Activation gating kinetics of GIRK channels are mediated by cytoplasmic residues adjacent to transmembrane domains

Rona Sadja and Eitan Reuveny*

Department of Biological Chemistry; Weizmann Institute of Science; Rehovot, Israel

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G-protein-coupled inwardly rectifying potassium channels (GIRK/Kir3.x) are involved in neurotransmission-mediated reduction of excitability. The gating mechanism following G-protein activation of these channels likely proceeds from movement of inner transmembrane helices to allow K⁺ ions movement through the pore of the channel. There is limited understanding of how the binding of G-protein βγ subunits to cytoplasmic regions of the channel transduces the signal to the transmembrane regions. In this study, we examined the molecular basis that governs the activation kinetics of these channels, using a chimeric approach. We identified two regions as being important in determining the kinetics of activation. One region is the bottom of the outer transmembrane helix (TM1) and the cytoplasmic domain immediately adjacent (the slide helix); and the second region is the bottom of the inner transmembrane helix (TM2) and the cytoplasmic domain immediately adjacent. Interestingly, both of these regions are sufficient in mediating the kinetics of fast activation gating. This result suggests that there is a cooperative movement of either one of these domains to allow fast and efficient activation gating of GIRK channels.

Introduction

Control of resting membrane potential in excitatory cells is mediated, in part, by inwardly rectifying potassium (IRK; Kir) channels. These channels operate near the potassium equilibrium potential, being blocked by internal Mg²⁺ ions and polyamines at more depolarized values (reviewed in ref. 1). Channels of the G-protein gated inwardly rectifying potassium subfamily (GIRK; Kir3.x) are gated directly by Gβγ subunits of the G-protein, released upon receptor stimulation.²⁴ There are four mammalian GIRK channels, found primarily in the brain, where they are involved in generating postsynaptic slow inhibitory potentials,⁵⁻⁸ and in pacemaker cells of the heart, where they increase the time between consecutive heartbeats.⁹⁻¹³ GIRK channels are composed of heterotetramers, generally of GIRK1 and either GIRK2 or GIRK4, or homotetramers of GIRK2,¹⁴,¹⁵ or GIRK4,¹¹,¹² (reviewed in refs. 16 and 17). GIRK3 may be involved in downregulating GIRK expression by directing channels to lysosomes.¹⁸

Each subunit consists of two transmembrane domains with a pore region in between, containing the GYG K⁺ selectivity motif.¹⁷ Activation gating likely proceeds by movement of the inner transmembrane helices (TM2s),¹⁹⁻²¹ but the transduction pathway is unclear. Many different regions are necessary for Gβγ-mediated activation gating have been identified,²²⁻³⁰ but it is still not apparent how these G protein binding sites translate the binding event to channel opening.³¹ In the past few years some very important advances have been made in understanding the structure/function aspects of Kir gating, mainly by the elucidation of the crystal structure of most of the GIRK1 cytosolic domains,³² as well as that of bacterial inward rectifying K⁺ channels, KirBac1.1 and KirBac3.1.³³ These studies have contributed greatly to our understanding of the lowest energetic state of these channels, but can only provide hints pertaining to the movement of this channel in opening of the permeation pathway. Furthermore, the structures currently available lack much of their N-termini and the distal C-termini, regions which in GIRK1 not only bind Gβγ subunits,³⁴ but also show large conformational rearrangements upon gating.³⁵

Previously, we created a chimeric construct that displays slow activation gating, in which the cytosolic domains of GIRK1 were replaced with those of IRK1 in a tandem GIRK1/4 tetramer.³⁶ Based on this initial observation, in this study, we set out to test the molecular elements responsible for the kinetics of channel activation gating using a chimeric approach. Here we found that both a region in the N-terminus proximal to the first transmembrane domain, as well as a region in the C-terminus proximal to the second transmembrane domain of GIRK1, functioned independently to affect kinetics of activation gating. Further discussion will follow to show that the effect on channel activation gating kinetics was independent of known channel modulators such as PIP₂, the type of Gα modulator, RGS, which is involved in activation and/or GTP hydrolysis.
Results

