RNA editing in *eag* potassium channels

Biophysical consequences of editing a conserved S6 residue

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RNA editing at four sites in *eag*, a Drosophila voltage-gated potassium channel, results in the substitution of amino acids into the final protein product that are not encoded by the genome. These sites and the editing alterations introduced are K467R (Site 1, top of the S6 segment), Y548C, N567D and K699R (Sites 2–4, within the cytoplasmic C-terminal domain). We mutated these residues individually and expressed the channels in Xenopus oocytes. A fully edited construct (all four sites) has the slowest activation kinetics and a paucity of inactivation, whereas the fully unedited channel exhibits the fastest activation and most dramatic inactivation. Editing Site 1 inhibits steady-state inactivation. Mutating Site 1 to the neutral residues resulted in intermediate inactivation phenotypes and a leftward shift of the peak current-voltage relationship. Activation kinetics display a Cole-Moore shift that is enhanced by RNA editing. Normalized open probability relationships for 467Q, 467R and 467K are superimposable, indicating little effect of the mutations on steady-state activation. 467Q and 467R enhance instantaneous inward rectification, indicating a role of this residue in ion permeation. Intracellular tetraethylammonium blocks 467K significantly better than 467R. Block by intracellular, but not extracellular, tetrathysoammonium interferes with inactivation. The fraction of inactivated current is reduced at higher extracellular Mg2+ concentrations, and channels edited at Site 1 are more sensitive to changes in extracellular Mg2+ than unedited channels. These results show that even a minor change in amino acid side-chain chemistry and size can have a dramatic impact on channel biophysics, and that RNA editing is important for fine-tuning the channel's function.

**Introduction**

An organism's genetic code is deciphered according to the central dogma of genetics: DNA to RNA to protein. However, the enzyme adenosine deaminase acting on RNA (ADAR) encroaches upon this process during transcription. ADAR binds to specific double-stranded pre-mRNA structures and deaminates particular adenosine (A) nucleotides to inosines (I;1). Inosines are then read as guanosines by the translational machinery. This single-nucleotide change can have various consequences such as the creation of an alternative splice site or alteration of RNA stability.2 The A→I conversion can also produce single amino acid changes in the resultant protein, thus potentially altering protein function. Following a systematic screen for RNA editing activity in varied protein types, Hoopengardner et al. found that this type of editing occurs in voltage-gated and ligand-gated ion channels, and in synaptic vesicle release proteins.3 Voltage-gated potassium (Kv) channels are a part of this group of proteins and play an important role in neuronal excitability.

With a growing list of naturally occurring amino acid substitutions due to RNA editing, ion channel researchers are presented with another layer of complexity in interpreting their data, especially because RNA editing itself may be regulated. Recent studies in the Kv channels of *Drosophila melanogaster* have shown that the distribution of editing isoforms varies among specific tissue types, e.g., fly wing vs. head, and varies substantially during fly development from the larval to adult stages.4,5 Moreover, environmental temperatures may also regulate RNA editing of Kv channels in octopus.6

In this study, we characterize the effects of four RNA editing sites in the Kv channel *eag* from *Drosophila melanogaster*.7 We mutated these sites one at a time to their edited form on the background of a completely unedited channel. We found editing site changes that affect activation kinetics, ion permeation, inactivation, pore block by quaternary ammonium cations and gating alterations caused by extracellular Mg2+. Our biophysical examination focused largely on Site 1 mutations, which had the most pronounced consequences on channel function.

**Results**

RNA editing of *eag* channels. We characterized the effects of amino acid alterations introduced by RNA editing at four sites identified in the *Drosophila eag* potassium channel. The *eag* locus

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with its 16 exons is shown in Figure 1A. The four RNA editing sites are positioned as follows (unedited: edited): Site 1 K467R (Exon 11) is at the top of the S6 segment, Sites 2 and 3, Y548C and N567D, (Exon 13) are in the C terminus and in the cyclic nucleotide binding domain and Site 4, K699R, (Exon 14) is in the distal C terminus (Fig. 1A and B). Figure 1C shows developmental changes in the percentage of editing at each of the four sites. Editing decreases with developmental stage for Site 1 and increases for Site 4. Sites 2 and 3 show more complicated developmental changes in editing with a reduction of editing at the early pupa stage. Although these four sites were identified in an earlier study, the developmental regulation and consequences of these mutations on channel function were previously unknown.

