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Intracellular linkers are involved in Mg^{2+}-dependent modulation of the Eag potassium channel

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Abbreviations: *eag, ether-à-go-go; sh, shaker*

Modulation of activation kinetics by divalent ions is one of the characteristic features of Eag channels. Here, we report that Mg^{2+}-dependent deceleration of Eag channel activation is significantly attenuated by a G297E mutation, which exhibits a gain-of-function phenotype in Drosophila by suppressing the effect of *shaker* mutation on behavior and neuronal excitability. The G297 residue is located in the intracellular linker of transmembrane segments S2 and S3, and is thus not involved in direct binding of Mg^{2+} ions. Moreover, mutation of the only positively charged residue in the other intracellular linker between S4 and S5 also results in a dramatic reduction of Mg^{2+}-dependent modulation of Eag activation kinetics. Collectively, the two mutations in *eag* eliminate or even paradoxically reverse the effect of Mg^{2+} on channel activation and inactivation kinetics. Together, these results suggest an important role of the intracellular linker regions in gating processes of Eag channels.

The Drosophila *ether-à-go-go* channel (Eag) is the founding member of an evolutionarily conserved subfamily of voltage-gated K^+ channels,[1-5] which includes HERG (human *eag*-related gene), a channel that plays an important role in regulating cardiac excitability and maintaining normal cardiac rhythm.[6] As demonstrated by previous studies from many groups, this family of channels is critical to the function of a variety of biological processes, including memory formation,[7] signal transduction,[8,9] cell proliferation and tumor progression.[10-12]

In flies, mutation of *eag* increases neuronal excitability. When combined with a loss-of-function mutation in another voltage-gated K^+ channel, *shaker* (*sh*), the *eag* phenotype is further enhanced, suggesting that both K^+ channels contribute to the repolarization of presynaptic nerve terminals.[13-16] Recently, Gardnell et al., identified a gain-of-function mutation in the Eag channel that suppresses the effect of *sh* mutation on behavior and neuronal excitability in Drosophila.[17] The mutation is caused by a single amino acid substitution (G297E) in the S2-S3 linker of the Eag channel protein. Interestingly, they have determined that extracellular Mg^{2+} is required for the gain-of-function *eag* mutant to suppress *sh* phenotypes. It thus raises the possibility that the G297E mutation may affect Eag channel properties regulated by the divalent ion.

Eag channels are uniquely regulated by a variety of external divalent cations, including Mg^{2+}, Mn^{2+}, and Ni^{2+}.[18-21] The major effect of these divalent ions is to slow activation kinetics, which presumably reflects a switch of Eag channels from a fast to a slow mode of activation gating.[22] Based on previous studies, the divalent ions appear to modulate activation process of the Eag K^+ channel through binding to acidic residues located in an extracellular-facing crevice between transmembrane segments S2 and S3 of the voltage sensor domain.[20,23,24] However, the molecular and physical mechanisms underlying divalent ion-dependent switch of gating modes in the Eag channel, particularly how ion binding affects voltage sensor and its coupling to the activation gate, are yet to be determined.[24,25]

In this study, we carried out detailed analysis of the G297E mutant Eag channel in a heterologous expression system. Our data reveal that this mutation resulted in a significant decrease in Mg^{2+}-induced deceleration of Eag channel activation kinetics, which provided a mechanistic basis for its gain-of-function phenotype in flies. In addition, we identified another mutation in the other intracellular linker that, in conjunction with G297E, eliminated Mg^{2+}-dependent regulation of channel activation. Our findings set a stage for further determination of molecular detail of Eag channel activation processes.

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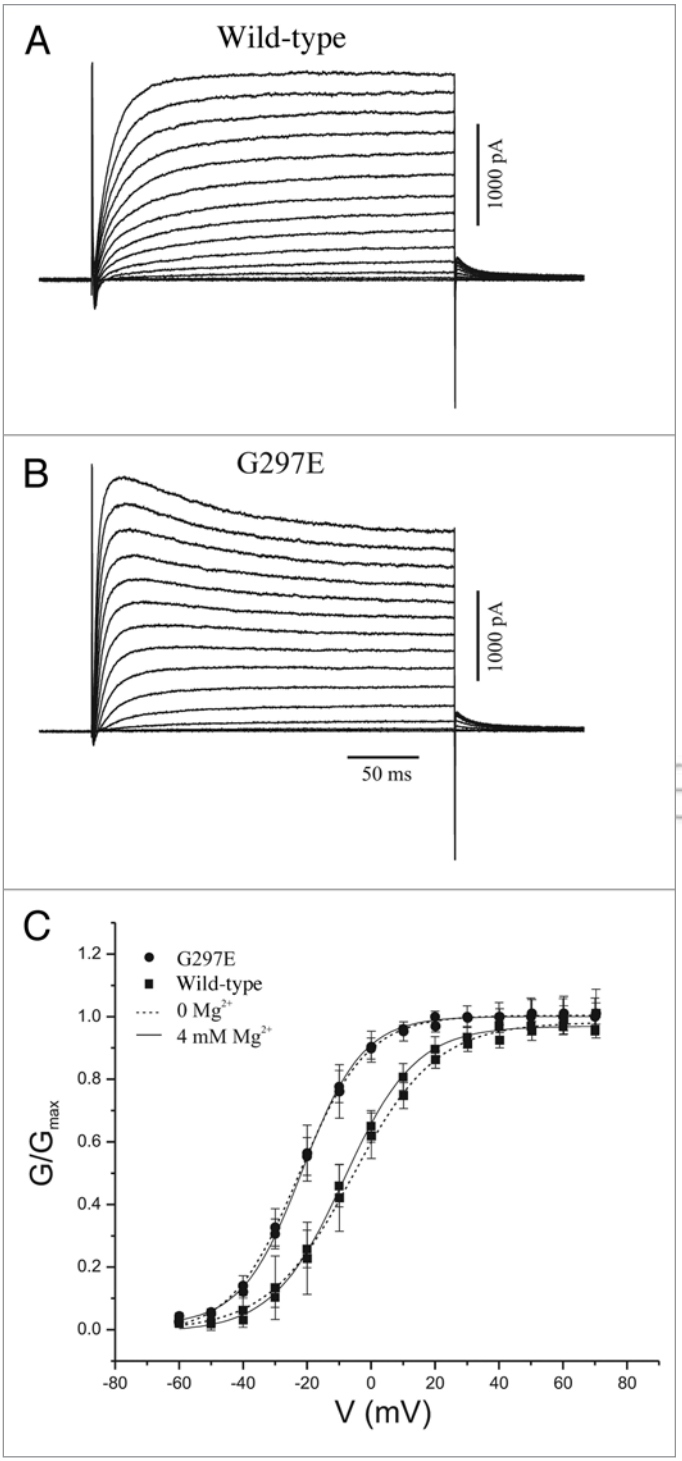