Measuring activation gating kinetics. In order to accurately measure channel activation times at high resolution, we developed a method for measuring half-times of activation ($t_{1/2}$). The perfusion system of the two-electrode voltage clamp setup was automated so that solution changes occur at a predetermined time point. This time-resolved perfusion change takes into account the delay (dead time) between the actual switching of solution to the point at which the activation gating response is seen. This dead time is mainly due to the time it takes for the solution to enter the recording chamber. Since the GIRK activation response seen electrophysiologically is delayed, it is very difficult to estimate the time once the receptor agonist reached the oocyte’s membrane. The difficulty lay in the fact that the time dependence of GIRK channel activation begins relatively slowly ($t_i$; see Fig. 1). To estimate the exact time it takes for solution exchange on the oocyte membrane, we expressed the ionotropic receptor GABA$_A$, which displays rapid activation of a few milliseconds, and thus accurately reports the time when the appropriate solution reaches the oocyte’s membrane. Figure 1 shows an example of the time course of activation of GABA$_A$ upon saturating agonist stimulation (20 μM GABA), in which $t_0$ is the time at which the agonist-containing solution was switched on and $t_i$ is the time at which the channel opens. The dead time was thus determined to be 590 ± 30 ms ($n = 10$). Since $t_0$ is preset and the dead time of solution flow is known (590 ms), we can easily calculate $t_j$, which is taken as the initial time of agonist introduction to the GPCR receptor. The half-time of activation ($t_{1/2}$) is then calculated as the time at which currents are half-maximal ($I_{1/2} = (I_f - I_i)/2$) (Fig. 1). Since GIRK1/4 activation upon muscarinic type 2 receptor (m2R) activation (at saturating agonist concentration) is slower than that of GABA$_A$, we conclude that the rate limiting step for GIRK1/4 channel activation is not dependent on solution exchange.

Chimeric dimers behave like the parent tetramers. We have previously shown that a tandem tetramer consisting of two wild-type GIRK4 subunits and two chimeric subunits, where GIRK1 cytosolic domains were replaced with the corresponding domains of IRK1, exhibited dramatically slowed m2R activation. To simplify the data interpretation and to have complete control of subunit stoichiometry, we constructed tandemly linked dimers consisting of GIRK4 and GIRK1 wild-type channels, Dim1C, and compared the activation kinetics with the expressed wild-type monomers. This dimer showed m2R-mediated activation of 2.1 ± 0.1-fold compared to 1.5 ± 0.1-fold for channel formed from wild-type monomers (data not shown), and half-time of activation ($t_{1/2}$) of 1.3 ± 0.1 s compared to 1.8 ± 0.1 s for wild-type monomers (Fig. 2A and B). We then set out to test the effect of replacing the cytosolic domains of GIRK1 with the corresponding domains of IRK1, similar to the tandem tetramer we previously described. Comparing the wild-type dimer (Dim1C) to the chimeric dimer (Dim2) half-times of activation following m2R stimulation, Dim2 displayed a much slower $t_{1/2}$ of 7.2 ± 0.7 s (Fig. 2). This initial set of experiments demonstrates that the
like residues in both the N- and C-termini. Following the observed slow activation gating of the dimer containing the N- and C-termini of IRK1 instead of the corresponding GIRK1 termini (Dim2), we decided to determine whether the N- or the C-terminus of GIRK1 was responsible for fast activation gating kinetics. We created a series of dimers in which various cytoplasmic regions of GIRK1 were switched with those of IRK1. (See Suppl. Fig. 1 for a listing of the exact residues involved). Adding back the C-terminus of GIRK1 to the slow dimer to create Dim2C, we were able to reconstitute fast activation gating, with $t_{1/2}$ of 1.9 ± 0.1 s, similar to what had been observed for the wild-type dimer. Surprisingly, when the N-terminus of GIRK1 was added to the slow Dim2, creating Dim1, a fast kinetic of activation ($t_{1/2}$ of 1.8 ± 0.1 s) was observed as well, similar to the wild-type dimer. These results suggest that both the N- and the C-termini of GIRK1 can support fast activation gating independently. In order to further localize the residues important for fast activation gating in both the N- and the C-terminal domains of GIRK1, more dimers were made in which smaller pieces of the termini were added back into the slow dimer (Fig. 2A). The minimal region that was responsible made in which smaller pieces of the termini were added back into the N- and the C-terminal domains of GIRK1, more dimers were able to reconstitute fast activation gating, with $t_{1/2}$ of 1.8 ± 0.1 s. This channel displayed impaired kinetics of activation ($t_{1/2}$ of 4.9 ± 0.7 s). This is in contrast to the cases where the dimers contain either the N-terminus (Dimers 1, 3, 5) or the C-terminus (Dimers 2C, 4C, 6C) of GIRK1 residues, to display normal channel activation gating kinetics. These results thus suggest that the part of the N-terminus adjacent to TM1, including the first six residues of TM1, as well as the last six residues of TM2 and the region post the TM2 of GIRK1, are responsible for fast activation gating kinetics independently.

