Research Paper

Potassium Channel Gating in the Absence of the Highly Conserved Glycine of the Inner Transmembrane Helix

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NOTES
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ABSTRACT

Potassium channel activation regulates cellular excitability, such as in neuronal and cardiac cells. Regulation of ion channel activity relies on a switching mechanism between two major conformations, the open and closed states, known as gating. It has been suggested that potassium channels are generally gated via a pivoted mechanism of the pore-lining helix (TM2) in the proximity of a glycine that is conserved in about 80% of potassium channels, even though about 20% of the channels lack a glycine at this position. Yet, as we show in G-protein gated potassium (Kir3) channels that lack a glycine at this position, the βγ subunits of G-proteins can still stimulate channel activity. Our results suggest that the effect of mutation of the central glycine (at position 175 in Kir3.4) on βγ-induced whole-cell currents is related to the extent of the interaction between residues located at the position of the central glycine and two residues, one located in the signature sequence of the selectivity filter (T149 in Kir3.4) and the other in the pore helix (E147 in Kir3.4). Our results also suggest that interactions with position 149 are more detrimental to channel function than interactions with position 147. The ability of Gγ to overcome such restraining interactions is likely to depend on a combination of characteristics specific to each residue.

INTRODUCTION

Potassium channel activation modulates cellular excitability in several physiological systems including that of neuronal and cardiac cells. A switching mechanism between channel open and closed states known as gating regulates activity and is triggered by a stimulus such as a change in membrane voltage or ligand binding. Upon activation, channels undergo conserved conformational changes. Recent structural and functional studies have suggested that a highly conserved glycine in the center of the inner α-helices (TM2) of the pore of potassium and sodium channels plays a critical role in the gating mechanism.1-5 Although this glycine is conserved in 81% of potassium channels, 19% of the channels include residues other than glycine at this position. In fact, the structural study of the bacterial KirBac1.1 K⁺ channel6 (PDB code 1P7B) suggested that another conserved glycine located nine residues below the central glycine functions as a gating hinge. Amongst 478 potassium channels,2 there is an asparagine in 7.5% of the channels instead of the glycine, an alanine in 4.0% and a serine in 3.1%. Table 1 displays the frequency of residues that occupy this position in the 478 potassium channels examined.

Examination of the subgroup of inwardly rectifying potassium channels shows that also within this subgroup, 80% of the channels possess a glycine at the center of the inner helix. The only inwardly rectifying potassium channels that do not possess a glycine are Kir4.1 and Kir4.2 that have a threonine in the equivalent position, and Kir5.1 that has a serine. The G-protein gated inwardly rectifying K⁺ (Kir3 or GIRK) channels are intracellular ligand-gated K⁺ channels, which are activated by the βγ subunits of Pertussis toxin-sensitive (PTX) G proteins.7,8 Kir3 channels are homomers or heteromers of four different subunits (Kir3.1–4) that have been identified so far.9-13 Heteromers of Kir3.1 and Kir3.4 channels constitute KACh14 that controls atrial cell excitability and the slowing of heart rate. The vagus nerve releases AcH to stimulate Muscarinic type 2 (M2) receptors and trigger the PTX-sensitive G proteins that activate KACh.

Kir3.4* is a mutated Kir3.4(S143T) channel that functions as a homomer,15 thus simplifying the experimental approach of structure-function studies. The central glycine of the Kir3.4* channel that was used in this study was mutated to all other amino acids.2
Single channel analysis showed that in most cases the mutants exhibited low-level basal activity with characteristic long bursts of activity and even longer silent periods, suggesting that the flexibility of the glycine at this position is required for channel opening. This leads to the question of whether potassium channels that possess a residue other than glycine at the center of the inner helix of the transmembrane domain are activated via the same mechanism that involves pivoted bending in the vicinity of the position of this glycine. In the case of Kir3.4*, the question could be rephrased as: can the βγ subunits of the G-protein enhance the activity of Gly mutant Kir3.4* channels, in which the highly conserved central glycine of the inner helix, position 175, is mutated to other residues?