In order to systematically study the effects of editing, we created constructs that contained different combinations of edited (E) and unedited (U) residues at each of the four sites. The construct UUUE, for example, is unedited (genomic) at Sites 1–3 and edited at Site 4. We created six such constructs, editing one site at a time on a background of a completely unedited, genomic channel. We used both fully edited (EEEE) and fully genomic
(UUUU) constructs as controls. For initial characterization, currents were recorded from *Xenopus* oocytes using two-microelectrode voltage clamp. Despite the fact that *eag* shares amino acid sequence similarity with cyclic nucleotide-gated channels, there is some debate as to whether the function of the *eag* channel is influenced by cAMP levels.\(^{7,4}\) Recording for each construct was therefore performed on three different days using three different oocyte harvests to control for any differing levels of cAMP or other modulatory factors.

**Biophysical properties of editing mutants.** Voltage-dependent activation was initially examined from families of currents as shown in Figure 2. The peak current-voltage relationships, normalized to +80 mV, do not differ significantly among the constructs (data not shown). However, each of the constructs possesses varying degrees of partial inactivation at more depolarized voltages. This partial inactivation is nearly absent in the all-edited construct, EEEE. The fraction of inactivated current is displayed in Figure 3 as a function of the depolarizing voltage. Editing at Site 1, position 467, plays a marked role on the extent of the partial inactivation. There is a 54% difference in the extent of inactivation at +80 mV when comparing EUUU to UUUU; an arginine at 467 vs. a lysine in the unedited construct (Fig. 3, inset). In terms of the extent of inactivation, the residue at position 467 (Site 1) is the most important of the four RNA editing sites.

One striking feature of voltage-gated potassium channels is the sensitivity of their activation kinetics to the pre-pulse potential. Hyperpolarizing pre-pulses delay the onset of potassium channel activation.\(^{9}\) This delay is known as the Cole-Moore shift. Although first reported for Kv currents in squid giant axon, *Drosophila eag* and its mammalian homologs share this biophysical phenotype.\(^{10,11}\) We observed a ~2–4-fold effect of prepulse duration on activation kinetics measured as activation rise-time at +50 mV (Fig. 4). Moreover, the magnitude of the Cole-Moore shift depends on RNA editing. The genomic construct (UUUU) has the lowest sensitivity to pre-pulse potential, and the fully edited EEEE construct is most sensitive. The editing mutation displaying the largest effect on activation kinetics is at Site 2 (Fig. 4).

**Biophysical phenotype of residue 467.** Position 467 is located at the top of the S6 segment (Fig. 1). This residue is highly conserved, and is either a lysine or an arginine in potassium channels (Fig. 5). In the rat Kv1.2-Kv2.1 chimera crystal structure, the four lysine side-chains, one from each subunit, are situated at the external perimeter of the pore, ~14 Å from the central axis of the permeation pathway (Fig. 5).\(^{12}\) The same is true in the prokaryotic cyclic nucleotide-gated channel, MloTIK1, whose primary amino acid sequence is very similar to the *eag* sequence (Fig. 5).\(^{13}\) In both structures, the aligned residue, an arginine in MloTIK1, appears to interact closely with a membrane phospholipid molecule resolved in the crystal.

The equivalent residue is a lysine in Kv channels, including *Shaker* (Fig. 5). We attempted to characterize mutants with differing amino acid side-chains at this position in *Shaker* and hERG to explore the importance of this site for channel function. However, mutating residue 638 to arginine in hERG and mutating the homologous residue 456 in *Shaker* to arginine, glutamine or glutamate produced non-functional channels (data not shown).

In order to examine the biophysical role of position 467 in *eag*, we mutated this residue to amino acids with varying side-chains: alanine, cysteine, glutamine and glutamate on the background of a completely unedited channel. Mutating this residue to either phenylalanine or aspartate resulted in non-functional channels. We discovered that when the peak current-voltage relationships of each of the mutants are normalized at +80 mV and compared, residues that are not positively charged produce a left-shifted curve (Fig. 6). Only the edited arginine mutant retains the shape of the peak I-V relationship observed with the unedited lysine construct. We also measured the differences in the fraction of inactivated current for these mutants (Fig. 3B). Each construct produced a phenotype more similar to the arginine at 467 than to the unedited lysine. The identity of the residue at Site 1 is therefore important for both inactivation and the peak current-voltage relationship.

