Tyrosine Residues from the S4-S5 Linker of Kv11.1 Channels Are Critical for Slow Deactivation*

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Claudine Ng†, Andrée E. Gravel†1,2, Matthew D. Perry†5, Alexandre A. Arnold†, Isabelle Marcotte*2,3, and Jamie I. Vandenberg†4  
From the †Victor Chang Cardiac Research Institute, 405 Liverpool Street, Darlinghurst and the §St. Vincent’s Clinical School, University of New South Wales, Victoria Street, Darlinghurst, New South Wales 2010, Australia and the ¶Department of Chemistry, Université du Québec à Montréal, Montreal H3C 3P8, Québec, Canada

Slow deactivation of Kv11.1 channels is critical for its function in the heart. The S4–S5 linker, which joins the voltage sensor and pore domains, plays a critical role in this slow deactivation gating. Here, we use NMR spectroscopy to identify the membrane-bound surface of the S4S5 linker, and we show that two highly conserved tyrosine residues within the KCNH subfamily of channels are membrane-associated. Site-directed mutagenesis and electrophysiological analysis indicates that Tyr-542 interacts with both the pore domain and voltage sensor residues to stabilize activated conformations of the channel, whereas Tyr-545 contributes to the slow kinetics of deactivation by primarily stabilizing the transition state between the activated and closed states. Thus, the two tyrosine residues in the Kv11.1 S4S5 linker play critical but distinct roles in the slow deactivation phenotype, which is a hallmark of Kv11.1 channels.

The rhythm of the heartbeat is primarily controlled by the coordinated activity of a range of voltage-gated ion channels (1, 2). Kv11.1, which is encoded by the human ether-á-go-go-related gene (official name, KCNH2) (3), is responsible for the delayed rectifier current (I_{\text{Kr}}), which plays a critical role in cardiac repolarization (4, 5). Kv11.1 channels have unusual kinetics of gating, namely slow activation and deactivation but fast inactivation and recovery from inactivation (6). Consequently, these channels pass relatively little current during the plateau phase of the action potential but deliver a large current during repolarization (7). The slow kinetics of deactivation during the early diastolic period also enable Kv11.1 channels to play a critical role in terminating ectopic beats that may otherwise lead to cardiac arrhythmias and sudden cardiac arrest (6, 8).

Kv11.1 channels have the typical topology of voltage-gated K\(^+\) channels, which includes cytoplasmic N- and C-terminal domains, a voltage sensor domain (VSD)⃦ composed of the first four transmembrane segments (S1–S4), and a pore domain composed of the 5th and 6th transmembrane segments (S5 and S6). Pore domains from each of the four subunits co-assemble to form the ion conduction pathway, as well as the gates that control activation and inactivation. The slow activation and deactivation kinetics of Kv11.1 channels are due in part to the slow movement of the VSD (9, 10). Motion of the VSD, in response to changes in the membrane electric field, facilitates opening and closing of the activation gate at the intracellular end of the pore domain by pulling on the S4S5 linker (residues 539–550), which connects the VSD to the activation gate at the cytoplasmic end of the pore (11). Numerous studies have emphasized the importance of the S4S5 linker for Kv11.1 gating (11–17). In addition to providing a direct link between the voltage sensor and pore domains, the S4S5 linker interacts with the cytoplasmic domains and is thought to mediate the influence of cytoplasmic domains on the kinetics of deactivation gating (12, 13, 15). Recently, Lörinczi et al. (18) showed that when the S4S5 linker in Kv11.1 channels was disrupted (i.e. co-expressing a construct composed of residues 1–545 with another composed of residues 546–1159), the channels can still assemble and retain their slow activation kinetics, as well as rapid inactivation and recovery from inactivation kinetics, but they have much faster deactivation kinetics compared with full-length wild-type (WT) channels (18). These data indicate that an intact S4S5 linker is not required for normal activation gating, but once the channels have opened, an intact S4S5 linker is required to stabilize the activated state and thereby contributes to slow deactivation. Another important implication of this study is that activation and deactivation gating are not simply reciprocal processes, but rather the transition from the closed state to the open state likely follows a different pathway to the transition from the open state back to the closed state.

In the crystal structure of the Kv1.2/2.1 chimeric channel, which is thought to have captured the channel in the activated

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† Both authors contributed equally to this work.

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3 Member of the Centre Quebécien sur les Matériaux Fonctionnels and the Groupe de Recherche Axe sur la Structure des Proteines (GRASP). To whom correspondence may be addressed: Département de Chimie, Université du Québec à Montréal, P. O. Box 8888, Downtown Station, Montréal, Québec H3C 3P8, Canada. Tel.: 1-514-987-3000 #5015; Fax: 1-514-987-4054; E-mail: marcotte.isabelle@uqam.ca.

4 To whom correspondence may be addressed: Mark Cowley Lidwll Research Programme in Cardiac Electrophysiology, the Victor Chang Cardiac Research Institute, 405 Liverpool Street, Darlinghurst, New South Wales 2010, Australia. Tel.: 61-2-9295-8600; Fax: 61-2-9295-8601; E-mail: j.vandenberg@victorchang.edu.au.