In order to answer this question we have examined the effect of the coexpression of the βγ subunits of the G-protein on whole-cell currents of several Kir3.4* mutants, and studied the mechanism of action of these mutations via molecular dynamics using the homology model of the open conformation of Kir3.4* based on the structure of the MthK channel.1

The structure of the MthK bacterial K+ channel includes its gating machinery (the gating ring) and contains a bound ligand (a Ca2+ ion) that causes the channel to reside in an open state (PDB code 1LNQ). Comparison of the structure of this channel with the structure of the closed KcsA bacterial K+ channel (PDB code 1K4C)16 revealed large differences in the inner helices: in KcsA they are almost straight whereas in MthK they are bent approximately 30°. It has been suggested that MthK represents the open conformation of potassium channels. Previous Electron Paramagnetic Resonance (EPR) studies of the conformational changes of the KcsA transmembrane domain that occur when the channel opens17,18 are consistent with the crystallographic structure of MthK. Similarly to the MthK structure, the EPR studies showed that TM2 undergoes tilting away from the permeation pathway and twisting around its helical axis with a pivot point near the middle of TM2. Mutational effects in the Shaker voltage-gated potassium channel seemed to be consistent with close and open structures that are similar to the KcsA and MthK structures, respectively.19 Studies concerned with Kir3.4*2,20 are also consistent with a pivoted bending mechanism of the inner helix, which is in agreement with the bending in the corresponding helix in MthK. Modeling of the open state of KirBac1.1 on the basis of electron microscopy data on two-dimensional crystals of the inwardly rectifying K+ channel KirBac3.1 are also in agreement with the open configuration of MthK.21 On the basis of the above studies, we have chosen MthK to serve as the template for the open conformation of the transmembrane domain of Kir3.4* in the presence of the βγ subunits of the G-protein.

Previously, we showed that although mutation of the highly conserved glycine greatly reduces channel activity, its role lies in that it prevents constraining interactions with critical neighboring residues rather than in acting itself as a hinge. Here, we show that even in the absence of the conserved glycine, the activity of G protein-gated K+ channels can be enhanced by the Gβγ subunits. Our results suggest that on the one hand replacement of the central Gly with other residues leads to interactions with critical residues in the selectivity filter and the pore helix that can interfere with normal channel gating. Yet, the activity of mutated G protein-gated K+ channels can be enhanced by the appropriate stimulus, the βγ subunits of the G-protein. Furthermore, our results also suggest that the conformational change that results from activation of the channel by its physiological stimulus relieves interactions of the residues that replace the central glycine with the crucial residues in the selectivity filter and the pore helix in most cases, thus allowing the mutated channels to function significantly better, and in some cases, as well as, the control wild-type channel. Thus, absence of the highly conserved glycine in 3 out of 15 of Kir channels does not imply that these channels use a distinct gating mechanism in order to open.

**MATERIALS AND METHODS**

**Molecular modeling via restrained molecular dynamics.** MD simulations were performed using CHARMM version 26. The initial structure of Kir3.4* was constructed as a homology model of the KcsA crystallographic structure17,16 or of the MthK crystallographic structure.1 The missing side-chains in the homology models of Kir3.4* and its mutants were constructed and minimized using CHARMM. We applied a protocol that was previously used successfully to predict interactions in Kir3.4* between a lysine in position 175 and positions 149 and 179.20 Accordingly, we used structurally restrained molecular dynamics simulations that maintain the structural integrity of the helical TM domain. Specifically, we imposed NOE type restraints on α-helical backbone distances as well as harmonic restraints on the Cα atoms of residues in TM1. Following a minimization stage using the Steepest Descent and the adopted-basis Newton Raphson algorithms, a 1.2 nsec MD simulation at room temperature was carried out with a time step of 1 fsec. The environment was modeled by a distance-dependent dielectric. The force field for the energy calculation is the CHARMM force field.22 Structures in the trajectory were recorded every 1 psec. The analysis included all four subunits of the channel. The distributions of the structures according to the minimal distances between specific residues were plotted in terms of the percentage of structures of the total number obtained in the trajectory that exhibited a specific range of distances.