Because the cysteine mutation at position 467 produced a functional channel, we used many different methanethiosulphonate (MTS) reagents to try to modify the cysteine side chain; however, none of the reagents were apparently able to modify the side chain because they had no effect on function (data not shown...
In order to investigate if a change in channel gating produces the shifted I-V relationship, we collected both peak I-V and instantaneous I-V data from the same macroscopic oocyte patches. Using these data and the equation $I(V) = N \cdot i(V) \cdot P_0(V)$ (see legend to Fig. 7) we were able to calculate normalized open probability curves for three 467 position mutants (Fig. 7A). The three constructs do not significantly differ from one another, suggesting that the identity of the residue at 467 (arginine, lysine or glutamine) does not directly affect the voltage dependence of activation.

In order to further elucidate why non-positively charged residues at 467 cause a leftward shift in the peak I-V relationship, we used instantaneous current-voltage protocols and non-stationary noise analysis to determine whether the shift is due to a change in instantaneous rectification of open-channel conductance or to shifted activation gating. The single-channel currents of the eag channel are very small and difficult to resolve reliably. Therefore, we used non-stationary noise analysis for data from cell-attached patches in Xenopus oocytes 2 d after cRNA injection to obtain mean current vs. variance plots for three 467 mutants (Fig. 7B–D). All recording was performed on the same harvest of eggs and on the same day to obviate oocyte variability. We then used the single channel current at +40 mV, estimated from these plots, to normalize the instantaneous current-voltage relationship (Fig. 7E). Potassium permeation in the arginine and glutamine mutants was altered, as shown by changes in rectification at voltages more depolarized than +40 mV, when compared with the unedited lysine (Fig. 7E). The altered permeation might be explained by a change in the channel’s susceptibility to fast block by an endogenous cytoplasmic cation or by an allosteric conformational change in the vicinity of the channel’s selectivity filter.

In order to test if internal cation block is a factor in the permeability change, we recorded currents from both cell-attached and inside-out patches in a bath solution devoid of divalent ions, to obviate any open-channel block by these cations. Previous experiments have shown that the eag current runs down in inside-out patches; however, the rundown is on the order of minutes and does not affect the currents immediately after excision.8 In Figure 8, the relative difference between the current level at +50 mV and +80 mV, i.e., the relative instantaneous conductance, does not change significantly, whether the patch is in the cell-attached configuration or 20s after achieving the inside-out configuration. If internal cations were present and blocked the channel, they should have largely diffused away from an inside-out patch after 20 sec. The null consequence of patch excision on either current level or rectification suggests that the change in rectification seen in the instantaneous current-voltage relationship in Figure 7E is not due to different sensitivities of the 467 mutants to block by endogenous cytoplasmic cations. To test this possibility more directly, however, we examined channel block by exogenously applied cations.

Figure 3. Inactivation. The fraction of inactivated current with respect to peak current was measured at the end of a 750-ms depolarization. (A) Edited and unedited sites. The construct containing lysine (unedited) at Site 1 (UUUU) undergoes 54% more inactivation than the construct containing an arginine (EUUU) at +80 mV. At +80 mV all constructs were significantly different from UUUU (p < 0.01, t-test) except for UUUE and UEEE. Inset: Representative traces of the UUUU (red) and EUUU (violet) constructs at +80 mV. Currents normalized to the peak current. Scale bar: 150 ms. (B) Site 1 mutants.

shown). This was surprising because in the chimera crystal structure this residue is at the top of the S6 segment near the extracellular mouth of the channel,12 where it is expected to be accessible to extracellular cysteine reagents.

The shift in the peak I-V curves in the 467 mutants (Fig. 6) could be the result of a change in either gating or permeation.
Block. Intracellular or extracellular application of quaternary ammonium cations has long been used to probe the pore of potassium channels. We tested the effect of intracellular tetrabutylammonium (TBA) on currents from three 467 constructs (arginine, lysine and glutamine) by injecting TBA into *Xenopus* oocytes expressing the construct of interest (Fig. 9A). We found that at the -100 μM intracellular concentration channels with a lysine at 467 are more susceptible to block than those with either a glutamine or arginine; this difference remains significant at -250 μM when comparing lysine with arginine (Fig. 9A). These data allow us to estimate apparent inhibition constants for intracellular TBA block of 128 ± 10 μM (UUUU), 282 ± 79 μM (EUUU, p = 0.027) and 164 ± 39 μM (K467Q, p > 0.05 with respect to UUUU).