Figure 1. Voltage-dependent activation of wild-type and G297E Eag K^+ channels in transfected cells. Representative traces of whole-cell K^+ currents recorded from CHO cells transfected with either wild-type (A) or G297E mutant (B) *eag* cDNAs. Currents were evoked by a series of 250 msec depolarizing voltage steps to +70 mV in 10 mV increments from a holding potential of -80 mV. Bath solution contained 4 mM Mg^{2+}. (C) Conductance and voltage relationship (G-V curve) for wild-type and G297E Eag in the absence or presence of 4 mM Mg^{2+}. Conductance values were measured from isochronal tail currents recorded at -60 mV after the depolarizing steps and normalized to maximal conductance. Lines were fitted with Boltzmann function.

Results

G297E mutation enhances voltage-dependent activation of the Eag channel. We have recently discovered that functional expression of the Eag channel is sensitive to cultivation temperature. When *eag* cDNA is transfected into mammalian cell lines, it failed to generate significant macroscopic K^+ current in cells maintained at normal cultivation temperature of 37°C. However, cultivation of *eag*-transfected cells at lower temperature (26°C) for 24 h resulted in production of voltage-dependent macroscopic K^+ currents that are similar to Eag K^+ currents recorded from *Xenopus* oocytes.[2,26] Using this heterologous expression system, we analyzed biophysical properties of the gain-of-function Eag channel mutant (G297E) identified from an in vivo mutagenesis screen.[17] In these experiments, whole-cell K^+ currents were elicited by depolarizing voltage steps from a holding potential of -80 mV (**Fig. 1A and B**), and voltage dependency of channel activation was determined by measuring relative conductance at various depolarizing potentials. Compared to the wild-type, G297E mutant channels exhibited a significant increase in voltage sensitivity. This was evidenced by a shift of G-V curve to more hyperpolarizing voltage (**Fig. 1C**). However, the enhancement of G297E channel activity is not dependent on extracellular Mg^{2+}. The difference in half-activation potential ($V_{1/2}$) between the G297E and wild-type Eag channels was -12.89 mV in the presence of 4 mM Mg^{2+}, and -13.30 mV in the absence of Mg^{2+}.

G297E mutation decreases Mg^{2+}-dependent modulation of activation kinetics. Extracellular Mg^{2+} slows activation kinetics of Eag channels. For wild-type channels, the rate of deceleration in activation kinetics by Mg^{2+} was greatest from a concentration of 0 to 5 mM (**Fig. 2B**). In the previous report, the G297E mutant channel suppresses *sh* mutation induced neuronal hyperexcitability only in standard saline solution containing 4 mM Mg^{2+}.[17] Thus, we recorded whole-cell K^+ currents from CHO cells, which expressed either the wild-type or G297E mutant Eag channels in a bath solution containing the same concentration of Mg^{2+} (4 mM). As is clearly evident from a comparison of macroscopic K^+ current traces, activation kinetics of I_K is considerably faster in G297E channels than that in the wild-type control under this condition (**Fig. 1A and B**). Given that activation kinetics of wild-type and G297E channels was not significantly different in the absence of Mg^{2+} (**Fig. 2A and B**), these results suggested that the G297E mutation reduced the effect of Mg^{2+} on activation kinetics of the Eag channel.

To assess the change of Mg^{2+}-dependent slowing in activation kinetics resulted from the G297E mutation, we recorded whole-cell K^+ currents in cells perfused with solutions containing various concentrations of $MgCl_2$. Since wild-type Eag channel K^+ currents display sigmoid kinetics in higher concentrations of Mg^{2+}, we measured time to half maximal current amplitude ($t_{1/2}$) as an index of activation kinetics.[20] Compared to wild-type Eag, the G297E mutant channel had a much reduced sensitivity to Mg^{2+} in concentrations up to 20 mM. Most noticeably, its activation kinetics was only moderately slowed by Mg^{2+} up to 10 mM (**Fig. 2B**).