**Expression of recombinant channels in Xenopus oocytes.** Point mutations on the background of the control Kir3.4 (S143T) (Kir3.4*) channel23 were generated using the Quickchange site-directed mutagenesis kit (Stratagene, La Jolla, CA). RNAs were transcribed in vitro using the “Message Machine” kit (Ambion, Austin, TX). RNA concentration of Kir3.4* was estimated from two successive dilutions.
of Kir3.4*, position 175, a substantial decrease in whole-cell basal currents is observed in most cases. In view of the fact that 19% of potassium channels lack a glycine in the center of the inner helix, we examined whether the appropriate stimulus could activate the channel without a glycine at position 175. As Kir3.4* channels are activated by the βγ subunits of G proteins, we have examined the effect of coexpression of Gβ1y2 with several Gβ175 mutants of Kir3.4*. As can be seen in Figure 1A, the extent of the effect of coexpressing Gβ1y2 with Gβ175 mutants of Kir3.4* varied with the mutant. For example, in the case of Kir3.4*G175S, coexpression with Gβ1y2 resulted in currents that were close to the currents obtained for the control Kir3.4* channel. Even more striking was the effect of coexpressing Gβ1y2 with Kir3.4*G175S. Whereas the basal currents exhibited by Kir3.4*G175S were only about 20% of the control currents, in the presence of the stimulus, Gβ1y2, complete recovery was obtained. On the other hand in the case of Kir3.4*G175K, the currents obtained upon coexpression of the channel with Gβ1y2 were significantly smaller than in the case of the control Kir3.4*. Other cases, including the Kir3.4*G175E, Kir3.4*G175Q, and Kir3.4*G175N exhibit partial recovery of the currents. And yet, the exogenous Gβ1y2 enhanced the activity of the majority of the cases that lacked glycine at the center of the inner helix more than it enhanced the activity of Kir3.4* itself. Only for the Kir3.4*G175N, the effect of Gβ1y2 was comparable to its effect on Kir3.4*.

**Interaction pattern between residues in position 175 and residues in the signature sequence of the selectivity filter and its vicinity.** We have previously shown that in several Gβ175 mutants of Kir3.4* including the G175D, G175E, G175K and G175Q, basal currents are significantly smaller than in the control channel.20 We have shown that for all these cases, this was not due to a decrease in conductance. Furthermore, in the case of G175K our data suggested that interaction of the lysine at position 175 with the threonine in position 149 was detrimental. Position 149 in Kir3.4* is located at the signature sequence of the selectivity filter. Mutation of the threonine to serine resulted in an increase in basal current for Kir3.4*G175K despite a decrease in basal current of the control Kir3.4* following the T149S mutation. These results lead to the question whether in the absence of glycine at position 175, interactions of residues at this position with residues in the signature sequence of the selectivity filter and its vicinity also play a role in determining the extent of the effect of coexpressed Gβ1y2 on channel activation. In order to examine this possibility, we have studied the possible interactions of several different residues at position 175 with residues 147–149 in both the closed and open conformations of the channel as represented by the homology models of Kir3.4* that are based on KcsA and MthK. The alignment of the inner helix, the pore helix and the selectivity filter of Kir3.4*, KcsA and MthK on which the homology models were based is given in Figure 1B. This alignment is in agreement with the alignment previously published for the inner helices and the selectivity filter of Kir3.2, KcsA and MthK.1 Residues

**RESULTS**

The effect of coexpression with Gβ1y2 of Kir3.4* channels that are mutated at position 175 depends on the residue substituted for glycine. In the absence of a glycine at the center of the inner helix which were electrophoresed in parallel on formaldehyde gels and compared to known concentrations of an RNA marker (GIBCO, Gaithersburg, MD). Oocytes were isolated and microinjected as previously described.24 Expression of channel proteins in oocytes was accomplished by injection of the desired amount of RNA into Xenopus oocytes. Oocytes were injected with RNA, 2 ng of channel, and 2 ng of each G protein subunit when required. All oocytes were maintained at 17°C. Two-electrode voltage clamp recordings were performed two days following injection.