5 The abbreviations used are: VSD, voltage sensor domain; WT, wild-type; NMR, nuclear magnetic resonance; DMPC, dimerystylophosphatidylcholine; DPC, dodecylphosphocholine; MLV, multilamellar vesicle; TOCSY, total correlation spectroscopy; ANOVA, analysis of variance.
state (19), the S4S5 linker forms an α-helix that is located at the cytoplasmic membrane interface, with one surface interacting with the membrane and/or transmembrane regions of the channel when the activation gates are in the open conformation. In this study, we sought to identify key S4S5 linker residues that interact with the membrane and/or intramembrane protein domains to stabilize the open state of the channel. A combination of nuclear magnetic resonance (NMR) and electrophysiology experiments suggest that the two tyrosine side chains (Tyr-542 and Tyr-545) point toward the membrane interior. Thermodynamic mutant cycle analysis experiments indicate that Tyr-542 interacts with both pore domain and voltage sensor residues to stabilize the open state of the channel. Conversely, mutations to Tyr-545 cause marked changes to the kinetics of deactivation but have much smaller effects on the voltage dependence of the distribution between open and closed states, which suggests that Tyr-545 stabilizes an intermediate transition state in the pathway between the activated and closed states.

Results
An alignment of the S4S5 linkers of different voltage-gated potassium channels is shown in Fig. 1. The most highly conserved residues are the charged arginine residues from the distal S4 and a glycine residue in the middle of the S4S5 linker. There are also two leucine residues in the S4S5 linker that are highly conserved in the Kv1–9 channels but are replaced by tyrosines in Kv10–12 channels. In the crystal structure of the Kv1.2/1.2 chimera, the leucine residues in the S4S5 linker are oriented toward the lipid membrane. It has been shown previously that the S4S5 linker is critical for channel activation and deactivation in Kv11.1 (12, 14, 16, 17) and that many of the S4S5 residues contribute to the slow gating kinetics. Previous studies, however, have largely focused on how the S4S5 linker interacts with cytoplasmic domains. For example, Asp-540 interacts with the bottom of the S6 transmembrane domain to stabilize the closed state of Kv11.1 channel (16), and cysteine scanning studies have suggested that the S4S5 domain interacts with the intramembrane tail (13). The tyrosine residues, however, have not been extensively studied. Our sequence homology analysis, coupled to the location of the S4S5 linker in crystal structures of the activated state, suggest that the two tyrosine residues are likely to contribute to interactions with the membrane and/or intra-membrane domains of the channel.

To determine whether the equivalent residues in Kv11.1 (Tyr-542 and Tyr-545) are likely to be oriented toward the lipid membrane, we studied the interaction of a peptide (Leu-532–Phe-551) that contains the S4S5 linker of the Kv11.1 channel with dimyristoylphosphatidylcholine (DMPC) or DMPC/dodecylphosphocholine (DPC) model membranes (Fig. 2). First, a
solution NMR was used to gain insight into whether the S4S5 linker interacts with the lipid membrane. We recorded one-dimensional $^1$H NMR spectra of the S4S5 peptides in water (Fig. 2A, panel i) and in the presence of DMPC/DPC bicelles (Fig. 2A, panel ii). Comparison of the spectra shows changes in the chemical shifts and significant broadening of the peptide resonances, consistent with an interaction between the peptide and the model membranes (20). Phosphorus ($^{31}$P) and deuterium ($^2$H) solid-state NMR experiments were then employed to further investigate the interaction of the S4S5 peptide with the membrane using DMPC multilamellar vesicles (MLVs). Because phospholipids harbor a phosphate in their polar headgroup, $^{31}$P NMR spectra were acquired to monitor change at the membrane surface. Broad power spectra are characteristic of non-oriented MLVs and represent the superposition of all chemical shifts from the distribution of orientations in the magnetic field (Fig. 2B, panel i, with the 90° edge on the right and 0° edge on the left.

To probe the bilayer’s hydrophobic core, phospholipids with perdeuterated acyl chains (DMPC-$d_{55}$) were used. The spacing between each doublet on the $^2$H NMR spectrum is known as the quadrupolar splitting ($\Delta v_{Q}$) and gives insight on the degree of organization of the acyl chains (Fig. 2B, panel ii). The largest splitting corresponds to the CD$_2$ bonds closer to the headgroup, whereas the smaller splitting corresponds to the most mobile CD$_2$ group at the end of the lipid chain. Pure lipids $^{31}$P (Fig. 2B, panel i, gray) and $^2$H (Fig. 2B, panel ii, gray) spectra were recorded as a reference. The peptide disrupts model DMPC membranes suggesting the formation of fast-tumbling lipid assemblies, as revealed by the isotropic resonance on the $^{31}$P and $^2$H spectra (Fig. 2B, black). Moreover, the $^3$P spectra for the peptide (Fig. 2B, panel i, black) shows a change in intensity between the 0 and 90° edges of model membranes (Fig. 2B, panel i, gray), with less lipids on the 90° edge, which can be ascribed to a change in vesicle shape or the transverse relaxation ($T_2$) effect caused by an increase in slow motions. The peptide mainly interacts with the surface of the membrane because no changes in $\Delta v_{Q}$ of $^2$H spectra are observed, indicative of no deep insertion in the hydrophobic core of the membrane (21). Although these data suggest that the S4S5 linker is directly involved in membrane interaction, the orientation of the tyrosine residues was further probed by adding soluble paramagnetic gadolinium lanthanide ions (Gd$^{3+}$) to the samples. The Gd$^{3+}$ ions attenuate the proton signals of the peptide residues that are solvent-exposed (22). In agreement with solid-state NMR, the $^1$H total correlation spectroscopy (TOCSY) spectrum of the linker (Fig. 2C) shows that the tyrosine ring protons are protected from the effect of Gd$^{3+}$. This suggests that Tyr-542 and Tyr-545 are located facing the membrane.