As described above, activation kinetics is mainly due to the presence of both N- and C-termini of the GIRK1 subunit. We were interested to test whether these domains also affect deactivation (closing) of the channel upon removal of receptor agonist. Example of current trace of Dim1C and Dim2 are shown to demonstrate that although channel activation kinetics is greatly affected, the deactivation rates are not (Fig. 3C). These results may suggest that upon agonist removal, the rate limiting step for channel closing is not dependent of channel structure that affect gating, but probably reflect the rate of GTP hydrolysis and the reassembly of the G protein trimer (for more details see discussion).

Since the C-terminal region implicated in fast activation gating overlaps with the region previously identified to constitute part of the PIP$_2$ binding pocket,37,38 we examined whether PIP$_2$ binding strength dictates the speed of channel activation. In particular, L222 of IRK1 is an important component of PIP$_2$ binding. When mutated to the isoleucine of GIRK4, PIP$_2$ interaction is significantly weakened.37 However, when we made the mutation L222I on the background of the slow dimer Dim6 (Fig. 2), we did not see a change in kinetics of activation. PIP$_2$ binding strength is therefore not the apparent key determinant in affecting channel activation kinetics of these dimers. It is important to note that in all dimeric constructs tested, $t_{1/2}$ of activation was not correlated with the basal and/or carbachol-induced current densities (Fig. 2C) or with the ability to gated the channel (Suppl. Fig. 2). This implies that the activation kinetics is not dependent on the amount of functional channels in the plasma membrane.

In attempting to further identify residues involved in activation gating kinetics, we tried to narrow down the N-terminus and C-terminus regions mentioned above. Of the 11 N-terminal residues (77–87), seven are conserved between GIRK1 and IRK1 (Fig. 4A). Independently mutating the remaining four on the background of Dim1 (178L/R79K, M83N and V86F) resulted in mutants which were all as slow as Dim11, even though these four residues accounted for all the difference between GIRK1 and IRK1 in this region (Fig. 4B). It is therefore seems likely, that the region as a whole, is important for activation gating rather than any single residue. The C-terminal region (residues 180–249) was quite large, but two constructs in which smaller regions of IRK1 were substituted, failed to yield any useful information. Dimers 12 and 13 (see Suppl. Fig. 1) resulted in channels with no induced currents (N.I.C.). It is interesting to note that the channels were functional, albeit with very low basal currents, and perhaps the lack of induced activation gating may be considered a type of slow gating.