Two-electrode voltage-clamp recording and analysis. Whole-cell currents were measured by conventional two-microelectrode voltage clamp with a GeneClamp 500 amplifier (Axon Instruments, Union City, CA), as previously reported.24 A high-potassium (HK) solution was used to superfuse oocytes (in mM: 96 KCl, 1 NaCl, 1 MgCl₂, 5 KOH/HEPES [pH 7.4]). Basal currents represent the difference of inward currents obtained (at -80 mV) in the presence of 3 mM BaCl₂ in HK solution from those in the absence of Ba²⁺. Each experiment shown was performed on 3–5 oocytes of the same batch. A minimum of two batches of oocytes was tested for each normalized recording shown. Recordings from different batches of oocytes were normalized by the mean of whole-cell basal currents from oocytes expressing the control channel Kir3.4*. Statistics (i.e., mean and standard error of the mean) of each construct were calculated from all of the normalized data recorded from different batches of oocytes.
147, 149 and 175 of Kir3.4* are highlighted in the figure green, cyan and red, respectively.

Patterns of interactions between threonine 149 and residues substituted for the central glycine 175. When the position of the central glycine in Kir3.4* was occupied by an aspartate, a glutamate, or a lysine, we examined the interactions between these residues and residues in their vicinity. The results obtained from molecular dynamics simulations suggested that these residues interact with the threonine at position 149. The details of these interactions are as follows.

Interaction pattern of an aspartate at the center of the inner helix. Four hydrogen-bonding pairs were observed between D175 and T149. These include the interactions between the side-chain OD1 and OD2 of the aspartate and the side-chain HG1 and backbone HN of the threonine, as can be seen in Figure 2A and B. Supplementary Figure 1A displays the histograms of the distances between the four hydrogen bonding pairs obtained from MD simulations for models based on the closed KcsA and the open MthK structure. In the open conformation, the distance between the hydrogen bonding pairs increases and the centers of the distributions shift to larger distances. In order to take into account all the structures that exhibit hydrogen bonding between positions 175 and 149, we calculated the minimal distance between D175 and T149. The cutoff for hydrogen bonding was set at 2.5Å between a hydrogen-acceptor pair. Figure 2F portrays the histogram of the minimal distance between D175 and T149 in the MthK-based open conformation. The histogram includes several distributions of distances, and among them only 6% are within hydrogen bonding range. Comparison between the percentages of structures in which there is at least one hydrogen bond between positions 175 and 149 in the KcsA- and in the MthK-based models (Table 2) suggests that there is a significant decrease of H-bonding between these positions with the conformational change. Whereas in the KcsA-based structure there are hydrogen bonds between D175 and T149 in 69% of the structures, in the MthK-based structure, there are hydrogen bonds in only 6% of the structures.

Interaction pattern of a glutamate at the center of the inner helix. Four hydrogen-bonding pairs were also observed between E175 and T149. These include the interactions between the side-chain OE1 and OE2 of the glutamate and the side-chain HG1 and backbone HN of the threonine, (Fig. 2C and D). As can be seen in Supplementary Figure 1B, there is much less hydrogen bonding between the hydrogen bonding pairs in the closed KcsA-based conformation compared with the G175D mutant (Supplementary Fig. 1A). However, the conformational change from the KcsA-based closed structure to the MthK-based open structure has a much smaller effect on the hydrogen bonding pattern between E175 and T149.
compared to its effect in the case of the G175D mutant, probably due to the longer side-chain of the glutamate compared with that of the aspartate. Figure 2F (middle panel) depicts the histogram of the minimal distance between E175 and T149 in the MthK-based open conformation. Accordingly, in 17% of the structures there is hydrogen bonding. In comparison, in the closed conformation 25% of the structures (Table 2) exhibit hydrogen bonding between positions 175 and 149. In other words, the number of structures that exhibited interactions between positions 175 and 149 in the open conformation was 32% less than the number of structures that exhibited interactions in the closed conformations. This difference is primarily a result of a significant reduction in the population that exhibited strong hydrogen bonding between positions 175 and 149 following the conformational change from the closed to the open structure. Specifically, there is a reduction of about 75% in the population in which the distance between OE2 of E175 and HG1 of T149 is less than 1.5Å (Supplementary Fig. 1B). In addition, there is about 8% of reduction in the population in which the distance between positions 175 and 149 is between 1.5Å and 2.5Å.