We first performed alanine-scanning mutagenesis on the S4S5 residues (Asp-540–Val-549) to investigate how individual residues in the S4S5 linker contribute to the voltage dependence of the distribution between closed and open states (activation) as well as the rate of activation of Kv11.1 channels. Fig. 3A, panel i, shows the 3-s activation protocol and exemplar traces for WT Kv11.1 channels. The $V_{0.5}$ of activation for WT is $-23.9 \pm 1.3$ mV, $n = 5$. Most of the mutants altered the $V_{0.5}$ of activation when compared with WT with the largest shifts seen for S543A, G546A, and A548V (Fig. 3A, panel ii). The changes observed for Y542A ($-7.2 \pm 1.5$ mV, $n = 4$) and Y545A ($-36.0 \pm 1.2$ mV, $n = 5$) were more modest. Fig. 3B, panel i, shows the protocol, and typical family of current traces, used to measure the rate of activation at 20 mV (see under “Experimental Procedures” for more details). The time constants for WT

![Tyrosine Residues within the S455 Linker of Kv11.1](image_url)
TABLE 1
Summary of the values for the 3-s isochronal activation $V_{0.5}$ and time constant of activation at 20 mV for S4S5 alanine mutants

Data are presented as mean ± S.E. ($n$) and $n$ denotes number of individual recordings. Asterisk indicates $p < 0.05$ versus WT using one-way ANOVA with Bonferroni post-test.

| Mutant  | Activation $V_{0.5}$ (mV) ($n$) | Time constant of activation at $+20$ mV (ms) ($n$) |
|---------|---------------------------------|-----------------------------------------------|
| WT      | $-23.9 ± 1.3$ (5)               | $102.5 ± 6.1$ (8)                             |
| D540A   | $-23.1 ± 1.7$ (5)               | $55.2 ± 2.2$ (5)                              |
| R541A   | $-33.2 ± 1.3$ (4)               | $87.0 ± 3.6$ (6)                              |
| Y542A   | $-7.2 ± 1.5^*$ (4)              | $128.0 ± 11.0$ (6)                            |
| S543A   | $-45.5 ± 1.2^*$ (4)             | $64.2 ± 1.6^*$ (7)                            |
| E544A   | $-25.7 ± 0.7$ (3)               | $97.8 ± 2.6$ (6)                              |
| Y545A   | $-36.0 ± 1.2^*$ (5)             | $97.3 ± 4.3$ (7)                              |
| G546A   | $-65.2 ± 2.2^*$ (3)             | $63.0 ± 2.8^*$ (5)                            |
| A547V   | $-13.0 ± 1.8^*$ (3)             | $157.2 ± 6.0^*$ (6)                           |
| A548V   | $-55.3 ± 0.8^*$ (5)             | $55.0 ± 5.1^*$ (7)                            |
| V549A   | $-13.3 ± 1.1^*$ (4)             | $225.8 ± 13.0^*$ (7)                          |

(102.5 ± 6.1 ms, $n = 8$) and mutants are summarized in Fig. 3B, panel ii, and in Table 1. Notably, both Y542A and Y545A did not significantly perturb the rate of activation at 20 mV compared with WT.

Previous studies have suggested that activation and deactivation are not simply reciprocal processes but rather occur via different pathways (18, 23). We therefore investigated whether any of the S4S5 mutants preferentially altered the rate of deactivation. Consistent with the data at $-120$ mV, the change in the rates of deactivation at $-70$ mV for the S4S5 alanine mutants can also be explained by changes in the thermodynamics of deactivation gating.

As alanine mutagenesis of the two tyrosine residues appears to have quite distinct phenotypes in terms of effects on activation/deactivation, both resulted in accelerated rates of deactivation with minimal perturbation on the rate of activation. We thus focus our investigation on the deactivation phenotypes of Tyr-545 and Tyr-542. Each tyrosine residue was mutated to Ala, Leu, Ile, Phe, or Trp. All of the Tyr-542 and Tyr-545 mutants resulted in accelerated rates of deactivation, with the exception of Y545F (Fig. 6A). Mutations at Tyr-542 tended to cause greater perturbations to the $V_{0.5}$ of deactivation than mutations at Tyr-545 (Fig. 6B). A plot of the perturbation to the rate of deactivation (measured at $-70$ mV versus the change in $V_{0.5}$ of deactivation, for the groups of mutants at Tyr-542 and Tyr-545, highlights that mutations to Tyr-542 predominantly affect the thermodynamics of channel deactivation, whereas mutations to Tyr-545 cause relatively more perturbation to the kinetics of deactivation gating (Fig. 6C).