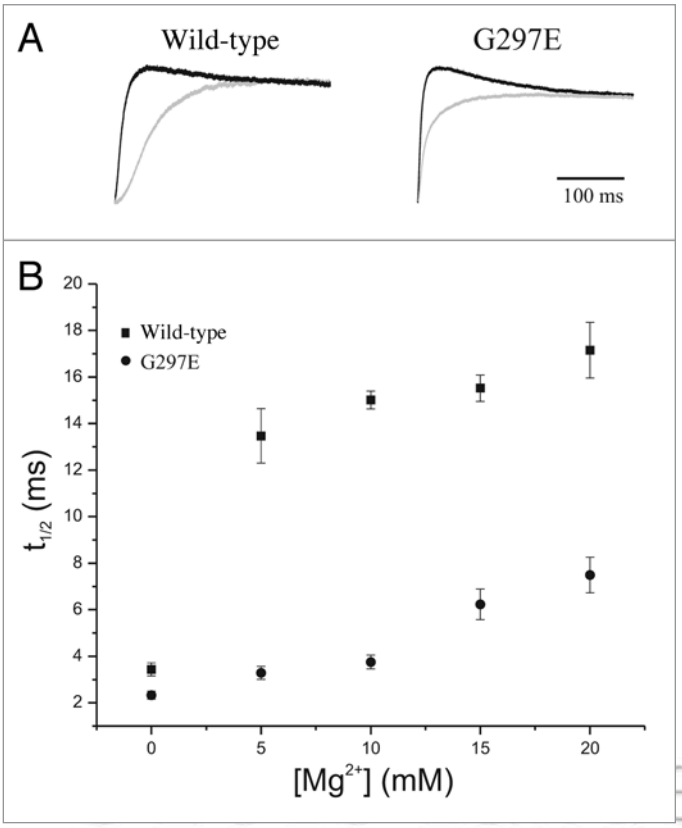

Figure 2. G297E mutation reduces slowing of Eag channel activation by Mg^{2+}. (A) Representative whole-cell K^+ current traces recorded from CHO cells transfected with either wild-type (left) or G297E mutant (right) *eag* cDNAs. Currents were evoked by test pulses to +60 mV from a holding potential of -80 mV in the absence (dark trace) or presence of 10 mM Mg^{2+} (gray trace). (B) Difference in Mg^{2+}-induced deceleration of activation between wild-type and G297E, as measured by $t_{1/2}$ at +60 mV in various concentrations of Mg^{2+}. Values are shown as mean ± SEM (n = 5 for wild-type; n = 6 for G297E).

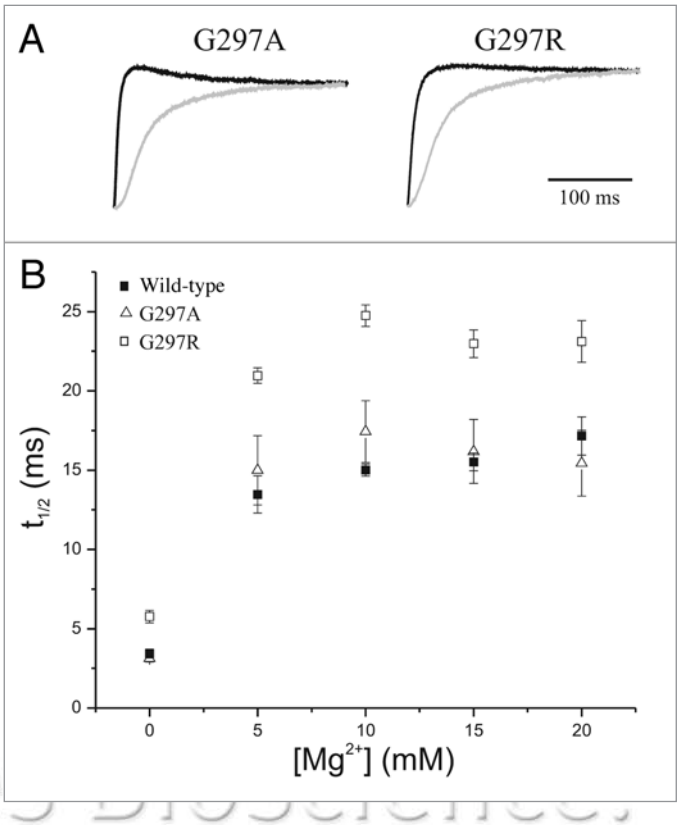

Figure 3. G297 mutation involves charge substitution. (A) Representative whole-cell K^+ current traces recorded from CHO cells transfected with either G297A (left) or G297R mutant (right) *eag* cDNAs. Currents were evoked by test pulses to +60 mV from a holding potential of -80 mV in the absence (dark trace) or presence of 10 mM Mg^{2+} (gray trace). (B) Mg^{2+} sensitivity is preserved in G297A and enhance in G297R, as measured by $t_{1/2}$ at +60 mV in various concentrations of Mg^{2+}. Values are shown as mean ± SEM (n = 5 for wild-type; n = 4 for G297A; n = 3 for G297R).

One explanation for the decrease of Mg^{2+}-sensitivity in the G297E mutant channel could involve an alteration of electrical charge at the amino acid side chain. To test this possibility, we generated a conservative mutation G297A and a charge reversing mutation G297R. When expressed in CHO cells, the G297A mutant was as sensitive to Mg^{2+} as wild-type Eag channels. Values of $t_{1/2}$ measured in the absence and presence of various concentrations of Mg^{2+} were very similar between G297A and wild-type channels (**Fig. 3A**, left panel, **and 3B**). The mutant G297R, however, exhibited slower activation kinetics than wild-type Eag in the absence of Mg^{2+} (**Fig. 3A**, right panel). In addition, Mg^{2+} exerted a bigger effect on G297R, as values of $t_{1/2}$ were considerably larger in G297R than in wild-type or G297A Eag in various concentrations of Mg^{2+} (**Fig. 3B**). Together, these data demonstrated that a change in electrical polarity of residue G297 is responsible for the attenuation in Mg^{2+}-dependent modulation of activation kinetics.