**Interaction pattern of a lysine at the center of the inner helix.** Three hydrogen-bonding pairs were observed between K175 and T149. Due to the different nature of the side-chain of the lysine compared with the aspartate and the glutamate, the interacting pairs change accordingly, and include interactions between the side chain HZ1, HZ2 and HZ3 of the lysine and the side-chain OG of the threonine, see Figure 2E. As summarized in Table 2 and can also be seen in Supplementary Figure 1C, there is a decrease in the extent of hydrogen bonding between K175 and T149 from 60% to 25% with the conformational change from the closed KcsA-based structure to the open MthK-based structure. In addition to its interactions with position 149, HZ1, HZ2 and HZ3 of K175 also interact with the backbone carbonyl of position 147, see Figure 2E. Comparison of these interactions in the closed and open conformations of the mutated channel shows that also in this case, there is a decrease in the percentage of structures that exhibit interactions, see Supplementary Figure 1D. In the closed conformations 86% of the structures exhibit interactions between positions 175 and 147, in the open conformation only 3% of the structures exhibit interactions between these positions. Examination of the structures that exhibit interactions with either position 147 or position 149 shows that there is significant overlap between these populations. In the closed conformation, only 4% of the structures that exhibit interactions between positions 175 and 147 are not included in the population that exhibits interactions between positions 175 and 149. In other words, 56% of the structures exhibit both interactions, 30% of the structures exhibit interactions between position 175 and position 147 alone, and 4% of the structures exhibit interactions between position 175 and position 149 alone. Figure 2F depicts the histograms of the distributions of the minimal distances between position 175 and positions 147 and 149 as obtained from the MD simulation of the open conformation of the mutated channel. In the open conformation, just 3% of the structures exhibit interactions between positions 175 and 147 and these are included in the population that exhibits interactions between positions 175 and 149 (25% of the structures). Thus, in the open conformation, the dominant interaction is the one between positions 175 and 149.

**Patterns of interactions between glutamate 147 and residues substituted for the central glycine 175.** When the position of the central glycine in Kir3.4* was occupied by a glutamine, a serine or an asparagine, the results obtained from molecular dynamics simulations suggested that these residues interact with the glutamate at position 147. The details of these interactions are as follows.

**Interaction pattern of a glutamine at the center of the inner helix.** Two hydrogen-bonding pairs were observed between Q175 and E147. These include the interactions between the side-chain HE21 and HE22 of the glutamate at position 175 and the backbone carbonyl O of the glutamate at position 147 (Fig. 3A). The decrease in the population that exhibits interactions between Q175 and E147 upon conformational change from the closed KcsA-like conformation to the open MthK-like conformation is from 66% to 18% respectively, as summarized in Table 2, and as can be seen in Supplementary Figure 2A.

**Interaction pattern of a serine at the center of the inner helix.** S175 and E147 interact via hydrogen bonding between the backbone HG1 of S175 and the backbone carbonyl O of E147, as can be seen in Figure 3B. Similarly to the case of the Q175 mutation, also in this case there is interaction with the backbone carbonyl of the glutamate. Comparison of the population that exhibits interactions between these residues in the closed and open conformations shows a remarkable decrease in the size of this population from 80% to 2%; see Table 2 and Supplementary Figure 2B. Following the conformational change to the open MthK-based structure, the short side-chain of the serine is rarely able to extend sufficiently to interact with position 147, see Figure 3D.

**Interaction pattern of an asparagine at the center of the inner helix.** The G175N mutant shows a combination of two kinds of interactions (Fig. 3C): interaction of the side-chain HD21 and HD22 of the asparagine with both the backbone carbonyl O and the side-chain OE1 and OE2 of the glutamate. In both cases, there is a moderate decrease in the population that exhibits hydrogen bonding with the conformational change from the closed KcsA-like conformation to the open MthK-like one (Supplementary Fig. 2C and D). There is a decrease from 49% to 30% in the interactions between N175 and the backbone carbonyl of E147, and a decrease from 61% to 54% in the interactions between N175 and the side-chain of E147. Overall, there is a decrease from 85% to 57% in the population that displays interactions between N175 and E147 (Table 2). This implies that there is considerable overlap between the population that exhibits interactions of N175 with the backbone of E147 and the population that exhibits interaction with the side-chain of E147 in the closed KcsA-like conformation. In the open MthK-like conformation almost all structures that exhibit interactions between N175 and the backbone of E147 also exhibit interactions with the side-chain of E147. Only 3% of the structures that exhibit interactions between N175 and the backbone of E147 do not display interaction with the side-chain of E147.

