not contribute significantly to formation of functional channels. Currents for WT/C140R and WT/G77R were significantly less than the haplo-insufficient mimic (WT/H2O), consistent with a partial dominant-negative effect. In contrast, WT/R65P, WT/T164I, WT/A167V, and WT/R297C had currents indistinguishable from WT/WT. Decreased protein expression can account for some, but not all, of the decreased current seen with WT/G77R, whereas WT/C140R displayed ample protein expression (Figure 3C).

Mutant Subunits Show a Further Decrease in Function when Coexpressed with Kir5.1

In addition to forming homomeric channels, Kir4.1 also forms functional heteromeric channels with Kir5.1 in the brain, inner ear, retina, and distal nephron. To test for the effect of Kir5.1 on mutant function, Kir5.1 and mutant channels were coinjected in a 10:1 ratio. An excess of Kir5.1 was used to minimize the contribution from Kir4.1 homomeric channels. Kir5.1 does not form functional homomeric channels in oocytes (although these have been reported in brain), and indeed, no current was detected when Kir5.1 was coinjected with H2O (Figure 4A). The increased pH sensitivity of the Kir5.1/Kir4.1 channel relative to the Kir4.1 homomeric
channel was used to confirm the formation of heteromeric channels with coexpression (Figure 4B). All of the coexpressed channels had very reduced function with only Kir5.1/A167V and Kir5.1/R65P having measurable currents. In contrast to the enhancement of current seen for many of the mutants when coexpressed with WT Kir4.1 (Figure 3B), currents for R65P, R65P/R199X, A167V, and A167V/R297C had a stronger decrease in function when coexpressed with Kir5.1 than when expressed alone (Figure 4C). The decreased function seen when mutant subunits were coexpressed with Kir5.1 suggests that heteromeric channels may also be affected by this syndrome. Although decreases in protein expression relative to WT are seen for some of the coexpressed mutants (Figure 4D), this reduction alone cannot account for the marked decreased function observed (Figure 4A).

Mutant Channels Have Increased pH Sensitivity
Because Kir4.1 is known to be regulated by pH, we studied the effect of mild intracellular acidification on mutant and WT subunits at two different pH levels. A cell-permeable, acetate buffer was used to nominally set pHi to either 7.4 or 6.8. Oocytes expressing WT or mutant subunits were incubated for 1 hour in one buffer or the other, and the resulting currents were then compared (Figure 5). WT channels were unaffected by this degree of acidification, consistent with published reports. In contrast, currents from R65P, A167V, R65P/R199X, and A167V/R297C were essentially abolished at the lower pH (Figure 5A). This likely reflects a shift in pH sensitivity to more alkaline values and may contribute to the decreased currents seen with these mutants under control pH conditions (Figure 1C). The pH of DCT in rabbit has been shown to be in the 7.2 to 7.3 range on the basis of fluorescent-dye measurements. However, in a rodent model of hypokalemic metabolic alkalosis, of which SeSAME/EAST syndrome is an example, evidence for paradoxical uptake of protons and efflux of K⁺ from cells systematically has been reported. If DCT cells and glia participate in such a process, an exacerbation of the SeSAME/EAST phenotype would be expected. The heterozygous mimics (except for WT/R199X) also displayed substantially increased sensitivity to pH (Figure 5B), suggesting that under conditions associated with decreased intracellular pH, such as hypokalemia or metabolic acidosis (as with diarrhea), they too may show a phenotype.