G297E/R363E double mutations abolish Mg^{2+}-dependent deceleration of activation kinetics. G297 is located in the intracellular S2-S3 linker, and thus should not directly involve in Mg^{2+} binding. Considering that Mg^{2+}-dependent modulation was influenced by electrical polarity of the residue, it is possible that presence of an acidic residue at this position may facilitate electrostatic interaction between the S2-S3 linker and other intracellular region(s), and thereby altering configuration of the channel protein. To test this hypothesis, we constructed a G297E/R363E double mutant, in which the only positively charged amino acid in the S4-S5 linker was changed to an acidic residue (**Fig. 4A**). Interestingly, instead of enhancing Mg^{2+} sensitivity, the G297E/R363E double mutation resulted in a complete elimination of Mg^{2+}-dependent slowing of activation kinetics. Quantitatively, values of $t_{1/2}$ in the G297E/R363E mutant were even slightly less in higher concentration of Mg^{2+} (**Fig. 4C**).

To determine how the R363E mutation might further reduce Mg^{2+}-dependent slowing of activation kinetics in G297E channels, we measured activation kinetics of the R363E single mutant channel in various concentrations of extracellular Mg^{2+}. As shown in **Figure 4**, sensitivity to $Mg^{2+}$was also reduced in the R363E mutant channel. Quantified using $t_{1/2}$ values, the extent of change in Mg^{2+}-induced slowing of activation kinetics was less in R363E than that in G297E (comparing **Fig. 2B to Fig. 4C**).

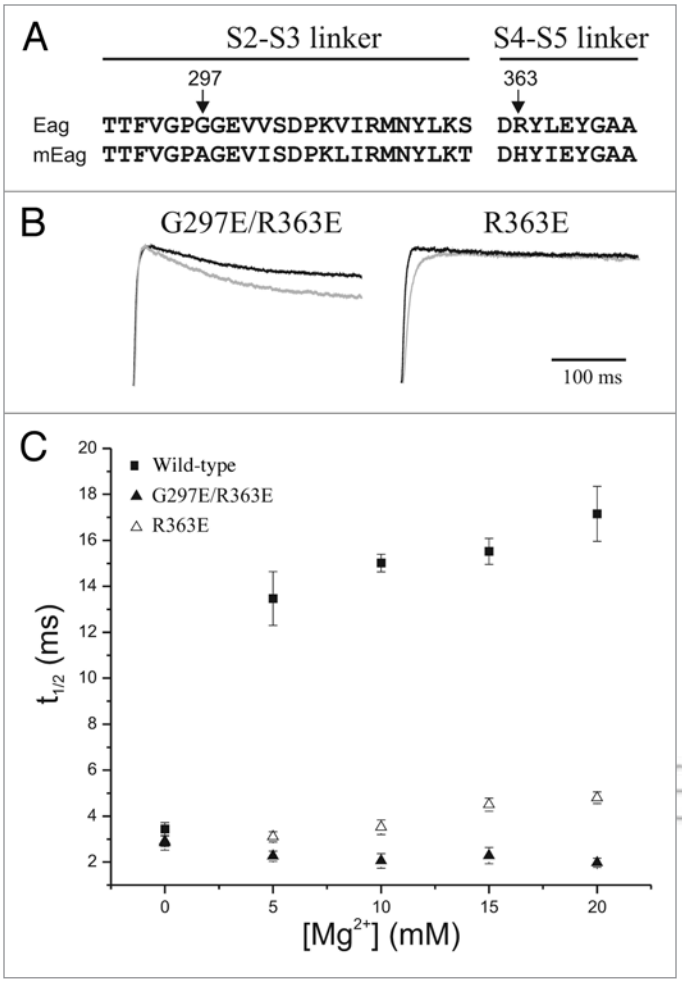

Figure 4. Effect of G297E/R363E mutations on Mg^{2+}-dependent modulation of Eag channels. (A) Amino acid sequences corresponding to S2-S3 and S4-S5 linkers of Eag and its mouse homolog, mEag. Note a substantial homology between them in the two regions. (B) Representative whole-cell K^+ current traces recorded from CHO cells transfected with either G297E/R363E (left) or R363E mutant (right) *eag* cDNAs. Currents were evoked by test pulses to +60 mV from a holding potential of -80 mV in the absence (dark trace) or presence of 10 mM Mg^{2+} (gray trace). (C) Change in Mg^{2+}-induced deceleration of activation in G297E/R363E double and R363E single mutant channels, as measured by $t_{1/2}$ at +60 mV in various concentrations of Mg^{2+}. Values are shown as mean ± SEM (n = 5 for wild-type; n = 5 for G297/R363E; n = 4 for R363E).

Thus, mutation of either G297E or R363E resulted in a profound reduction in Mg^{2+}-dependent deceleration of activation kinetics, and combining the two mutations could collectively eliminate or maybe reverse the effect of Mg^{2+} on activation kinetics of the Eag channel.