Both extracellular and intracellular tetrabutylammonium (TEA) blocked *eag* currents, but the consequences were different. A high concentration of extracellular TEA (50 mM) reduced the magnitude of current by ~70% without effect on the kinetics of inactivation and no difference in affinity between UUUU and EUUU (data not shown). Besides reducing the peak current amplitude, 250 μM intracellular TEA in the unedited lysine construct significantly reduced the fraction of inactivated current at +80 mV, whereas the same concentration of TEA did not significantly affect EUUU inactivation (Fig. 9B). The estimated inhibition constants for intracellular TEA block were significantly different between lysine (56.1 ± 0.8 μM) and arginine (59.4 ± 0.8 μM) at Site 1 (p = 0.02).

Because TEA is typically a fast blocker, this effect of TEA on inactivation kinetics is consistent with a foot-in-the-door inhibition of inactivation in the UUUU construct. For this unedited lysine construct, the inactivation could be due to the closing of a gate near the cytoplasmic end of the permeation pathway, because intracellular, but not extracellular, TEA has this effect.

Taken together, these results indicate that the change from lysine to arginine decreases intracellular blocking affinity and also the sensitivity of inactivation kinetics to intracellular channel block.

**Effect of extracellular Mg**2+. The identity of the residue at 467 also has an effect on the channel’s interaction with another cation, Mg2+. Previous studies have shown that extracellular Mg2+ can dramatically slow the activation kinetics of the *eag* channel family. However, the effect of Mg2+ concentration on *eag* inactivation has not been studied. Our experiments show that increasing extracellular Mg2+ from 0–10 mM decreases the fraction of inactivated current in both the EUUU and UUUU channels (Fig. 10). Note that all previous experiments in this study were performed in a bath solution containing 2 mM Mg2+. The effect on inactivation is more dramatic in the EUUU construct than in UUUU, as demonstrated by the difference in inactivation free energies of low (0 mM) vs. high (10 mM) extracellular Mg2+, especially at more depolarized voltages (Fig. 10).

**Discussion**

Over the course of evolution, excitable cells have armed themselves with a variety of mechanisms to regulate their firing properties. Most notably, this includes control of the expression and function of ion channels in the plasma membrane. Dynamic diversity of function is achieved using an abundance of strategies, including the regulation of biogenesis, trafficking, turnover and posttranslational modification. Among a cell’s arsenal of strategies is RNA editing, allowing the genomic sequence of channels to be preserved while enzymatically altering the mRNA to produce controlled alterations of the primary amino acid sequence of the channel protein. As we and others have found, point mutations induced by RNA editing can lead to a number of different functional consequences on ion channels, including effects on gating and permeation. Although gating effects may be more prevalent, a dramatic and well-known consequence of RNA editing is to suppress Ca2+ permeability of the open channel. This effect on permeation is due to a point mutation that is essential for normal brain function. If editing of this site is prevented in transgenic mice, the consequences are lethal.

Cis elements that control RNA editing of *Shaker* potassium channels have been shown to be evolutionarily conserved, as well as the editing itself. Likewise, we have determined by comparative genomics that the *eag* gene from all 12 Drosophila genomes possesses such cis elements that are predicted to fold into dsRNA structures consistent with ADAR-mediated RNA editing. Deletion of these elements in vivo resulted in lack of editing of *eag* (data not shown). Thus, it would appear that *eag* RNA editing has been conserved at least throughout the course of Drosophila evolution.

RNA editing is ubiquitous in Kv channels, with reported consequences on tetramerization of channel subunits and a variety of
we observe changes in open-channel rectification and susceptibility to pore block by small cations when Site 1 (residue 467) is mutated. If the genomically encoded lysine is mutated either to the edited arginine or to neutral glutamine, instantaneous inward rectification is enhanced at membrane potentials greater than approximately +40 mV. There are two likely explanations for this effect. The first is that mutating residue 467 might alter the free-energy landscape experienced by a K⁺ ion traversing the pore. The second is that the observed rectification might be due to fast voltage-dependent pore block, presumably by cytoplasmic cations. However, if pore block is the cause of inward rectification, then we might expect that a neutral residue at position 467 would, for electrostatic reasons, enhance pore block, compared with a positively charged side-chain. In fact, we observe exactly the opposite consequence when testing the effect of cytoplasmic pore blockers (Fig. 9). The genomic residue lysine is associated with the greatest sensitivity to block by cytoplasmic tetraalkylammonium cations.

Our collected results show that residue 467 affects the permeation pathway in at least two different ways. It modulates pore block and the free-energy landscape experienced by permeant cations as they cross the membrane. The relationship between these biophysical phenotypes is not clear at the moment. It is also unclear whether the dramatic effect of the editing mutation K467R on inactivation gating is a downstream consequence of changes in the permeation process.