**Relationship between the experimental data and the computational results.** Comparison of the currents obtained following coexpression with Gβγ of the Kir3.4* G175D, G175E and G175K mutants (Fig. 1A), and the interactions displayed between positions 175 and 149 (Fig. 2F) in the open conformation suggests that the larger the percentage of structures that exhibit hydrogen bonding with position 149 is, the smaller are the currents obtained. Similarly, comparison of the currents obtained following coexpression with Gβγ (Fig. 1A) of the Kir3.4* G175N, G175Q and G175S mutants, and the interactions displayed between positions 175 and 147 (Fig. 3D),
suggests that the larger the percentage of structures that exhibit hydrogen bonding with position 147 is, the smaller are the currents obtained. This can be more clearly seen in Figure 4, which depicts the normalized current obtained for the various Kir3.4* mutants following coexpression with Gβγ as a function of the fraction of structures that lack interaction between position 175 and either position 147 or position 149. Basal currents of Kir3.4* have been shown to be completely inhibited by the Gβγ scavenger PH domain of the β-adrenergic receptor kinase (βARK-PH), and are thus a result of endogenous Gβγ present in the oocytes. The concentration of endogenous Gβγ in oocytes enables some of the channels to open but is not sufficient to achieve maximal whole-cell currents. We have therefore chosen to plot the current obtained following coexpression with Gβγ since the βγ subunits are required for activation of Kir3.4*, and thus provide a better manifestation of the channel being in its open state.

Examination of the relationship between the currents obtained following coexpression with Gβγ and the interactions displayed between positions 175 and 147 or 149 in the open conformation as depicted in Figure 4, results in the following three observations:

(a) In view of the importance of positions 147 (pore helix) and 149 (selectivity filter), interactions with these positions can interfere with normal channel function, and are therefore detrimental.

(b) Interactions with position 147 are less detrimental than interactions with position 149. Within the group that displays interactions with position 149, almost all currents were abolished when only 25% of the structures exhibited interactions. On the other hand, within the group that displays interactions with position 147, significant currents (about 40% Gβγ enhanced Kir3.4* currents) were observed even when about 60% of the structures exhibited interactions.

(c) Within each group of mutants (i.e., those that display interactions with position 147 and those that display interactions with position 149) the current displayed by the mutant becomes smaller as the population that displays interactions increases.

Mutations of position 175 of Kir3.4* on the background T149S. The above results suggest that interactions with position 149 are detrimental to channel function. We thus mutated this position to serine and tested several of the G175 mutants on the background of the T149S mutation. Kir3.4*T149S displays basal currents of approximately 50% compared to Kir3.4*. The T149S mutation, however, does not affect the selectivity properties of the channel or its conductance characteristics, and does not have a significant effect on its ACh dose-response curve. We have thus examined the G175D, G175E, G175N and G175S mutations on the background of the T149S.

The effect of coexpression with Gβ1γ2 of Kir3.4*T149S channels that are mutated at position 175. Similarly to the effect of the G175D, G175E, G175N and G175S mutations on Kir3.4*, mutation of Gly175 to these amino acids in Kir3.4*T149S resulted in a substantial decrease in whole-cell basal currents (Fig. 5A). The extent of the decrease in current, however, was generally different compared to the effect of the mutations on the current of Kir3.4*. Examination of the effect of coexpressing Gβ1γ2 with G175 mutants of Kir3.4*T149S varied with the mutant as obtained also for the G175 mutants of Kir3.4*, but the details were different. For example,
in the case of the Kir3.4*G175D mutation, coexpression with Gβ1γ2 resulted in currents that were only about 40% of the currents obtained for the Kir3.4*T149S channel (Fig. 5A), which is significantly less than the effect of coexpression of Gβ1γ2 on Kir3.4*G175D (Fig. 1A). In the case of Kir3.4*T149S-G175E, the increase in current upon coexpression of the channel with Gβ1γ2 was even smaller. On the other hand, for Kir3.4*T149S_G175S—complete recovery was obtained in the presence of Gβ1γ2. Also, similarly to the general trend that was obtained for Kir3.4*, the exogenous Gβ1γ2 enhanced the activity of the majority of the Gly175 mutants more than it enhanced the activity of the Kir3.4*T149S itself.