Furthermore, we examined the effect of these mutations on deceleration of activation kinetics induced by hyperpolarizing prepulses, which is another characteristic feature of Eag channels.[20,26-28] Consistent with previous reports, slowing of Eag activation kinetics by hyperpolarizing prepulses in wild-type Eag was more pronounced in the presence of extracellular Mg^{2+} (**Fig. 5A**, left panel; **and 5B**). In the mutant G297E channel, a further deceleration of activation kinetics was revealed by hyperpolarizing prepulses in the presence of 10 mM Mg^{2+} (**Fig. 5A**, middle panel; **and 5B**). However, relative to the wild-type, the $t_{1/2}$– V_{PP} curve was shifted to more hyperpolarizing potentials in G297E (**Fig. 5B**), confirming a decrease in Mg^{2+} sensitivity of the G297E mutant channel. On the other hand, hyperpolarizing prepulses did not decelerate activation kinetics of the G297E/R363E double mutant (**Fig. 5A**, right panel). Judged by the $t_{1/2}$ values, activation rate of I_K after various prepulse potentials was noticeably faster in 10 mM Mg^{2+} than that in the absence of Mg^{2+} (**Fig. 5C**). These results are consistent with data obtained at a normal holding potential of -80 mV (**Fig. 4**), and provided addition evidence in support of the role of the two residues in channel activation processes.

G297E/R363E mutations also eliminate deceleration of activation kinetics by Mn^{2+}. In addition to Mg^{2+}, several other divalent ions, including Mn^{2+}, have qualitatively similar effects on Eag channels.[23] To examine whether G297E/R363E mutations also affect deceleration of activation kinetics induced by other divalent cations, we recorded whole-cell K^+ currents in transfected CHO cells perfused with solutions containing either 0 or 10 mM $MnCl_2$. Similar to what was observed in Mg^{2+} (**Fig. 2A**, left panel), macroscopic I_K of wild-type Eag in 10 mM Mn^{2+} exhibited sigmoid kinetics, which reflects a delayed onset and decelerated activation time course (**Fig. 6A**, left panel). In contrast, presence of 10 mM Mn^{2+} in the bath solution did not alter activation kinetics, but led to an accelerated inactivation time course in the G297E/R363E double mutant channel (**Fig. 6A**, right panel; **and 6B**). Likewise, the G297E mutant retained some sensitivity to extracellular Mn^{2+}, whereas the extent of change induced by 10 mM Mn^{2+} is smaller than that in wild-type (**Fig. 6A**, middle panel; **and 6B**). Thus, these results indicate that mutations of the two residues are sufficient to abolish modulation of Eag channel activation kinetics by divalent ions, including both Mg^{2+} and Mn^{2+}.

G297E/R363E mutations enhance inactivation of the Eag channel. Other than affecting activation kinetics, the G297E and/or R363E mutations also led to changes in Eag channel inactivation, which is characteristic of Eag channels cloned from Drosophila and some other invertebrate species, but has not been observed in their mammalian homologs.[2,26,29,30] In the presence of 4 mM Mg^{2+}, however, macroscopic currents of wild-type Eag channels did not exhibit inactivation at depolarization potentials up to +70 mV (**Fig. 1A**). In contrast, the G297E mutant channel retained an apparent inactivation component at positive membrane potentials under this condition (**Fig. 1B**), indicting a significant augmentation in channel inactivation property as a result of the mutation. Moreover, there was a further acceleration of inactivation kinetics for the G297E/R363E double mutant channel, which was more pronounced in the presence of high concentration of extracellular Mg^{2+} or Mn^{2+} (**Fig. 4B**, left panel; **Fig. 6A**, right panel).

As an index of channel inactivation, we measured the amplitude of whole-cell K^+ currents at the end of a 250 ms test pulse and normalized it to the peak current (I_{250}/I_{max}). As shown in **Figure 7A**, inactivation of wild-type or G297R mutant Eag channels was absent in the presence of high concentrations of