As mutations to Tyr-542 significantly perturbed the voltage dependence of the equilibrium distribution of the open and closed states, we probed for residues that might interact with Tyr-542 in the open or closed state by using double mutant cycle analysis. Based on our homology model (Fig. 7A), Tyr-542 lies in close proximity to Val-535 and Arg-537 in S4 and Ile-560 in S5. Fig. 7B, panel i, shows the 3-s isochronal deactivation curves for WT (black circle), I560A (black square), Y542A (white circle), and I560A/Y542A (gray circle). I560A is WT-like, whereas Y542A has a large depolarizing shift. The double...
mutant I560A/Y542A has an intermediate phenotype between I560A and Y542A. The method for calculating interaction energies and a plot of the $\Delta \Delta G^0$ values for I560A, Y542A, and I560A/Y542A are shown in Fig. 7B, panel ii. The $\Delta \Delta G^0$ value for I560A/Y542A (Fig. 7B panel ii, black bar) is less than the sum of the $\Delta \Delta G^0$ values for the two individual mutants, indicative of an energetic interaction between these two residues. In addition to Ile-560, the double mutant cycle analysis indicates that Tyr-542 is energetically coupled to Val-535 and Arg-537 (Fig. 7C). In contrast, double mutant cycle analysis of Y545A with three residues (Ile-560, Ser-654, and Asn-658) did not reveal any significant energetic interactions (Fig. 7C). A summary of the experimental $\Delta \Delta G^0$ values for all single and double mutants and their interaction energies are shown in Table 3.

Discussion

Kv11.1 channels have unusual kinetics, which include rapid recovery from inactivation and slow deactivation. In the heart, the slow deactivation kinetics play a particularly important role in the suppression of propagation of ectopic beats that occur during the early diastolic interval (6). The S4S5 linker clearly plays a critical role in deactivation gating of voltage-gated $\mathrm{K}^+$ channels, including Kv11.1 channels (12, 15, 16). Here, we have shown that two tyrosine residues in the S4S5 linker, Tyr-542 and Tyr-545, play important, but distinct, roles in stabilizing the open state of the Kv11.1 channel and thus in mediating the slow deactivation gating of Kv11.1 channels.

In this study, mutations to Tyr-542 resulted in large shifts in the voltage dependence of the equilibrium distribution between the open and closed states, with all mutants shifting the equilibrium in favor of the closed state (Fig. 6B, panel i). All Tyr-542 mutants also resulted in an acceleration of the rates of deactivation (Fig. 6A, panel i). These results are consistent with a role for Tyr-542 in stabilizing the open state of the channel. Mutating Tyr-542 to alanine caused a marked acceleration of the rates of deactivation with a shallow slope (Fig. 4D). The fact that the slope of the voltage dependence of rates of deactivation for Y542A is much shallower than that of WT or Y545A suggests that the native Tyr-542 interacts with, and so influences, the motion of the VSD during deactivation. This conclusion is consistent with our mutant cycle analysis experiments indicating an energetic interaction between Tyr-542 and Arg-537, as well as with Val-535 and Ile-560 (Fig. 7), although only the interaction with Ile-560 exceeds the 1 kcal mol$^{-1}$ threshold that is often used to indicate biologically relevant interactions (25–30).

In contrast to Tyr-542, mutations to Tyr-545 caused less perturbation to the voltage dependence of the equilibrium distribution between the open and closed states (Fig. 6B, panel ii) but still caused a marked acceleration of the rates of deactivation (Fig. 6A, panel ii). This suggests that Tyr-545 might form

![FIGURE 4. Kinetics of deactivation for S4S5 alanine mutants. A, cells were depolarized to 40 mV for 1 s and followed by repolarization to $-70$ and $-120$ mV for 3 s. Example deactivation current traces acquired at $-70$ (black) and $-120$ mV (gray) for WT (panel i), Y545A (panel ii), and Y542A (panel iii). x and y axes are 1 s and 0.25 pA, respectively. B, comparison of time constants of deactivation for WT (circle), Y545A (triangle), and Y542A (square) corresponding to the test voltage range of $-150$ to $-70$ mV. C, time constants for the fast component of deactivation at $-70$ mV (black) and $-120$ mV (gray) for S4S5 alanine mutants (mean ± S.E.). D, summary of the voltage dependence of the rates of deactivation (mean ± S.E.) for WT and S4S5 mutants. Statistical comparisons were made using one-way ANOVA with Bonferroni post test. Significant differences ($p < 0.05$) are highlighted with asterisks. The values for all mutants are summarized in Table 2.](thefile:///repo/fig4.png)
Tyrosine Residues within the S4S5 Linker of Kv11.1

**TABLE 2**
Summary of the values for the 3-s isochronal deactivation $V_{0.5}$, time constants of deactivation at $-70$ and $-120 \text{ mV}$ for S4S5 alanine mutants

Data are presented as mean ± S.E., and $n$ denotes number of individual recordings. Asterisk indicates $p < 0.05$ versus WT using one-way ANOVA with Bonferroni post-test.