**GDP/GTP exchange is not the rate-limiting step in activation of slow dimers.** In order to determine whether activation kinetics of the slow dimers was impaired due to weakened interaction with G$_\text{G}_{\text{q/11}}$ in general, we expressed several dimers with RGS4 (regulator of G-protein signaling). RGS4 has been shown to directly bind G$_\alpha^{\text{GTP}}$ to accelerates activation kinetics,40,41 perhaps through supporting the assembly of a complex involving receptor, G-protein, and RGS,40,42-44 or possibly by accelerating GDP for GTP exchange.45-47 When co-injected with m2R and RGS4,
the wild-type dimer (Dim1C) and two other fast dimers, Dim1 and Dim2C, displayed a decrease in half-time of activation, as expected (Fig. 5). In contrast, the slow dimers, Dim2, Dim4, Dim6 and Dim7, did not display a reduction in $t_{1/2}$ in the presence of RGS4. This suggests that channel conformation leading to activation of the slow dimers is the rate-limiting step, rather than GDP/GTP exchange. This result also support the observation described above that shows that deactivation kinetics are unaffected in the slow dimers (Fig. 3C).

Slow dimers do not have altered regulation by members of the $G_{\alpha/o}$ family. $G_{\alpha}$ provides a crucial link in the formation of a complex of receptor with $G$-protein subunits, channel, and RGS. $G_{\alpha}$ is well-known to bind GPCRs (reviewed in ref. 50). $G_{\alpha}$ binds RGS independently of the state of the G-protein; and $G_{\alpha}$ binds GIRK channels directly. Different $G_{\alpha/o}$ subunits have different affinities for GIRK channels, as determined by examining activation kinetics and induced current levels. We therefore wanted to investigate whether the slow dimers exhibited altered regulation by $G_{\alpha/o}$ subunits.

With wild-type GIRK channels, $G_{\alpha/o}$ isoforms have been shown to be more efficient than $G_{\alpha/i}$ isoforms in inducing m2 receptor-mediated activation gating, at least in Xenopus oocytes. Zhang et al. were able to determine this by using a series of PTX-insensitive $G_{\alpha/o}$ subunits expressed in PTX treated cells. In order to address the issue of whether the dimers, in particular the slow dimers, retain this preference, we obtained the PTX-insensitive $G_{\alpha/o}$ subunits: $G_{\alpha/i1(C351G)}, G_{\alpha/i2(C352G)}, G_{\alpha/o3(C351G)}$, and $G_{\alpha/o8(C351G)}$. (These constructs will hereby be referred to with an asterisk, as in $G_{\alpha/i1*}$.) We expressed Dim1C and Dim6 individually with the m2 receptor, the catalytic subunit of PTX, PTX-S1, and each of the PTX-insensitive $G_{\alpha/o}$ subunits. It should be noted that the PTX-insensitive $G_{\alpha/o}$ subunits induce slower activation than that seen with endogenous $G_{\alpha/o}$ subunits. This has been proposed to be due to either the cysteine to glycine mutation altering coupling to the channel, or the fact that overexpression of $G_{\alpha/o}$ subunits may lead to a relative deficit of endogenous RGS proteins. In any case, we means that we cannot directly compare $t_{1/2}$ of Dim1C when coupled to endogenous $G_{\alpha/o}$ subunits with Dim1C, when expressed with the PTX-insensitive subunits. We can, however, compare the effect of $G_{\alpha/o}$ versus $G_{\alpha/i}$ subunits on the dimers to see whether there is a change in preference of the channel. By examining the half-times of activation for Dim1C, it is apparent that the $G_{\alpha/o}$ subunits are not as efficient in transducing the gating signal as are the $G_{\alpha/i}$ subunits (Fig. 6A). These results are in agreement with Zhang et al. The slow Dim6 showed a similar pattern, indicating that the slow activation gating is not due to altered coupling preferences. Although $G_{\alpha/o}$ subunits were able to speed $t_{1/2}$ of Dim6, they were not able to push $t_{1/2}$ to the levels seen in the wild-type dimer, suggesting, that the slow activation gating is intrinsic to the channel.
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Figure 6. $G_\alpha_z$ isoforms are more effective in inducing m2 receptor-mediated activation gating than $G_\alpha_i$ isoforms, in both slow and fast dimers. Dim1C (fast) and Dim6 (slow) were expressed with the m2 receptor alone or with each of the pertussis toxin insensitive $G_\alpha_i/o$ isoforms in the presence of PTX. (A) $G_\alpha_oA^*_a$ and $G_\alpha_oB^*_a$ expression resulted in faster $t_{1/2}$ both for wild-type dimer and the slow dimer Dim6, than did expression of $G_\alpha_i$ isoforms. $p < 0.01$ using one-way ANOVA to compare Dim1C $G_\alpha_i$’s to Dim1C $G_\alpha_o$’s and Dim6 $G_\alpha_i$’s to Dim6 $G_\alpha_o$’s. (B) The $G_\alpha_o^*$ isoforms were also more efficient in inducing carbachol-activated current than the $G_\alpha_i^*$’s, both for Dim1C and Dim6. $p < 0.01$ using one-way ANOVA to compare Dim1C $G_\alpha_i$’s to Dim1C $G_\alpha_o$’s and Dim6 $G_\alpha_i$’s to Dim6 $G_\alpha_o$’s.