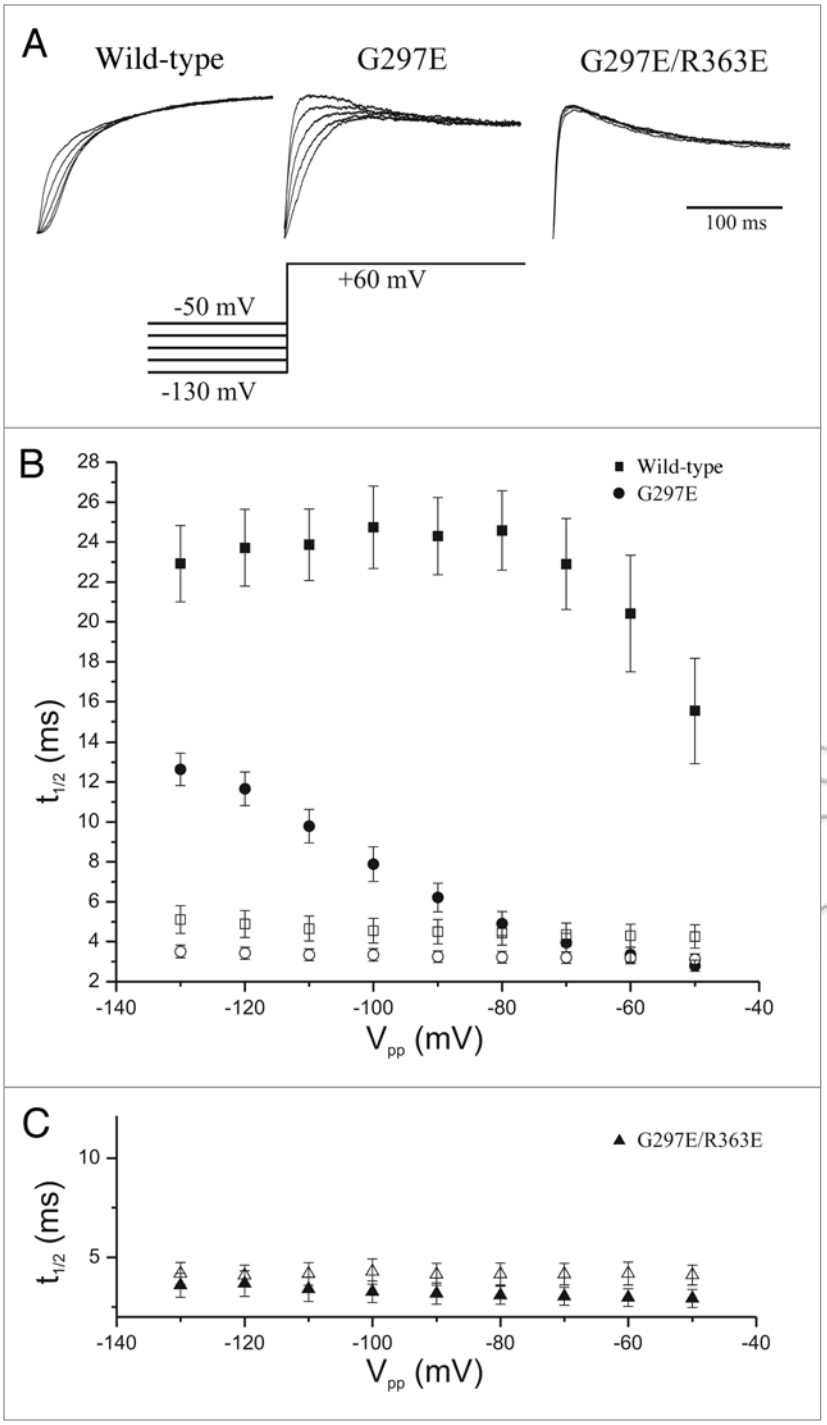

Figure 5. Effect of G297E/R363E mutations on Eag activation kinetics modulated by hyperpolarizing prepulses. (A) Representative whole-cell K^+ current traces recorded from CHO cells transfected with wild-type (left), G297E (middle), or G297E/R363E (right) *eag* cDNAs. Currents were evoked by test pulses to +60 mV following a series of 200 ms prepulse voltage steps between -130 mV and -50 mV in 20 mV increments in the presence of 10 mM Mg^{2+}. Prepulse-dependent deceleration of activation kinetics for wild-type and G297E (B), or G297E/R363E (C) Eag channels in the absence (open symbols) or presence of 10 mM extracellular Mg^{2+} (closed symbols), as measured by $t_{1/2}$ at +60 mV following various prepulse potentials. Values are shown as mean ± SEM (n = 5 for wild-type; n = 6 for G297E; n = 5 for G297E/R363E).

extracellular Mg^{2+}, while the G297E mutant channel exhibited inactivation in extracellular Mg^{2+} concentrations up to 20 mM (**Fig. 7A**). Interestingly, the extent of inactivation in the G297E/R363E double mutant channel was slightly enhanced with increased concentrations of Mg^{2+} (**Fig. 7A**).

Given that activation kinetics was also affected by these mutations, we sought to determine whether the presence of inactivation component is associated with changes in channel activation kinetics. **Figure 7B** illustrated the relationship between inactivation and activation kinetics for wild-type and mutant Eag channels in the absence of extracellular Mg^{2+}. Compared to wild-type Eag, the extent of inactivation was larger in both the G297E and G297E/R363E mutant channels, which also had faster activation kinetics (**Fig. 7B**). Conversely, the slow-activating G297R mutant channel did not inactivate as much as the wild-type channel (**Fig. 7B**). A similar correlation between the extent of inactivation and activation kinetics was observed for both wild-type and mutant channels in the presence of extracellular Mg^{2+} (**Fig. 7C**). Together, these analyses suggest a likely association between inactivation and the fast activating component of the Eag channel currents.

Discussion

K^+ channels play a major role in regulating neuronal excitability. In Drosophila neuromuscular junctions, loss-of-function mutations in certain types of K^+ channels cause prolonged depolarization and/or spontaneous action potentials at the nerve terminals.[13,15,31-34] As K^+ channels shape action potentials and set the resting membrane potential, involvement of a particular subtype of neuronal K^+ channels in the regulation of neuronal excitability is mainly determined by its biophysical properties, including voltage dependency and kinetics of activation and inactivation.[35,36] In this study, we have characterized a gain-of-function mutant of Drosophila Eag channel in a heterologous expression system. We show that the G297E mutation in Eag channel increased voltage sensitivity, which will likely lead to channel opening at more hyperpolarized potentials, as well as greater K^+ ion fluxes at a given membrane potential. More significantly, mutation of G297E resulted in a dramatic decrease in Mg^{2+}-dependent deceleration of Eag activation kinetics. In the physiological concentration of Mg^{2+}, the G297E channel activates much more rapidly than wild-type Eag, and thus should make a greater contribution to the fast-activating component of K^+ currents, which would have been mediated by *shaker* channels.