Interaction pattern between residues in position 175 and residues in the signature sequence of the selectivity filter and its vicinity on the background of the T149S mutation. We thus examined the effect of these mutations on the interaction pattern between position 175 and residues in the signature sequence of the selectivity filter and in its vicinity. The first two, the G175D and G175E mutations, exhibit interactions between position 175 and position 149, while the last two, the G175S and the G175N mutations, exhibit interactions primarily with position 147.

Interaction pattern of an aspartate at the center of the inner helix. Two hydrogen-bonding pairs were observed between D175 and S149. These include the interactions between OD1 and OD2 of the aspartate and HG1 of the serine. Figure 5B portrays the histogram of the minimal distance between D175 and S149 in the MthK-based open conformation. Comparison between the percentages of structures in which there is at least one hydrogen bond between positions 175 and 149 in the KcsA and in the MthK-based models (Table 3) suggests that there is a decrease of H-bonding between these positions with the conformational change from 46% in the KcsA-based structure to 20% in the MthK-based structure.

**Table 3**

| Percentage of Structures Exhibiting Interactions with T149 | Percentage of Structures Exhibiting Interactions with E147 |
|----------------------------------------------------------|----------------------------------------------------------|
| G175D | G175E | G175S | G175N |
| KcsA  | 46%  | 25%  | 100% | 83%  |
| MthK  | 20%  | 26%  | 7%   | 37%  |

The population that exhibits these interactions in the closed and open conformation, shows a significant decrease in the size of this population from 100% to 7%, see Table 3.

Interaction pattern of an asparagine at the center of the inner helix. In the closed KcsA-based structure, the G175N mutant interacts with both positions 147 and 149. The interactions with position 147 include hydrogen bonding of HD21 and HD22 of the asparagine with both the backbone carbonyl and the side-chain OE1 and OE2 of the glutamate. The interactions of position 175 with position 149 include hydrogen bonding between HD21 and HD22 of the asparagine with OG of the serine, and between OD1 of the asparagine and HG1 and HN of the serine. The total percentage of structures that exhibit interactions of position 175 with position 147 is 83% (Table 3). Among these, 16% also exhibit interactions with position 149.

To summarize, molecular dynamics simulations for the Kir3.4*T149S G175 mutants on the basis of the homology model of MthK suggest that on the background of the T149S the interaction pattern between position 175 and position 149 is altered compared to the Kir3.4*G175 mutants, see Figure 5B. Both Kir3.4*T149S_G175D and Kir3.4*T149S_G175E exhibited higher percentages of interactions with position 149 compared to Kir3.4*G175D and Kir3.4* G175E. Also, Kir3.4*T149S_G175S exhibited a slightly higher percentage of interactions with position 149 than Kir3.4* G175S.

Figure 5C depicts the normalized currents obtained following coexpression with Gβ1γ2 of the Kir3.4*T149S G175 mutants as a...
function of the fraction of structures that lack interaction between position 175 and either position 147 or position 149. As can be seen in this figure, the additional points obtained for the Kir3.4*T149S-G175 mutants fall along the curves that illustrate the trend of the data obtained for the Kir3.4* G175 mutants.

**DISCUSSION**

The results presented above suggest that in general interactions of position 175 with either position 149 of the selectivity filter or position 147, which is located in its vicinity in the pore region, result in reduced Kir3.4* whole-cell current. This is probably due to the interference of such interactions with the normal function of the channel. The percentage of the occurrence of these interactions in the open state determines to what extent the conformational change due to Gβγ will be able to relieve these interactions and achieve maximal activation. Our results also suggest that interactions of position 175 with position 147 are less detrimental than interactions with position 149.