Figure 7. $G_\alpha_z$ slows activation kinetics in both wild-type and the slow dimers. Dimers were expressed with the m2 receptor and with and without PTX-S1 and $G_\alpha_z$. $t_{1/2}$ of all dimers significantly increases in the presence of $G_\alpha_z$ (**$p < 0.01$ using student’s t-test for each pair).

Discussion

By creating a series of GIRK1/IRK1 chimeras tethered to GIRK4, we identified two regions, which are responsible for fast channel activation. One region is at the end of the N-terminus and the beginning of the outer transmembrane helix (TM1), corresponding to residues 77–87 of GIRK1. The second region encompasses residues 180–249, which are localized to the end of the inner transmembrane domain (TM2) and the proximal C-terminus. It is possible that the C-terminal area involved in kinetics of activation gating is smaller than the region we identified, but attempts to reduce the IRK1 segment in the upstream direction resulted in channels that displayed low basal activity and no induced currents (Dim12 and Dim13; see Suppl. Fig. 1). When we tried to extend the GIRK1 residues from TM2 in the COOH direction (Dim14), the channel displayed intermediate kinetics (Suppl. Fig. 1). The sequences of the two regions involved in channel activation gating kinetics, are shown in Figure 8A and B, and are modeled on the background of KirBac1.3/Chimera60 in Figure 8C. These regions affect channel activation gating independently, and each one by itself is sufficient to maintain fast activation gating.

In the tetrameric formation of the channel, GIRK1 subunits alternate with GIRK4 subunits in a 1:1 stoichiometry. Dimers that lack GIRK1 residues proximal to TM1 but contain the full-length C-terminus (Dimers 2C, 3C, 5C, 4C and 6C; Fig. 2) retain wild-type-like fast kinetics of activation. The alternate situation, in which the GIRK1 N-terminus is intact but the C-terminus proximal to TM2 is IRK1-like (Dimers 3 and 5; Fig. 2), allows fast activation gating. The lack of GIRK1 in either region alone within a subunit is not sufficient to impair activation gating, but rather both regions must be mutated in order to see slow kinetics of activation, as in the fourth group of dimers, Dimers 2, 4, 6, 8, 9, 10 and 11 (Fig. 2). The fact that each of these regions by itself is sufficient to support fast activation gating may suggest the possibility that each of these regions interacts with the adjacent subunit. This observation is supported by the recent crystal structures of both GIRK1 cytosolic domain, KirBac1.1 and IRK1,13,33,62 which show an interaction of the N-terminus of one subunit with the C-terminus of the adjacent one. Furthermore, recently it has been suggested that similar interaction occurs in IRK1 and mutations
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Comparison of two conformational states of KirBac3.1, indeed reveal a substantial movement of both the outer and the inner helices. This movement is mainly characterized by the bending of the inner helix and tilting the outer helix to a point that expands the permeation pathway. Other regions that affect channel gating in a different manner have been previously identified, specifically in the pore region of the channel, where strong interaction of cations with the channel protein occurs.