| Mutant (n) | Deactivation $V_{0.5}$ (mV) | Time constant of deactivation at $-120 \text{ mV}$ (ms) | Time constant of deactivation at $-70 \text{ mV}$ (ms) |
|------------|----------------------------|--------------------------------------------------|--------------------------------------------------|
| WT (5)     | $-61.3 ± 0.5$              | 27.8 ± 1.3                                       | 286.2 ± 32.4                                     |
| D540A (5)  | $-25.6 ± 2.0^*$            | 23.8 ± 1.4                                       | 41.7 ± 1.4                                       |
| R541A (4)  | $-46.3 ± 0.8^*$            | 20.4 ± 0.9                                       | 129.4 ± 7.5*                                    |
| Y542A (5)  | $-1.6 ± 1.1$               | 14.3 ± 0.5*                                      | 29.7 ± 0.7*                                     |
| S543A (4)  | $-78.6 ± 0.6^*$            | 49.3 ± 3.6*                                      | NA                                               |
| E544A (3)  | $-40.3 ± 1.4^*$            | 21.1 ± 1.8                                       | 113.7 ± 9.0 *                                   |
| Y545A (5)  | $-46.4 ± 1.1^*$            | 14.4 ± 1.1*                                      | 110.4 ± 18.6*                                   |
| G546A (4)  | $-82.0 ± 0.2^*$            | 64.7 ± 2.2*                                      | NA                                               |
| A547V (3)  | $-56.5 ± 1.1$              | 31.2 ± 2.8                                       | 217.3 ± 3.4                                     |
| A548V (5)  | $-64.8 ± 0.6$              | 45.1 ± 1.7 *                                     | 268 ± 21.8                                      |
| V549A (4)  | $-53.3 ± 1.1^*$            | 28.4 ± 3.1                                       | 208.7 ± 15.9                                    |

**Experimental Procedures**

**NMR Spectroscopy**

**Materials**—Peptide that contains the S4S5 linker (LVR-VARKLDRYSEYGAVLF) was synthesized by GL Biochem Ltd. (Shanghai, China) with $>98\%$ purity. Protonated and deuterated dymristoylphosphatidylcholine (DMPC, DMPC-$d_{34}$) were purchased from Avanti Polar Lipids (Alabaster, AL), and deuterated dodecylphosphocholine (DPC-$d_{38}$), deuterium-depleted water, and diethylenetriaminepentaacetic acid gadolinium (III) dihydrogen salt hydrate were purchased from Sigma. Deuterium oxide ($D_2O$) was obtained from CDN Isotopes (Pointe-Claire, Quebec, Canada). Phosphatidylcholines are abundantly found in biological membranes; consequently, DMPC is frequently used in model membranes, although DPC has been previously used to determine the Kv11.1 S4S5 linker structure by NMR (12). DMPC/DPC bicelles are a more biologically relevant alternative and have recently been introduced as a new membrane mimicking system for the NMR analysis of membrane proteins (37).

**NMR Sample Preparation**—Bicelle and MLV samples used for solution- and solid-state NMR, respectively, were prepared by mixing freeze-dried DMPC and/or DPC in water. The mixture was submitted to a series of at least three freeze (liquid $N_2$)/thaw (50 °C)/vortex shaking cycles. In all experiments, the peptide concentration was kept between 2 and 5 μM, and the lipid concentration was maintained well above the critical micelle concentration of the lipids. To ensure the stability of the model membranes, the hydration percentages were kept at 75% for solid-state NMR and 90% for high resolution NMR using deuterium-depleted water and 10% $D_2O$, respectively. Solution important interactions that stabilize the transition state complex; however, these interactions are destabilized by mutations, i.e. lower the energy barrier for channel deactivation. This suggestion is also consistent with the thermodynamic mutant cycle analysis experiments, which showed that there were no significant energetic interactions involving Tyr-545 and other residues in the stable end states. However, at this stage we cannot determine the nature of such interactions. They could be with transmembrane domain residues and/or with the membrane lipids. Given that the NMR experiments suggest that the peptide mainly interacts with the surface of the membrane (because there were no changes in $\Delta \delta_{\text{H}}$ of $^1$H spectra), if there are any specific interactions between Tyr-545 and the membrane, these would most likely involve interactions with the lipid headgroups.

The S4S5 linker is thought to be essential to transmit conformational changes of the voltage sensor to the pore domain, as evidenced by its proximity to the S6 helix in the crystal structure of the Kv1.2 channel, as well as its direct interaction with the S6 domain (16). Previous studies of the S4S5 linker in Kv11.1 channels have focused on its role in linking the voltage sensor domain to the activation gate at the intracellular end of the pore domain (11, 16), as well as its role in mediating interactions with cytoplasmic domains that can also modulate deactivation gating (13). Our NMR studies of a peptide that contains the Kv11.1 S4S5 linker in a model membrane indicate that both Tyr-542 and Tyr-545 are likely to point toward the membrane, which is consistent with the homology model based on the structure of the activated Kv1.2/2.1 channel (see Fig. 7A). This suggests that the S4S5 linker could also have important interactions with the membrane directly and/or with residues in the transmembrane domains of the channel. We have clear evidence that Tyr-542 interacts with other transmembrane domain residues. However, the relatively small magnitude of this interaction (e.g. $-4.6 \text{ kJ} \cdot \text{mol}^{-1}$ for Y542A/I560A) suggests that other factors might be involved, which could include interactions between Tyr-542 and the membrane. Tyr-545 interactions with the membrane contributing to the stabilization of the transition state in the pathway connecting the activated state with the closed states are also plausible, but we do not yet have definitive proof of such interactions.