While this manuscript was under review, two other reports appeared investigating SeSAME/EAST syndrome mechanisms. Reichold et al. studied R65P, G77R, R199X, and a new mutation, R175Q, in mammalian cell lines under patch clamp. They reported functional deficits similar to those described here, both when the mutants were expressed alone and when they were coexpressed with Kir5.1. They observed decreased open probabilities for the mutants and a robust alkaline shift in pH sensitivity, consistent with the results presented here, for R65P and R175Q in excised patches. Tang et al. investigated most of the published SeSAME/EAST mutations in HEK293 cells (G77R and the compound heterozygous mutations were not studied), and they showed decreases in current, qualitatively similar to our results. Although both our group and Reichold et al. saw a further decrease in fractional current when Kir5.1 was coexpressed...
Figure 3. Coexpressing mutants with WT subunits restores WT function for R65P, T164I, A167V, and R297C, but for G77R and C140R, a partial dominant negative effect is seen. (A) Normalized I-V curves for WT subunits expressed alone (WT/WT) or coexpressed in a 0.5:0.5 molar ratio with mutants (WT/mutant) or H2O (WT/H2O) to mimic the homozygous, carrier, and haplo-insufficient states, respectively. (B) Normalized currents derived from the I-V curves in A measured at +40 mV. WT/R199X, WT/C140R, and WT/G77R had currents that were 55, 40, and 20%, respectively, of WT/WT. *Significant versus WT (P < 0.05). WT/C140R and WT/G77R had currents significantly less than WT/H2O, indicated by a plus sign (P < 0.05). The error bars for A and B indicate SEM for at least 14 oocytes from at least two batches.
with mutants in a 10:1 ratio, Tang et al.\textsuperscript{38} saw less of a decrease in current using a 1:1 coexpression ratio. This group also found that coexpression of the mutants 1:1 with WT led to "rescue" of all currents (including those arising from WT/C140R and WT/ R199X) to WT/WT levels, whereas we found reductions relative to WT/WT of 40% and 55%, respectively, for these two constructs (Figure 3B). Whether their use of green fluorescent protein-tagged channel subunits (versus our untagged constructs) or whether differences in expression system led to this discrepancy is unclear. Tang et al.\textsuperscript{38} also showed a representative blot of cell-surface biotinylated proteins probed with anti-green fluorescent protein antibody. No surface expression was seen with R199X, consistent with our data which suggested that R199X does not contribute to the formation of functional channels. The other mutants showed expression that appeared less than WT on this single blot. In contrast, whereas we saw decreased surface expression for three of the mutants (G77R, C140R, and A167V) using quantitative immunofluorescence, the other mutants had surface expression that was equal to or greater than WT (Figure 2).

The occurrence of two compound heterozygous mutations within the six reported kindreds suggests that some of the Kir4.1 mutations may have appreciable allele frequencies within the general population where, given the tissue distribution of Kir4.1, they may contribute importantly to polygenetic traits such as epilepsy and hearing loss.\textsuperscript{39,40} Given the Na\textsuperscript{+} wasting observed in SeSAME/EAST syndrome patients, carriers of these mutations may also, on average, have lower BP. Consistent with this notion, lower BP levels were recently reported in carriers of Bartter’s and Gitelman’s syndrome mutations of the Na\textsuperscript{+}/K\textsuperscript{+}/2Cl\textsuperscript{−} cotransporter, the Na\textsuperscript{+}/Cl\textsuperscript{−} cotransporter, and Kir1.1 among Framingham Heart Study patients.\textsuperscript{41}

**Molecular Modeling of Mutant Channels**

To gain greater insight into the molecular mechanisms underlying the decreased function in the disease mutations, molecular modeling was performed. A homology model of Kir4.1 was constructed on the basis of the crystallographic structure of the chimera formed by the transmembrane domains of the bacterial inwardly rectifying K\textsuperscript{+} channel, KirBac1.3, and the cytosolic domains of the mammalian inwardly rectifying K\textsuperscript{+} channel, Kir3.1.\textsuperscript{42} The functional, tetrameric channel indicating the location of the disease-associated mutations, and the neighboring residues close enough to interact (i.e., within 4 Å), is shown in Figure 6A. The transmembrane residues R65 (which is highly conserved across the Kir family), T164, and A167 all cluster close to the cytosolic domain. It has been previously shown that K67 interacts through H\textsuperscript{+}-bonding with T164 and that mutation of K67 affects the pH sensitivity of the channel.\textsuperscript{23,25} Given the proximity of R65 to K67 and of A167 to T164, the altered pH sensitivity of these mutant channels may originate from the same source.