We demonstrated that molecular elements involved in normal channel activation gating, such as G proteins and RGS subunits, are not responsible for the slow activation kinetics. Both Huang and Anderson's disease patients that cause altered interactions between these two domains. Furthermore, mutations in Kir6.2 in patients with congenital hyperinsulinism or neonatal diabetes, also allocated in the slide helix region, affect channel gating affected the intrinsic open probability of the channel or channel activation gating kinetics, respectively.

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Figure 8. Alignment of several potassium channels and structure of the tetrameric KirBac1.1. (A) Alignment of part of the N-terminus of several potassium channels, and the beginning of the outer transmembrane helix, TM1. The slide helix of KirBac1.1 is highlighted in pink, the S4-S5 linker of Shaker is in orange, and the slide helix of KcsA is in yellow. The Shaker residues necessary to maintain normal voltage-sensor activation gating are shown in green. The residues identified in this study, which are involved in slow activation gating are in blue. (B) Alignment of the C-terminal regions is shown. The C-terminal ends of the inner transmembrane helices (TM2) are in blue. KirBac1.1 R148 (circled) was identified by Kuo et al. as making contact with the N-terminal slide helix. The residues printed in blue and green are as described above. (C) The regions responsible for slow activation gating kinetics are shown. Two opposing subunits representing GIRK1 are in green. Regions in blue represent IRK1. The model of GIRK1 was done after the solved crystal structure of Kirbac1.3/GIRK1 chimera for details see Materials and Methods section. (D) A cartoon depicting the proposed closed and open conformations of the slide helix (Red), TM1 (Blue) and TM2 (Green). The proposed mechanism is based both on modeling data described for KirBac3.1 and on our observation (see Discussion).
et al.\textsuperscript{23} and Peleg et al.\textsuperscript{53} demonstrated direct binding of G\(\alpha\) to the GIRK1 N-terminus. Clancy et al.\textsuperscript{48} identified a binding site for G\(\alpha\) and the G\(\alpha_{i/o}\) heterotrimer in the middle of the C-terminus of GIRK1 and GIRK2, but not IRK1. This includes residues 300–324 of GIRK1, which is downstream of the area we have identified. Ivanina et al.\textsuperscript{54} identified amino acids 320–370 of GIRK1 as a strong G\(\alpha_{i/o}\) binding site, although residues 181–254 bound more weakly. Despite the fact that the G protein trimer reside bound to the channel in an inactive conformation, where its ready for fast receptor mediated activation,\textsuperscript{49} it seems unlikely that the slow activation gating kinetics is somehow due to altered G protein binding, since the regions we identified to be responsible for fast activation gating lie at the membrane interface, and include part of the transmembrane domains a region that is unavailable for \(G\) protein binding.

Zhang et al.\textsuperscript{45} used several G\(\alpha_{i/o}\)'s which resulted in different kinetics of activation of GIRK channels because of differences in GDP release rates among the G\(\alpha_{i/o}\) subunits. The fact that the slow Dim6 still remains slow even in the presence of G\(\alpha_{i/o}\)'s, suggests that GDP release rate is not rate limiting. The G\(\alpha_{i/o}\) experiments (Fig. 7) indicate that G\(\alpha_{i/o}\) is not endogenously activating the dimers, for two reasons. First, the deactivation rate seen with G\(\alpha_{i/o}\) in the presence of PTX to result in kinetics which found no interactions of the slide helix with any other region of the protein.

In using these structural studies to analyze the results of the present study, it becomes more obvious how the N-terminus region following the slide helix and including part of the outer transmembrane helix, and the C-terminal end of the inner transmembrane helix and including part of the C-terminus, can result in slowly activating channels when mutated. Presumably, the physical link between N-terminal movement and channel activation gating has been distorted slightly enough that the channel can still gate, but with altered kinetics. In this context, it is interesting that these changes in the N-terminal region (I to L, R to K, M to N, and V to F) have such an effect. Since we have shown that neither one of these mutations alone can restore fast activation gating in the chimera that contains the IRK1 N- and C-terminal residues (Dim11) (Fig. 4), we suggest that they may work in concert to alter the position of the preceding slide helix so that it misaligns with the C-terminal end of the inner transmembrane helix (Fig. 8D).