Recently, Lorincz et al. (18) showed that cutting the S4S5 linker of Kv11.1 channels in half could still produce functional channels with normal activation but accelerated deactivation.

This indicates that the major role of S4S5 linker is to stabilize the open state, and it is conceivable that the split S4S5 linker in the Kv11.1 prevents the interaction promoted by the S4S5 linker with other parts of the channel in the activated state. It is also possible that the split S4S5 may disrupt its interaction with the lipid membrane. It is unlikely that a split S4S5 linker would maintain a continuous helix that lies parallel to the membrane in the activated state of the channel (31–36). Our functional studies provide complementary information that is consistent with recent findings (18) by showing that the two conserved tyrosine residues play important but distinct roles in stabilizing the open state of Kv11.1 channels.
NMR experiments used lipid molar ratios ($q_{\text{DMPC/DPC}}$) of 0.25 and 1, as well as a lipid/peptide (L/P) molar ratio ranging from 50:1 to 58:1. Solid-state NMR samples were prepared at a L/P molar ratio of 100:1.

**NMR Experiments**—All experiments, except high resolution solution experiments, were recorded on a hybrid solution/solid-state Varian Inova Unity 600 (Agilent Technologies, Mississauga, Ontario, Canada) spectrometer operating at frequencies of 599.95 MHz for $^1\text{H}$, 246.86 MHz for $^{31}\text{P}$, and 92.125 MHz for $^2\text{H}$. Solid-state NMR experiments used a 4-mm broad-band/1H dual-frequency magic-angle spinning probehead. The $^1\text{H}$ chemical shifts were internally referenced by adding 0.5 mM of 2,2-dimethyl-2-silapentane-5-sulfonic acid set to 0.0 ppm, and $^{31}\text{P}$ NMR spectra were externally referenced with respect to the signal of 85% phosphoric acid set to 0 ppm. All data were processed using MestReNova (Mestrelab Research, Santiago de Compostela, Spain).

$^1\text{H}$ total correlation spectroscopy (TOCSY) spectra were recorded on a hybrid solution/solid-state Bruker Avance III-HD narrow bore spectrometer (Milton, Ontario, Canada) operating at a frequency of 599.95 MHz for $^1\text{H}$ with a broad-band $^1\text{H}$ dual-frequency magic-angle spinning probehead. The $^1\text{H}$ chemical shifts were internally referenced by adding 0.5 mM of 2,2-dimethyl-2-silapentane-5-sulfonic acid set to 0 ppm, and $^{31}\text{P}$ NMR spectra were externally referenced with respect to the signal of 85% phosphoric acid set to 0 ppm. All data were processed using MestReNova (Mestrelab Research, Santiago de Compostela, Spain).

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$^1\text{H}$ total correlation spectroscopy (TOCSY) spectra were recorded on a hybrid solution/solid-state Bruker Avance III-HD narrow bore spectrometer (Milton, Ontario, Canada) operating at a frequency of 599.95 MHz for $^1\text{H}$ with a broad-band $^1\text{H}$ dual-frequency magic-angle spinning probehead. The $^1\text{H}$ chemical shifts were internally referenced by adding 0.5 mM of 2,2-dimethyl-2-silapentane-5-sulfonic acid set to 0 ppm, and $^{31}\text{P}$ NMR spectra were externally referenced with respect to the signal of 85% phosphoric acid set to 0 ppm. All data were processed using MestReNova (Mestrelab Research, Santiago de Compostela, Spain).

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**FIGURE 5.** Voltage dependence of the distribution between open and closed states for S4S5 mutants. A, 3-s isochronal deactivation protocol and example traces for WT. X and y axes are 1-s and 0.25 μA, respectively. B, peak tail currents (•) derived from 3-s isochronal deactivation were fitted with Boltzmann equation to derive $V_{0.5}$ values. The $V_{0.5}$ values are as follows: WT, −61.3 ± 0.5 mV (mean ± S.E., n = 5; circle); Y545A, −46.4 ± 1.1 mV (mean ± S.E., n = 5; triangle); and Y542A, −1.6 ± 1.1 mV (mean ± S.E., n = 5; square). C, summary of the deactivation $V_{0.5}$ of all S4S5 alanine mutants. * indicates $p < 0.05$ versus WT using one-way ANOVA with Bonferroni post-test. The 3-s isochronal deactivation $V_{0.5}$ values for all mutants are summarized in Table 2.

**FIGURE 6.** Tyr-545 (blue) is functionally different to Tyr-542 (red). A, time constants of deactivation at −70 mV for different Tyr-542 (panel i) and Tyr-545 (panel ii) side-chain residues. B, 3-s isochronal deactivation $V_{0.5}$ for Tyr-542 (panel i) and Tyr-545 (panel ii) side-chain residues. Data are presented as mean ± S.E. * indicates $p < 0.05$ versus WT using one-way ANOVA with Bonferroni post-test. C, relationship between time constants of deactivation for different WT (white), Tyr-542 (red), and Tyr-545 (blue) side-chain residues at −70 mV and their corresponding deactivation $V_{0.5}$. Linear regression with 95% confidence interval was fitted to each dataset.