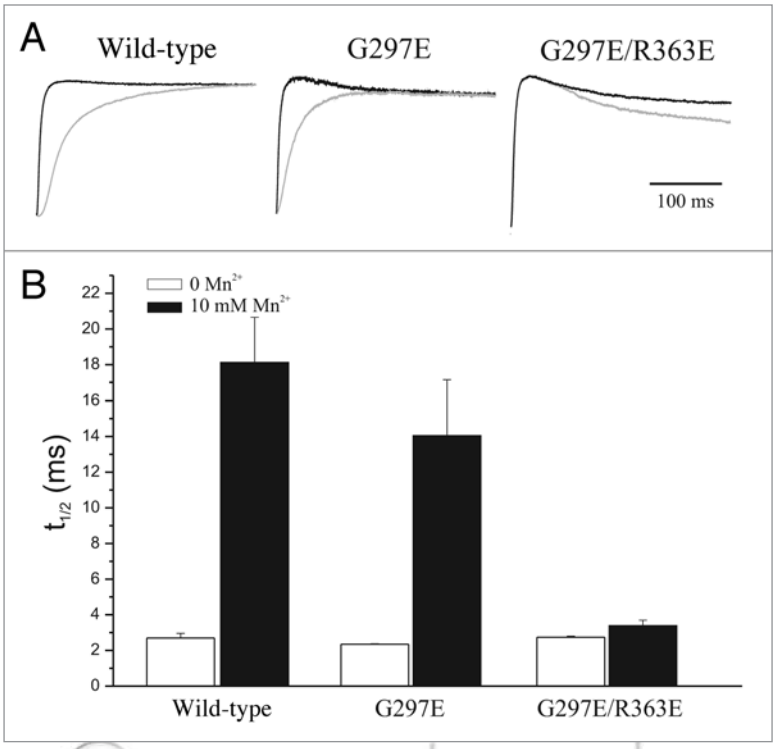

Figure 6. Effect of G297E/R363E mutations on Mn^{2+}-dependent modulation of Eag channels. (A) Representative whole-cell K^+ current traces recorded from CHO cells transfected with wild-type (left), G297E (middle), or G297E/R363E (right) *eag* cDNAs. Currents were evoked by test pulses to +60 mV from a holding potential of -80 mV in the absence (dark trace) or presence of 10 mM Mn^{2+} (gray trace). (B) Mg^{2+} modulation is reduced in G297E and eliminated in G297E/R363E, as measured by $t_{1/2}$ at +60 mV in various concentrations of Mg^{2+}. Values are shown as mean ± SEM (n = 3 for wild-type; n = 4 for G297E; n = 3 for G297/R363E).

In the absence of Mg^{2+}, however, both wild-type and G297E channels have a fast activating kinetics, and should compensate to a similar degree for the loss of *shaker* channels. These findings, therefore, provided a plausible mechanism for the different ability of the G297E mutant and wild-type Eag channels in suppression of *shaker* mutation-induced neuronal hyperexcitability in vivo in the presence, but not in the absence, of extracellular Mg^{2+} ions.[17]

Mg^{2+} regulates kinetics of Eag activation by binding to and thus neutralizing negative charges of acidic residues in the transmembrane segments S2 and S3. In previous studies, mutations that modify or eliminate Mg^{2+}-dependent modulation have been identified in either the S2 or S3 segment or the extracellular loop between S3 and S4.[19-22,28] Here, we have discovered two novel mutations in the intracellular linker of S2-S3 (G297E) or S4-S5 (R363E) that can independently reduce and jointly abolish Mg^{2+}-induced deceleration of Eag activation. Given their intracellular location and electrical polarity, mutations of the two residues are unlikely having a direct effect on Mg^{2+} binding. Thus, reduction of Mg^{2+} regulation in these mutants has to be caused by a change in channel configuration which may either indirectly alter Mg^{2+} binding site or hinder gating transitions modulated by Mg^{2+}. Intriguingly, the G297E/R363E double mutant exhibits an apparent acceleration of inactivation kinetics in the presence of high concentration of Mg^{2+}. Based on this observation, it is likely that these mutations may affect Mg^{2+}-modulated gating process, rather than binding of Mg^{2+} ions.[37]

In spite of recent progress in structure-function characterization of ion channels, positioning and the role of intracellular linkers in channel gating processes is relatively unknown.[25,38-41] We have determined that change in electrical polarity of residue 297 is responsible for the attenuation in Mg^{2+}-dependent modulation of activation kinetic. However, as indicated by results in the G297E/R363E double mutant, the G297E mutation is unlikely facilitating electrostatic interaction between the S2-S3 and S4-S5 linkers. This raises the possibility that electrical charge substitutions in key residues of the two linkers may lead to a change in their electrostatic interaction with other intracellular domains or their interface with the transmembrane domains of the Eag channel.[42,43] Interestingly, it has been known that amino (NT) and carboxyl (CT) terminal regions of the Eag channel play a significant role in channel function.[44,45] Our recent studies further revealed a critical function of the NT and CT domains in mediating temperature-dependent functional expression of Eag channels. In the future, it will be of interest to determine whether and how these intracellular terminal regions and inter-segment linkers may interact, and thus regulate Eag channel activation.

In summary, we have identified novel mutations in Eag channel that affect Mg^{2+}-dependent modulation of activation and inactivation kinetics. The resulting change in channel property may underlie the gain-of-function phenotype exhibited by the G297E mutant Eag channel in Drosophila.

Experimental Procedures

Molecular biology. The cDNA encoding Drosophila Eag (kindly provided by Dr. Gisela Wilson, UW Madison) was subcloned into a mammalian expression vector, pcDNA3. Site-directed mutations in Eag were generated by the Quickchange strategy (Stratagene) using appropriate primers. All mutated cDNA constructs were verified by DNA sequencing in the FSU Biology sequencing facility, Tallahassee, FL.