In the case of the Kir3.4*G175K and the Kir3.4*G175E_T149S mutants, examination of the trajectory of the interactions of position 175 with position 149 in the four channel subunits of the MthK...
homology-based open structure shows that only about 25% display interactions. Yet, the currents obtained were very small (Figs. 4 and 5C). As the channel is a tetramer, the 25% that display interactions may correspond to one of the four subunits. This suggests that even when just one of the four subunits exhibits interactions between positions 175 and 149, the channel becomes nonfunctional. In other words, this suggests that in the case of mutants that display interactions between position 175 and position 149, current would be obtained only in the absence of hydrogen bonding between positions 175 and 149 in all four subunits.

On the other hand, in the case of mutants that display interactions between position 175 and position 147, current would be obtained even when hydrogen bonding exists between positions 175 and 147. The $E147Q$ mutation has been shown to affect channel activity. This position is in very close proximity to the serine at position 143, mutation of which to a threonine dramatically increases homomeric channel activity of Kir3.4.\textsuperscript{15} On the background of Kir3.4 when coexpressed with Kir3.1, it has been shown that the $E147Q$ mutation leads to increased channel activity.\textsuperscript{26} On the other hand, on the background of Kir3.4\textsuperscript{4}, the $E147Q$ mutation has been found to decrease channel activity. Our results suggest that restraining interactions of this position result in decreased channel activity, but that the effect of these interactions is not as severe as restraining interactions on the selectivity filter that can abolish channel function. Our results also suggest that even when all the subunits that Kir3.4\textsuperscript{4} is comprised of are affected, the channel does not become completely nonfunctional (Figs. 4 and 5C).

This may explain the comparable effect of exogenous Gβ1γ2 on the activity of the G175N mutant compared with Kir3.4\textsuperscript{4}, and on the activity of Kir3.4\textsuperscript{4}T149S_G175E compared with Kir3.4\textsuperscript{4}T149S. Our results show that the βγ subunits of the G-protein can enhance the activity of mutated Kir3.4\textsuperscript{4} channels in which the highly conserved central glycine of the inner helix, position 175, is mutated to other residues. The extent to which Gβγ can enhance Kir3.4\textsuperscript{4} channel activity depends on the interactions of position 175 with residues that are crucial to channel function. In the control Kir3.4\textsuperscript{4} the native Gly at position 175 shows no interactions with position 147 (or 149) and Gβγ enhancement is modest. In the majority of the cases examined, the exogenous Gβ1γ2 enhanced the activity of the Gly175 mutants more than it enhanced the activity of the Kir3.4\textsuperscript{4} itself. For the G175N mutant, the percentage of structures that exhibit interactions between positions 175 and 147 was reduced from 85% to 57% with the conformational change from a closed structure to an open one. These results suggest that at this level of interactions, such a change would not have an effect on the normalized Gβγ enhanced currents (Figs. 4 or 5C, blue curve). Therefore, the effect of coexpression of the G175N mutant with Gβ1γ2 is similar to its effect for Kir3.4\textsuperscript{4}, in which the functional effect reflects a conformational change that is independent of the way residues at position 175 interact with E147. For Kir3.4\textsuperscript{4}T149S_G175E, following the conformational change from the closed conformation to the open one, the percentage of structures that exhibit interactions does not change significantly (Table 3), and is 25% and 26% respectively. As a result, the effect of coexpression of Kir3.4\textsuperscript{4}T149S_G175E with Gβ1γ2 is similar to its effect for Kir3.4\textsuperscript{4}T149S.

In contrast, the effect of removal of harmful interactions has been particularly manifested for the G175S mutant. In this case our analysis has shown reduction from 80% of structures that exhibit hydrogen bonding between positions 175 and 147 in the KcsA based homology model of Kir3.4\textsuperscript{4} to 2% in its MthK based homology model. This result is in agreement with the experimental data that show that when the channel is coexpressed with Gβγ, the currents obtained are not significantly different from those obtained for the wild type Kir3.4\textsuperscript{4}, indicating the removal of any detrimental interactions following the G175S mutation.

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