Minimization of the WT structure showed that K67 and T164 interact via intrasubunit hydrogen bonding in the closed state of the channel (Figure 6B, left panel). In the transition to the open state, K67 and T164 are thought to move away from each other.\textsuperscript{25} Because the bulky K168 appears to prevent downward movement of K67, it is likely that K67 moves in an upward direction when the channel opens (Figure 6B, left panel). Minimization of the T164I mutant structure (Figure 6B, right panel) suggests that this larger, more hydrophobic residue may impede movement of K67 away, thereby locking the channel in the closed state.

R297 is conserved across the Kir family. A robust alkaline shift in pH sensitivity was reported when this residue was mutated to lysine or glutamine in Kir4.1 or with mutation of the corresponding residue in Kir1.1 (R311Q/W), the latter being associated with Bartter’s syndrome.\textsuperscript{43} R297 is in the cytosolic domain, at the interface between two subunits, but within 4 Å of R175. Because A167 is within 4 Å of R171, R297 may be coupled to the pH gating residue at T164 through these intervening interactions. The increased pH sensitivity of R297C coexpressed with WT versus WT alone (Figure 5B) and the dominant-negative effect of R297C on A167V (Figure 1E) but not WT (Figure 3B) are consistent with such a model. In addition, mutation of the corresponding residue in Kir1.1, Kir2.1, and Kir6.2 has been shown to affect channel-PIP\textsubscript{2} interactions and result in decreased channel function.\textsuperscript{41,44,45} It is likely that R297C has a similar effect in Kir4.1. Altered PIP\textsubscript{2} interactions at the C terminus may be coupled to channel gating through intervening residues, as above, consistent with reports that pH and PIP\textsubscript{2} effects may be transduced through a common gate within this family.\textsuperscript{23,25} The recent report of R175Q causing SeSAME/EAST syndrome and showing increased pH sensitivity is not surprising given this model.\textsuperscript{37} Our model does not predict an intersubunit salt bridge between R297 and E288, although such an interaction has been reported between the corresponding residues (R311 and E302) in Kir1.1 on the basis of an older crystal structure.\textsuperscript{46} Functional studies must be done to test our model prediction, but if born out, the lack of interaction may reflect important structural differences between Kir1.1 and Kir4.1.

G77 is located in the center of TM1, facing the membrane. Mutation to a positively charged arginine would be unfavorable in the hydrophobic environment of the membrane and may lead to reorientation of TM1. A sufficiently perturbed helix may result in a subunit that is degraded or that does not form stable tetramers. Indeed, decreased surface expression of G77R was seen in HEK293 cells (Figure 2). The partial dominant-negative effect seen when G77R was coexpressed with WT (Figure 3B) may reflect the relative intolerance of the functional tetramer to assemble with such mutant subunits. Alternatively, the dominant-negative effect may reflect disruption of the channel pore because mutation of a leucine,
adjacent to the corresponding residue in KCNQ1, has been shown to affect the selectivity filter via interactions with the pore helix.47

C140 is located in the extracellular domain of the channel, between the selectivity filter and TM2, and is conserved among all Kir channels. C140 is located across from absolutely conserved A90 on the same subunit and L97 on an adjacent subunit. The complete loss of function seen with C140R (Figure 1C), as well as that reported with mutation of the correspond-