Effects on gating. There are, however, no reports of effects of RNA editing on K⁺ ion permeation until this study. In particular,
One of the more surprising observations in this study is that the charge-preserving mutation K467R has a more dramatic effect on inactivation than charge-changing mutations like K467A and K467C. This rules out a predominant role of the charge of the 467 side-chain, at least with respect to the extent of inactivation. An examination of the results in Figure 3B shows that an unedited lysine at this position differs significantly from any other residue with respect to inactivation. All mutations of this residue reduce the level of inactivation. In fact, the only mutation we examined in this study that had no effect on inactivation is the editing mutation at Site 4 (UUUE). All others reduced the extent of inactivation (Fig. 3). The reason that a lysine at position 467 plays such a unique role remains a mystery. It is unlikely that this is due to lysine’s interaction with phospholipids. If so, one would expect that K467 would be more similar to K467R than to K467A or K467C, but this is clearly not the case.

Although the charge of residue 467 is relatively unimportant for inactivation, this residue’s charge may play some role on permeation, because although K467R introduces a small amount of instantaneous inward rectification at depolarized potentials, the charge-neutralizing K467Q mutant has even stronger rectification (Fig. 7E). This could be explained by a rapid voltage-dependent block of the pore by intracellular cations, like Na+ ions. The affinity of the blocking site within the pore might be expected to be enhanced for the neutralizing mutation K467Q, allowing for greater voltage-dependent block and, therefore, more inward rectification in this mutant than in K467R, as observed in Figure 7E. This proposed mechanism is inconsistent, however, with the fact that instantaneous rectification remains relatively stable when a patch is excised (Fig. 8). Moreover, a simple electrostatic effect is ruled out by examining the inhibition constants for pore block by intracellular tetrabutylammonium ions, because block is weaker for K467Q than for the non-mutated K467 (Fig. 9A). Taken together, these results suggest that mutations of this residue cause propagated allosteric effects on both inactivation gating and ion permeation. The mechanism underlying these effects remains unknown.

Editing of Site 1 also affects the sensitivity of the inactivation process to modulation by extracellular Mg2+ ions (Fig. 10). For both unedited (UUUU) and edited (EUUU) constructs, raising (Mg2+)out reduces the amount of inactivation, although the effects are energetically larger for edited channels. This might reflect a higher affinity for Mg2+ binding in channels edited at Site 1. Alternatively, this result might instead reflect a
modulation of the coupling between Mg$^{2+}$ binding and the inactivation gating process.

This study primarily focused on mutations of Site 1. Mutations of the other three editing sites have less effect on channel function in our studies. Because these other sites are all intracellular and in the vicinity of the cyclic nucleotide binding domain, they may be involved with modulatory effects on gating or permeation. We have not attempted to test this possibility systematically because of the instability of inside-out patches of eag channels. Moreover, a role of cyclic nucleotide binding is controversial in eag channels.21,22 Future studies will elucidate whether these editing mutations affect the excitability of nerve cells in Drosophila and consequently affect fly behavior.

Materials and Methods

RNA analysis. RNA extractions were performed using TRIzol (Invitrogen) on whole flies. eag transcripts were amplified by RT-PCR using gene-specific primers at all steps. Levels of editing for individual editing sites were obtained by direct sequencing of RT-PCR products (gene-specific primer, three independent reactions); see refs. 4 and 23. Areas under the curves were determined from electropherogram traces and editing level expressed as percent editing = (area $G$/total area $A + G$) * 100. $A$ and $G$ represent the humps corresponding to adenosine and guanosine, respectively. Note that this peak ratio method produces results in good agreement with measurements obtained by sequencing large numbers of individual clones.4

Mutagenesis. All eag constructs were inserted into the pBSTA vector for expression in oocytes. Editing mutations at the four sites were generated using the QuikChange Site-Directed Mutagenesis Kit (Stratagene) and verified by direct sequencing. In each mutant construct, one edited site at a time was placed on a background of a completely unedited (genomic) channel. We also created a completely unedited (UUUU) construct and a completely edited (EEEE) construct for use as controls. cRNA was prepared by in vitro transcription (mMessage mMachine Ultra, Ambion).