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**FIGURE 7. Double mutant cycle analysis of Tyr-542 and Tyr-545.** A, orientation of Tyr-542 (red) and Tyr-545 (blue) within the S4S5 linker and the residues that were energetically coupled. B, 3-s isochronal deactivation curves for WT (black circle), I560A (black square), Y542A (white circle), and I560A/Y542A (gray circle). 0 mV was indicated by gray circle (WT), indicating the double mutant I560A and Y542A are shown on the right of panel ii. C, summary of the double mutant cycle analysis displayed as the $\Delta \Delta G^\circ_{\text{Mut1+Mut2 (additive)}}$ for the Tyr-542 double mutants (red) and Tyr-545 double mutants (blue). * indicates statistical differences, $p < 0.05$ using an unpaired $t$ test when comparing the experimentally derived $\Delta \Delta G^\circ$ for the double mutant with the sum of the $\Delta \Delta G^\circ$ values for the two individual mutants. Only the double mutant I560A/Y542A has an interaction energy greater than 4.2 kJ mol$^{-1}$, indicated by the dotted line. Data are presented as mean ± S.E.

**TABLE 3**

Summary of the values for the $\Delta \Delta G^\circ$ (mean ± S.E.) and $n$ denotes number of individual recordings

| Mutant (n) | $\Delta \Delta G^\circ_{\text{WT}(\text{exp})}$ | $\Delta \Delta G^\circ_{\text{Mut} - \text{WT}}$ | $\Delta \Delta G^\circ_{\text{Mut1} + \text{Mut2}}$ |
|------------|---------------------------------|---------------------------------|---------------------------------|
| WT (5)     | $-22.7 ± 0.6$                   | $22.3 ± 0.2$                    | $-0.2 ± 0.4$                    |
| Y542A/S(5) | $-0.3 ± 0.2$                    | $22.3 ± 0.2$                    | $-3.0 ± 0.4$                    |
| Y537A(3)   | $-20.8 ± 1.8$                   | $18.8 ± 1.8$                    | $-1.1 ± 0.5$                    |
| R537A(3)   | $-20.1 ± 0.6$                   | $2.6 ± 0.6$                     | $-3.5 ± 0.6$                    |
| I560A(3)   | $-22.1 ± 1.0$                   | $0.5 ± 1.0$                     | $-1.9 ± 0.8$                    |
| Y542A-V535A(5) | $-1.8 ± 0.1$                  | $20.8 ± 0.1$                    | $-3.4 ± 0.9^*$                  |
| Y542A/R537A(3) | $0.3 ± 0.1$                   | $22.9 ± 0.1$                    | $-2.0 ± 0.4^*$                  |
| Y542A/E660A(4) | $-4.4 ± 1.1$                 | $18.2 ± 1.1$                    | $-4.6 ± 0.8^*$                  |
| Y545A(S)   | $-19.5 ± 0.6$                   | $3.2 ± 0.6$                     | $-3.0 ± 0.4$                    |
| I560A(3)   | $-22.1 ± 1.0$                   | $0.5 ± 1.0$                     | $-1.1 ± 1.0$                    |
| S644A(4)   | $-23.7 ± 1.0$                   | $-1.1 ± 1.0$                    | $-2.0 ± 0.4$                    |
| N684A(4)   | $-17.2 ± 1.1$                   | $5.5 ± 1.1$                     | $-0.7 ± 0.4$                    |
| Y545A/E660A(4) | $-21.0 ± 1.2$                 | $1.7 ± 1.2$                     | $-4.0 ± 0.9$                    |
| Y545A/S644A(3) | $-20.1 ± 0.4$                 | $2.6 ± 0.4$                     | $0.5 ± 0.7$                     |
| Y545A/N684A(4) | $-13.7 ± 1.2$                 | $8.9 ± 1.2$                     | $0.3 ± 1.0$                     |

* indicates statistical differences, $p < 0.05$ using an unpaired $t$ test when comparing the experimentally derived $\Delta \Delta G^\circ$ for the double mutant with the sum of the $\Delta \Delta G^\circ$ values for the two individual mutants.
Oocyte Preparation—Female *X. laevis* frogs were purchased from Nasco (Fort Atkinson, WI). The Garvan/St Vincent’s Animal Ethics Committee granted approval (ID 14/30) for all animal experiments described in this study. Frogs were anesthetized by immersion in 0.17% w/v Tricaine. One ovarian lobe was mobilized, and ~5 ml were removed. The follicular cell layer was digested by using 1 mg/ml collagenase A (Roche Applied Science) in Ca2+−free ND96 solution containing (in mM): NaCl 96, KCl 2, MgCl2 1.0 and Hepes 5 (pH adjusted to 7.5 with 5 M NaOH) for ~2 h. After rinsing with ND96 (as above, plus 1.8 mM CaCl2) to remove collagenase A, stage V and VI oocytes were isolated and stored at 18 °C in ND96 supplemented with 2.5 mM pyruvic acid sodium salt, 0.5 mM theophylline, and 50 μg/ml gentamicin. *X. laevis* oocytes were injected with cRNA and incubated at 18 °C for 24–48 h prior to electrophysiological recordings.