Figure 4. Mutant subunits show a further decrease in function when coexpressed with Kir5.1. (A) Normalized I-V curves for Kir5.1 coexpressed (10:1) with either WT or mutant Kir4.1 subunits. Kir5.1/A167V and Kir5.1/R65P have 40 and 13%, respectively, of Kir5.1/WT current (at +40 mV), and the remaining coexpressed mutants, as well as Kir5.1 injected alone (Kir5.1/H2O), have currents similar to uninjected oocytes. The error bars indicate SEM for at least 14 oocytes from at least two batches. (B) pH sensitivity of WT Kir4.1 alone or coexpressed, as above, with Kir5.1. The oocytes were incubated for 1 hour in a cell-permeable buffer titrated to set pHi to either approximately 7.4 or 6.8. Shown are the mean IV curves normalized by the current for Kir4.1 with pHi 7.4 at +40 mV. The error bars indicate SEM for at least 10 oocytes from three batches. (C) Normalized currents (at +40 mV) derived from I-V curves as in A or Figure 1C for the mutants with currents large enough to measure when expressed alone. Coexpression with Kir5.1 led to a significant decrease in fractional current for all of the indicated mutant subunits beyond that seen when these mutants were expressed alone. The error bars indicate SEM for at least 14 oocytes from at least two batches. *Significant (P < 0.05). (D) Representative immunoblot (upper panel) and densitometric quantification (lower panel) of lysates from the coexpressed mutants shown above and analyzed as in Figure 1D. The error bars represent SEM for lysates from three oocyte batches.
ing residue in Kir1.1 underscores its critical role. The corresponding residue in Kir2.1 has been shown to interact with another conserved extracellular cysteine (corresponding to C108 in Kir4.1), forming a disulfide bond felt to be important for stabilizing the selectivity filter and for proper protein folding. Disruption of these processes may account for the decreased surface expression seen with C140R (Figure 2).

R199X results in a deletion of a large portion of the C terminus including a PDZ protein interaction motif shown to be important for basolateral localization of the channel. R199X displayed no current when expressed alone (Figure 1E), and when coexpressed with WT, its current magnitude (Figure 3B) was indistinguishable from WT/H2O, suggesting that it does not contribute to the formation of functional channels.

The results of these experiments provide potentially important insights into the molecular mechanisms of decreased Kir4.1 function in the SeSAME/EAST syndrome. However, we must be cautious, as always, in extrapolating results performed in heterologous systems to native tissue and human disease.

**Conclusion**

Mutations in Kir4.1 associated with the SeSAME/EAST syndrome have decreased function both as homomeric channels and as heteromeric channels formed with Kir5.1. For disease-associated mutants G77R and C140R, decreased surface expression may contribute to the reduced function seen. For R65P, R65P/R199X, T164I, and A167V/R297C, the markedly increased pHi sensitivity, which appears to arise from perturbed pH gating, likely underlies the phenotype. These results may have important physiologic implications for these patients, their parents, and carriers in the general population.

**CONCISE METHODS**

**Molecular Biology**

hKir4.1 (NM_002241) and rKir5.1 (AF249676), generous gifts from D. E. Logothetis and L. G. Palmer, respectively, were subcloned into the pGEMSH vector (modified from pGEMHE vector) for oocyte expression. Site-directed mutagenesis was performed using de-