Electrophysiology: Ionic currents of wild-type or mutant Eag channels were measured in the whole-cell configuration from CHO cells transiently transfected with either wild-type or mutant *eag* cDNAs along with the pEGFP-N1 vector cDNA (Clontech) in a 9:1 ratio using the Lipofectamine LTX (Invitrogen) transfection reagents. Following transfection, the cells were cultivated at 37^0C for 24 hrs, and then switched to an incubator maintained at 26^0C for at least 24 hours. Transfected cells bearing GFP fluorescence were identified with a FITC filter set on a TE2000U inverted fluorescence microscope (Nikon). Patch electrodes with resistances of 2-4 MΩ were pulled from borosilicate glass and fire-polished. Ionic currents were digitized at 20 kHz and filtered at 1 with an Axopatch 200B amplifier. Voltage command

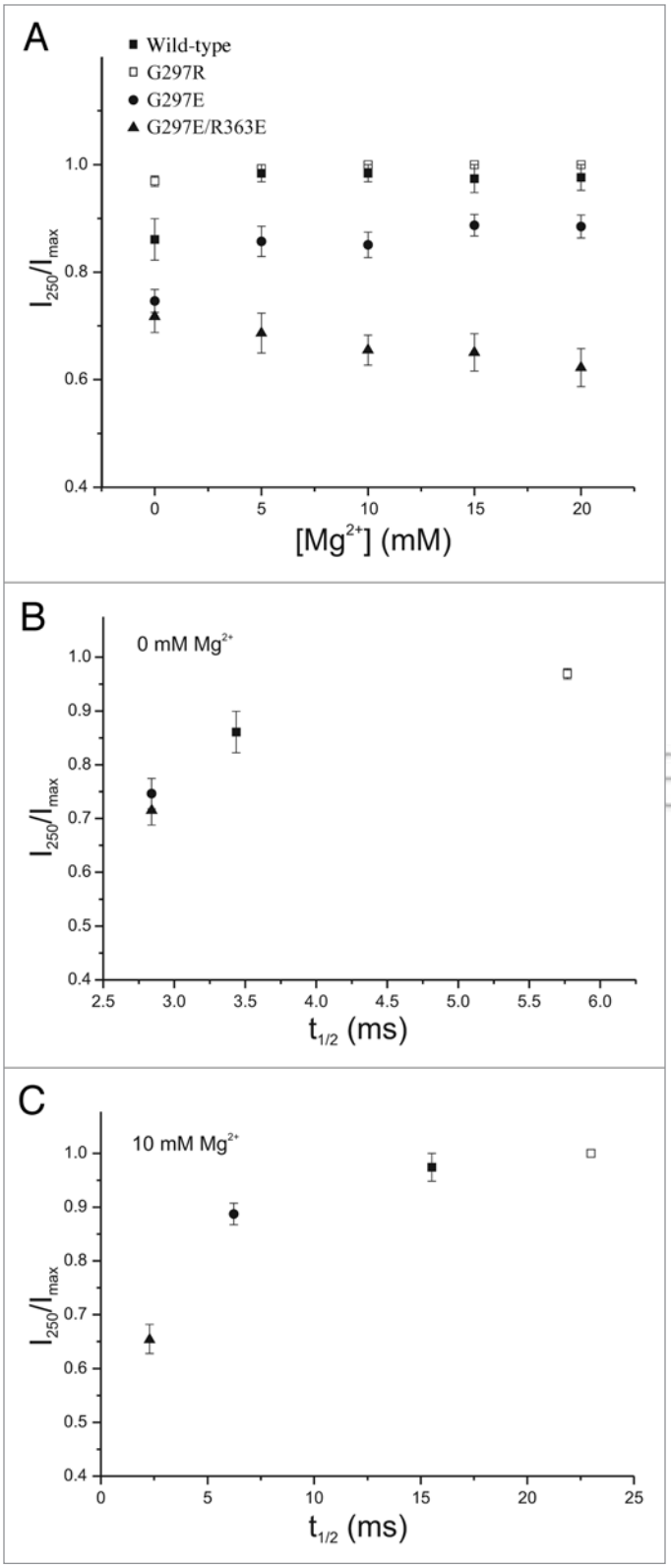

Figure 7. Effects of mutations and Mg^{2+} on inactivation property of Eag channels. (A) Effect of extracellular Mg^{2+} on inactivation of wild-type and mutant Eag channels. The extent of inactivation was measured by I_{250}/I_{max} at +60 mV in various concentrations of extracellular Mg^{2+}. Values are shown as mean ± SEM (n = 5 for wild-type; n = 3 for G297R; n = 6 for G297E; n = 5 for G297/R363E). Correlation between the extent of inactivation and activation kinetics for wild-type and mutant Eag channels in the absence (B) or presence of 10 mM extracellular Mg^{2+} (C). Values of I_{250}/I_{max} are shown as mean ± SEM, but $t_{1/2}$ is shown as mean value only.

and data acquisition were performed with Clampex 10 software (Molecular Devices).

To record whole-cell K^+ current, the pipette solution contained 130 mM KCl, 10 mM HEPES and 5 mM EGTA, pH 7.4. The standard bath solution contained 2 mM KCl, 148 mM NaCl, 2 mM $CaCl_2$, 10 mM HEPES and 1 mM EGTA, pH 7.4. In solutions with varying concentrations of $MgCl_2$ or $MnCl_2$, the concentration of extracellular NaCl was reduced accordingly to maintain equal osmolarity. The bath solution was exchanged using a gravity-driven perfusion system.

The channel conductance was determined by measuring the amplitudes of tail currents evoked by repolarizing to -60 mV from various depolarizing potentials. It was normalized to the maximum value and plotted verse depolarizing potentials. The data was fitted using a Boltzmann equation with Origin 7.0 (Origin Lab, Northampton, MA). Analysis of activation kinetics was performed with the Clampfit 10 program (Molecular Devices).

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