34 Mechanism of G-protein regulation of K^+ channels

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The βγ subunits of heterotrimeric G-proteins (Gβγ) were first demonstrated to directly participate in signaling by their activation of K^+ channels (I_{KACh}) underlying the acetylcholine (ACh)-induced decrease in heart rate. Outside of a membrane-targeting role, how Gβγ subunits specifically regulate the conformations of their effector proteins to alter activity is not understood at a molecular level. Several crystal structures of G-protein-gated inwardly rectifying K^+ (GIRK) channels were published in the past decade, but attempts to cocrystallize them with Gβγ failed until 2013, when the GIRK2/Gβγ complex structure was reported. A parallel computational approach aimed to develop a multistage docking algorithm that combines several known methods in protein–protein docking. Application of the docking protocol to Gβγ and GIRK1 structures produced a clear signal of a favored binding mode. Analysis of this binding mode suggested a mechanism by which Gβγ promotes the open state of the channel. The channel–Gβγ interactions predicted by the model could be disrupted by mutation of one protein and rescued by additional mutation of reciprocal residues in the other protein. These interactions were found to extend to agonist-induced activation of the channels as well as to activation of the native heteromeric channels. The complex structures of Gβγ with GIRK1 (computational) and GIRK2 (crystallographic) show not only remarkable similarities but also interesting differences. Future challenges include determination of three-dimensional structures of additional members of the receptor/G-protein/channel macromolecular complex that will reveal the structural basis of agonist-independent and agonist-dependent channel activation.

34.1 VAGAL INHIBITION OF HEART RATE

GIRK channels (or Kir3 channels) are known to play diverse roles including important regulation of cardiac, neuronal, and endocrine physiology.[28] The first known effect of GIRK channels was their role in underlying I_{KACh}, the cardiac current largely responsible for the negative chronotropic effects of vagally released ACh. The inhibitory action of the vagus nerve on the heart has been demonstrated since at least the nineteenth century when the brothers Eduard and Ernst Weber communicated their results to an Italian congress of scientists in 1845.[20,38] They showed that heart rate in a frog preparation could be slowed and brought to a stop by stimulation of the vagus nerves using a rotary galvano-magnetic apparatus. The sensitivity of this vagal inhibition to atropine and its accompaniment by the hyperpolarization of the heart muscle were recognized as early as 1886 by Gaskell using extracellular recordings.[21,22] Furthermore, through careful chemical quantitation of extracellular fluids, Howell and Duke had demonstrated by 1908 that vagal inhibition of the heart is accompanied by a small release of potassium.[29]

Otto Loewi ushered in modern neuroscience by the direct demonstration of chemical transmission of nervous impulses.[51] Extract from a frog heart which had undergone vagal stimulation contained a substance deemed *Vagusstoff*, which could be applied to a second heart and cause its inhibition (Figure 34.1). This substance was later identified to be ACh.[52] The advent of intracellular microelectrodes and voltage-clamp techniques allowed for more rigorous exploration of these phenomena. Burgen and Terroux revisited the vagal-induced hyperpolarization of heart muscle reported by Gaskell. Using microelectrodes, they confirmed that hyperpolarization is induced by ACh application.[6] By measuring the effect of external K^+ concentration on resting potential in the absence and presence of ACh, they also demonstrated that increased cell permeability to potassium may underlie hyperpolarization. Del Castillo and Katz used microelectrodes to directly show hyperpolarization of the sinus node upon vagal stimulation.[9] Voltage clamp allowed Trautwein and Dudel to directly confirm changes in cell potassium permeability by measuring K^+ reversal potentials.[82] Noma and Trautwein studied activation kinetics of ACh-induced K^+ currents and concluded that ACh binding activates a specific ion channel, K_{ACh}.[65] The introduction of the patch-clamp technique[62] led to the first single-channel recordings of K_{ACh} currents,[74] which