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**Figure 5.** Mutant channels display increased pH sensitivity. (A) Normalized I-V curves for oocytes incubated for 1 hour in a cell-permeable buffer titrated to nominally set pHi to either 7.4 or 6.8. Basal currents at pH 7.4 for R65P, A167V, R65P/R199X, and A167V/R297C are essentially abolished with acidification, whereas WT currents are unaffected. (B) Mutants with basal currents at pH 7.4 that were too small to evaluate were coexpressed 1:1 with WT (as in Figure 3) and then tested. Normalized I-V curves are shown as in A. With acidification, basal currents for WT/T164I and WT/R297C were reduced approximately 80%. Currents for WT/R65P, WT/A167V, WT/G77R, and WT/C140R were reduced approximately 50%, and WT/R199X was unaffected. The error bars indicate SEM for at least 14 oocytes from at least two batches.
Figure 6. Disrupted pH gating may contribute to the loss of channel function for several SeSAME/EAST syndrome mutants. (A) Homology model of the Kir4.1 tetramer, derived from the crystallographic structure of the chimera formed by KirBac1.3 (transmembrane domain) and Kir3.1 (intracellular N and C termini). The mutation locations are indicated by the small, lighter colored area near each label while the regions within 4 Å of the mutant residues are shown with the darker hue. R65 and G77 are on the outer transmembrane helix (TM1), and T164 and A167 are on the inner transmembrane helix (TM2). C140 is on the extracellular domain of the channel, between the selectivity filter and the activation may arise in part from the small increase in pHi that occurs approximately 30% (data not shown). This activation does not affect the any of the mutants except R65P, where currents were activated by ap- proximately 30% (data not shown). 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This activation does not affect the conclusion that R65P currents are blocked by mild intracellular acidifi- cation was determined (Figures 4B and 5) using a cell-permeable acetate buffering system validated with intracellular pH electrode measurements in Xenopus oocytes.32 The bath solutions contained 2 mM KCl, 2 mM potassium acetate, 53 mM NaCl, 53 mM sodium acetate, 2 mM CaCl2, and 1 mM MgCl2, with pH adjusted to 9.0 or 7.4 with NaOH to set intracellular pH nominally to 7.4 or 6.8, respectively. The latter solution maintained a normal extracellular pH of 7.4 while setting pHi to 6.8, suggesting that the nearly complete block of current observed for the mutants (Figure 5A) resulted from intracellular pH changes. However, the solution used to set pHi to 7.4 had a pH of 9.0, which could potentially activate the channel extracellularly. To control for this, currents were measured using standard bath solution (as above), containing the mini- mally cell-permeable HEPES buffer, adjusted to pH 9.0 or 7.4 with NaOH. No significant activation by extracellular pH was observed for any of the mutants except R65P, where currents were activated by approxi mately 30% (data not shown). This activation does not affect the conclusion that R65P currents are blocked by mild intracellular acidifi- cation. (In addition, because HEPES is not completely cell-impermeable, the activation may arise in part from the small increase in pH that occurs when extracellular pH is increased from 7.4 to 9.0.)
Immunoblotting of Oocyte Lysates

Oocytes lysates were prepared as described previously. Briefly, the oocytes were homogenized 48 hours after injection with cRNA. Ten oocytes were washed once in oocyte homogenization buffer (80 mM sucrose, 1 mM EDTA, 20 mM Tris/HCl, pH 7.4) and then homogenized in 20 μl/oocyte of homogenization buffer containing a protease inhibitor cocktail diluted 1:50 (Sigma). The oocytes were lysed by passage through a 25-gauge needle 20 times. The lysates were centrifuged twice at 200 × g for 5 minutes at 4°C. The supernatant was collected after each spin. It was then centrifuged at 14,000 × g for 20 minutes at 4°C. The pellet was resuspended in 4 μl/oocyte of Laemmli buffer (25 mM Tris, 192 mM glycine, 0.1% w/v SDS) containing the protease inhibitor cocktail as above. Immunoblots were performed in the standard fashion. 5 μl of oocyte lysate was mixed with 5 μl of sample buffer (66 mM Tris/HCl, pH 6.8, 26% glycerol, 2% SDS, 0.1% bromophenol blue) containing 100 mM dithiothreitol (final concentration), heated at 37°C for 10 minutes, resolved on a 10% polyacrylamide gel (Bio-Rad), and transferred to a nitrocellulose membrane (Bio-Rad). The membranes were blocked for 1 hour in 3% milk in TBST (20 mM Tris/HCl, pH 7.6, 137 mM NaCl, 0.1% Tween 20) and then probed with a C-terminal polyclonal antibody to Kir4.1 antibody (diluted 1:500) (Alomone Labs) followed by goat anti-rabbit IRDye 680 (diluted 1:20,000) (LI-COR). The membranes were imaged on an infrared imaging system (LI-COR), and densitometric analysis was performed using Image J software (National Institutes of Health).