Electrophysiology. Stage V to VI Xenopus oocytes were injected with cRNA. The oocytes were kept at 18°C for 1–2 d until they were used for recording. Potassium current was recorded with the two-microelectrode voltage clamp technique using an OC-725C voltage clamp (Warner Instruments) in a standard Ringers solution (in mM): 116 NaCl, 2 KCl, 1.8 MgCl$_2$, 2 CaCl$_2$, 5 HEPES, pH 7.6. Recording electrodes were filled with 3 M KCl. Perfusion experiments were performed with an ALA VM8 (ALA Scientific) gravity-flow delivery system.

Figure 8. Test for rapid intracellular cation block. Representative trace for EUUU using a protocol stepping from +50 mV to +80 mV from a holding potential of -100 mV. Scale bars: 20 ms and 20 pA. Each trace is an average of 10 sweeps, pulsing at 1 Hz. Black trace: cell-attached mode; red trace: just after achieving inside-out mode; blue trace: inside-out patch 20 sec after the patch was pulled. The relative change between the current level at +50 mV and the current level at +80 mV is not significantly different whether the patch is in the cell-attached or inside-out configuration (EUUU, n = 6; UUUU, n = 9; K467Q, n = 8), suggesting that the permeation changes seen in Figure 7 are not explained by internal cation block.

Figure 9. Residue at position 467 affects susceptibility to intracellular TBA and TEA block. (A) The fraction of blocked current at +20 mV is significantly greater for lysine at 467 than for arginine at 100 mM and 250 mM intracellular TBA. Both TEA and TBA were injected into oocytes. (B) 250 μM intracellular TEA significantly decreases the fraction of inactivated current at +80 mV for the lysine construct; however, there is no significant difference when an arginine is in position 467. * indicates statistical significance according to a two-tailed t-test (p < 0.05).
NaCl, 2KCl, 1.5 CaCl₂, 1 MgCl₂, 10 HEPES, pH 7.4. The bath solution contained (in mM): 152 KCl, 1.5 CaCl₂, 1 MgCl₂, 10 HEPES, pH 7.4 or 152 KCl, 10 HEPES, pH 7.4. Corrections were made for liquid junction potentials. All experiments were done at room temperature. Non-stationary noise analysis used previously described methods; 25 200 depolarizations were used for each test voltage. Summarized data are presented as mean ± SEM or mean ± propagated error where appropriate. 26

Figure 10. Effect of extracellular Mg²⁺ concentration on channel inactivation. Increased extracellular Mg²⁺ reduces the fraction of inactivated current for both the UUUU and EUUU constructs. Complete Mg²⁺ removal enhances inactivation dramatically. All previous experiments were recorded in 2 mM Mg²⁺. Below: ΔΔG is the difference in free energies between 0 mM and 10 mM Mg²⁺, using the equation ΔG = -kT ln(Pᵢ/(1 – Pᵢ)), where Pᵢ is the fraction of inactivated current. Extracellular Mg²⁺ has a greater effect on EUUU than on UUUU at voltages greater than +40 mV. Error bars are calculated by propagation.

Stock solutions (in H₂O) of either tetraethylammonium (TEA) chloride or tetrabutylammonium (TBA) chloride were stored at 4°C. TEA and TBA working dilutions were made fresh each day from these stocks, and oocytes were injected (maximum volume: 50 nl) to achieve the desired final concentration, assuming an oocyte internal volume of 1 μl. 24 We waited 5 min after injection to allow for diffusion before recording.

Patch currents, cell-attached or inside-out, were recorded from Xenopus oocytes using an Axopatch 200B amplifier (Molecular Devices) 1–3 d after oocyte injection. Electrode resistances were between 1.0–2.2 MΩ. Patch pipettes contained (in mM): 150 NaCl, 2KCl, 1.5 CaCl₂, 1 MgCl₂, 10 HEPES, pH 7.4. The bath solution contained (in mM): 152 KCl, 1.5 CaCl₂, 1 MgCl₂, 10 HEPES, pH 7.4 or 152 KCl, 10 HEPES, pH 7.4. Corrections were made for liquid junction potentials. All experiments were done at room temperature. Non-stationary noise analysis used previously described methods; 25 200 depolarizations were used for each test voltage. Summarized data are presented as mean ± SEM or mean ± propagated error where appropriate. 26

Instantaneous current-voltage relationships were measured in patches using a protocol that activated channels at +50 mV for 40 ms, then stepped from -70 mV to +80 mV in 10-mV increments. The instantaneous current was determined by fitting the current...
after the second voltage step to a single exponential decay and extrapolating back to the time of the step.

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No potential conflicts of interest were disclosed.