Electrophysiology—Two-electrode voltage-clamp experiments were performed at room temperature (~21 °C) using a Geneclamp 500B amplifier (Molecular Devices Corp., Sunnyvale, CA). We used glass microelectrodes with tip resistances of 0.3–1.0 megohms, when filled with 3 M KCl. Oocytes were perfused with ND96 solution (see above) during all experiments. A 50-ns 20-mV depolarization step from the holding potential of ~90 mV was applied at the start of each sweep to enable off-line leak-current subtraction. We assumed that the current leakage was linear in the voltage range ~150 to +40 mV. Data acquisition and analysis were performed using pCLAMP software (Version 10.2, Molecular Devices, Sunnyvale, CA), Excel software (Microsoft, Seattle), and Prism 6 (GraphPad Software Inc., La Jolla, CA). All parameter values are reported as means ± S.E. of the mean (S.E.) for *n* experiments, where *n* denotes the number of different oocytes studied for each construct.

Data Analysis—To measure the voltage dependence of activation, cells were depolarized from −70 to +60 mV for 3 s (exact voltage range depending on the mutant tested) followed by a step to −70 mV to measure tail current amplitude. Tail current amplitudes were normalized to the maximum tail current value and fitted with a Boltzmann function as shown in Equation 1,

\[
\frac{I}{I_{\text{max}}} = \left(1 + e^{(V_0 - V)/k}\right)^{-1} 
\]  
(Eq. 1)

where \(I/I_{\text{max}}\) is the relative tail current amplitude; \(V_{0.5}\) is the voltage at which 50% of channels are deactivated; \(V_t\) is the test potential, and \(k\) is the slope factor. To measure rates of activation, an envelope of tail protocol was used to obtain the rates of activation at 20 mV (42). The peak tail current amplitudes recorded at −70 mV using different activation duration at 20 mV were fitted with a single exponential function to obtain a time constant for activation.

Rates of deactivation were measured from the hooked tail current traces recorded at voltages in the range −70 to −150 mV, following a 1-s depolarization step to 40 mV from a holding potential of −90 mV. The decaying phase of the current traces was fitted with a double exponential component to derive both components of channel deactivation. However, only the fast component is reported in this study. To measure the voltage dependence of deactivation, cells were depolarized to +40 mV for 1 s to ensure channels were fully activated and then stepped to voltages between +60 and −100 mV (exact voltage range depending on the mutant tested) for 3 s, followed by a step to −70 mV to measure tail current amplitude (23, 24, 43). Tail current amplitudes were normalized to the maximum tail current value and fitted with a Boltzmann function using Equation 1. In experiments in which the free energies associated with voltage-dependent deactivation were examined, the same datasets were fitted with the thermodynamic form of the Boltzmann distribution as shown in Equation 2,

\[
\frac{I}{I_{\text{max}}} = \left(1 + e^{\Delta G^0 - zF(\theta)}\right)^{-1} 
\]  
(Eq. 2)

where \(\Delta G^0\) is the work done at 0 mV; \(z\) is the effective number of gating charges moving across the membrane electric field (\(F\)); \(F\) is the Faraday constant; \(\theta\) is the universal gas constant; and \(T\) is the absolute temperature. From Equation 2 we can also calculate the effect of a mutation on \(\Delta G^0\) as shown in Equation 3,

\[
\Delta \Delta G^0_{\text{mut}} = \Delta G^0_{\text{mut1}} - \Delta G^0_{\text{mut2}} 
\]  
(Eq. 3)

Double Mutant Cycle Analysis—To investigate whether two residues interact to stabilize the open and/or closed states we used thermodynamic mutant cycle analysis. The interaction energy, \(\Delta \Delta G^0_{\text{int}}\) between two mutants was calculated as the difference in free energy of the double mutant compared with the sum of the free energy differences of the individual mutants shown in Equation 4,

\[
\Delta \Delta G^0 = \Delta \Delta G^0_{\text{mut1}} + \Delta \Delta G^0_{\text{mut2}} - \Delta \Delta G^0_{\text{mut1+mut2}} 
\]  
(Eq. 4)

Absolute \(\Delta \Delta G^0_{\text{int}}\) values of greater than 4.2 kJ mol\(^{-1}\) (i.e. 1 kcal mol\(^{-1}\)) were considered significant (25–30).

Statistical Analysis—We used one-way ANOVA with Bonferroni post-test analysis to compare rates of deactivation and \(V_{0.5}\) of deactivation or an unpaired t test when comparing the \(\Delta \Delta G^0\) values of double mutants (\(\Delta \Delta G^0_{\text{mut1+mut2}}\)) compared with the sum of \(\Delta \Delta G^0\) values of individual mutants (\(\Delta \Delta G^0_{\text{mut1}} + \Delta \Delta G^0_{\text{mut2}}\)) in double mutant cycle analysis experiments. *p* values <0.05 were considered significant.

Homology Modeling—The crystal structure of a Kv1.2/2.1 channel chimera (19) was used to generate the homology model of Kv11.1 using Swiss PdbViewer (44), and the final structure was optimized by using SWISS-MODEL Workspace (45, 46).

Author Contributions—J. I. V. conceived and coordinated the study. C. A. N., A. E. G., I. M., and J. I. V. wrote the paper. C. A. N. designed, performed, and analyzed the experiments shown in Figs. 1 and 3–7. A. E. G. and I. M. designed, performed, and analyzed the experiments shown in Fig. 2. M. D. P. and A. A. A. assisted with the design of experiments and acquisition of data. All authors contributed to the preparation of the figures and approved the final version of the manuscript.

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