HEK Cell Immunofluorescence

HEK293 cells were grown to approximately 75% confluence on 3.5-cm dishes in DMEM high glucose (Mediatech), 1% GlutaMAX (Invitrogen), 10% FBS (Equitech-Bio). A total of 2 μg of DNA was used to transfect each dish. For compound heterozygous mutations, 1 μg of each component subunit was used. DNA was combined with 100 μl of 1 × Opti-MEM (Invitrogen). 5 μl of FuGENE HD transfection reagent (Roche) was added to the DNA/Opti-MEM mixture and incubated for 15 minutes at room temperature (RT). The samples were then added drop-wise to dishes. Four to 6 hours post-transfection, the cells were split 1:2 and plated onto 22 × 22-mm sterile glass coverslips. Twenty-four hours post-transfection, the coverslips were washed in ice-cold PBS. 2 ml of 3.7% formaldehyde solution in PBS was added to each dish and incubated at RT for 10 minutes. Formaldehyde solution was removed, and 2 ml of 0.5% Triton X-100 in TBST was added to each dish and incubated at RT for 10 minutes. The dishes were then washed five times, 3 minutes each time, in TBST. The coverslips were incubated with a C-terminal polyclonal antibody to Kir4.1 (1:200) (Alomone Labs) for 1 hour followed by Alexa-fluor 488 goat anti-rabbit IgG (1:1000) (Invitrogen). The coverslips were washed, mounted with Vectashield mounting medium (Vector Laboratories), and imaged by confocal microscopy. Plasma membrane localization of WT and mutant Kir4.1 was quantified by taking the ratio of membrane to cytoplasmic fluorescence intensity as follows: membrane/cytosolic intensity = [peak membrane intensity – background]/[mean cytosolic intensity – background]. The intensities were determined by line scan measurements through each cell using Image J software. The cytosolic intensity was calculated as the mean intensity over a distance two membrane thicknesses into the cell. Background was determined as the mean intensity two membrane thicknesses outward from the cell. A single membrane/cytosolic intensity measurement was made per cell in a blinded fashion, and the mean ratio for each mutant was then normalized by the mean WT ratio for that day.

Series Resistance

Oocyte batches had average expression of wild-type Kir4.1 current ranging from 11 to 42 μA, measured at + 40 mV. Robust expression was necessary to compare WT to mutant channel currents. At this level of expression, a significant voltage drop may occur from the series resistance of the bath. We estimated series resistance to be 250 to 500 Ω for our experimental configuration, corresponding to a maximal error of less than 20% in the estimation of the ratio between the wild-type and mutant currents measured at + 40 mV, and an average overestimation of mutant currents of less than 10%. This does not substantively change any of the results presented. The decrease in current observed for the mutants was not dependent on the level of channel expression (data not shown).

Statistical Analyses

Mutant and WT currents were always compared using oocytes from the same batch, and at least two oocyte batches were used for each study. The error bars represent SEM. The t test (two groups) or one-way ANOVA (multiple groups) followed by the Dunnett test (for single comparisons) were applied for the assessment of statistical significance using P < 0.05.

Molecular Modeling

The crystallographic structure at 2.2 Å resolution of the chimera formed by the transmembrane domain of KirBac1.3 and the cytosolic domain of Kir3.1 (Protein Data Bank accession number 2QKS) was used as the basis for the homology model of Kir4.1. An alignment between the chimeric and hKir4.1 shows 33% identical and 63% identical or similar residues (data not shown). The side chains were constructed using CHARMM. The orientations of the side chains of the structure obtained were then minimized using the Steepest Descent and the adopted-basis Newton Raphson algorithms as implemented in CHARMM. Minimization of the T164I mutant was carried out similarly. The environment was modeled by a distance-dependent dielectric. The force field for the energy calculation was the CHARMM force field.

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DISCLOSURES

None.
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