In Shaker, voltage-gated activation of the channel proceeds from the voltage sensor, which is N-terminal to the pore region and S6. In GIRK channels, however, both the N- and the C-termini are involved in binding G\(\beta\gamma\). Of interest is the study by Niu et al.\textsuperscript{75} in which they changed the length of a linker sequence immediately following S6 of the voltage- and Ca\(^{2+}\)-gated potassium channel BK. This linker connected the transmembrane region, which includes the gate, and the cytoplasmic Ca\(^{2+}\)-binding domains. They demonstrated that long linkers decrease channel activity, whereas short linkers increase channel activity, consistent with a passive spring that connects the two regions. If we hypothesize a similar
model in GIRQ channels, the region proximal to TM2 must be flexible enough to gate the channel, but not too flexible so as to “loosen” the spring. The length of IRK1 channel substituted into the dimers is the same as that of GIRK1, so it seems unlikely that length of this segment is a factor controlling activation gating kinetics. It may instead be due to different conformations or flexibility of the sliding helix, which, in IRK1 is more rigid toward the open state of the channel.

Although the picture is getting clearer, it is still not clear how and where Gβγ binds, or whether that binding occurs within subunits or across adjacent subunits. In addition, it is not clear if the activation gating signal is transduced via the N-terminus to the slide helix to TM2; or through the C-terminus to TM2 via a spring mechanism, which then pushes the slide helix and TM1 out of the way; or if there is a coordinated mechanism in whether both movements occur simultaneously to allow channel conduction. Answers for these questions will arise once we will obtain the crystal structure of GIRQ channels with and without the Gβγ dimer.

Materials and Methods

Construction of tandemly linked chimeric dimers. Dimers were constructed in the pGEMHE vector from rat GIRK1 and GIRQ4 and mouse IRK1. Dimers 2 and 1C were constructed by excising the middle two subunits of Tet(4-0-4-0) and Tet(4-1-4-1),36 respectively, with Age I. The remaining GIRK1/IRK1 chimeric dimers were made by standard molecular biology techniques and verified by sequencing. All oocytes expression constructs were made in pGEMHE plasmid.

Oocytes expression and electrophysiology. *Xenopus laevis* oocytes were isolated and treated as previously described.19 Using a microinjector (Nanoject II, Drummond Scientific, Broomall, PA), oocytes were injected with a 50 nl solution containing 0.2–5 ng of microinjector (Nanoject II, Drummond Scientific, Broomall, PA), buffer containing (in mM): 96 NaCl, 2 KCl, 1 CaCl2, 1 MgCl2 and 5 HEPES (pH 7.6), and 50 μg/ml penicillin/streptomycin (Sigma). All recordings were made 2–5 days after injection.

Macroscopic currents were recorded from oocytes with a two-electrode voltage clamp (Dagan CA1 amplifier) and constant perfusion with 90K solution containing (in mM): 90 KCl, 2 MgCl2, 10 HEPES (pH 7.4 with KOH), with or without 3 μM carbachol (applied for 30–120 s) (Sigma) to maximally activate m2R. Following the activation protocol for each oocyte, 5 mM BaCl2 in 90K was perfused to block the channel and to determine leak currents. A small chamber (4 x 20 mm) with fast perfusion was used. Kinetics of activation was determined as described in Results. Unless otherwise mentioned, all measurements were done at -80 mV. Acquisition hardware and software and analysis programs used were from Axon Instruments (Molecular Devices).

Modeling. Modeling the GIRK1 channel after the 2.2 Å KirBac1.3/GIRK1 chimera structure60 (PDB 2qks) was performed by using 3D-JIGSAW program (http://bmm.cancerresearchuk. org/~3djigsaw/).76 All data are presented as mean ± SEM. Statistical significance (p < 0.05) was tested using one-way ANOVA or student’s t-test.

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Note

Supplementary materials can be found at: www.landesbioscience.com/supplement/ SadjaCHAN3-3-Sup.pdf

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