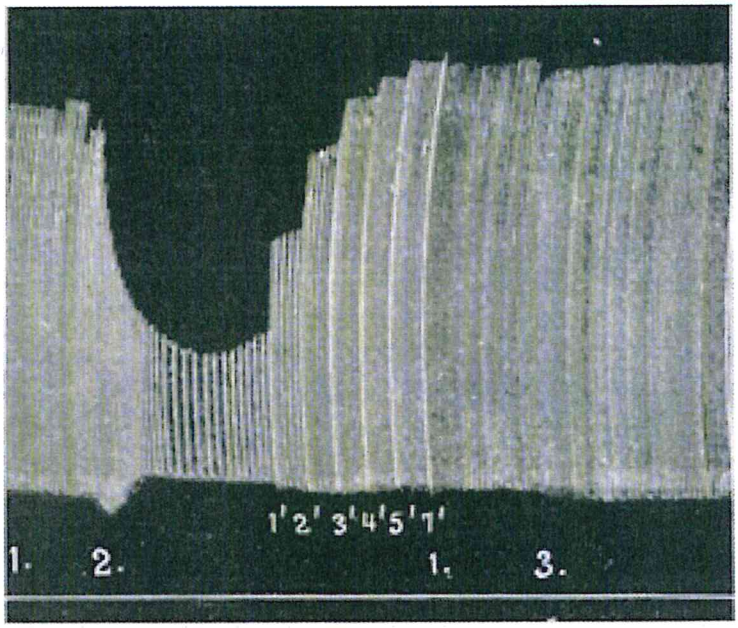

Figure 34.1 Frog heart contractions in a perfused frog heart, measured by suspension-lever. 1. Marks application of Ringer's solution. 2. Marks the application of *Vagusstoff* (extract from a separate heart after 15 min of vagal stimulation with esterases inactivated). 3. Marks the application of inactive *Vagusstoff* (just as in 2 but with the esterases active-not inactivated). Negative ionotropic (vertical amplitude) and chronotropic (horizontal frequency) effects can be seen. (1'–7') represent increasing dilution of the applied heart extract in Ringer's solution demonstrating the concentration dependence of the effects. (Adapted with permission from Loewi, O. and Navratil, E., *Pflügers Archiv Eur. J. Physiol.*, 214(1), 678, 1926.)

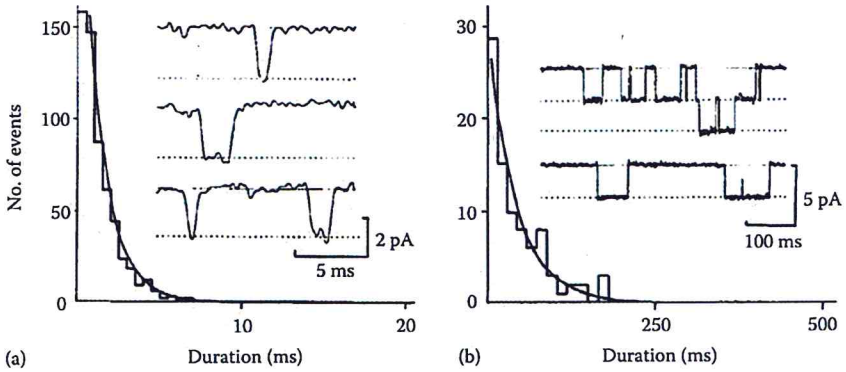

Figure 34.2 Single-channel recordings were performed on dispersed nonbeating AV nodal cells under identical conditions in (a) and (b). Frequency histograms of observed channel opening durations are displayed. Although the single-channel conductances were similar between (a) and (b) (see inset traces), the kinetics clearly differentiated the two classes of potassium channels. The channels in (a) were shown to be ACh sensitive and correspond to I_{KACh}, while those in (b) were ACh insensitive and correspond to background I_{K1} channels. (Adapted with permission from Sakmann, B. et al., *Nature*, 303(5914), 250, 1983.)

clearly demonstrated kinetic properties distinct from other background potassium channels (Figure 34.2).

Thus, the negative chronotropic effect of vagal stimulation is due to hyperpolarization of the sinus node due to the activation of a specific K^+ channel in response to ACh released at the vagal termini. This I_{KACh} current has been shown to play important roles in cardiac physiology. In vivo loss of function of this current was shown to cause an almost complete loss of variability in the heart's beat-to-beat frequency and a large reduction in heart rate vagal response.[89] Loss of its function by mutations in its component subunits have also been associated with long QT syndrome in humans.[93] Excessive I_{KACh} activity is associated with atrial fibrillation in humans.[8,39]

34.2 MOLECULAR COMPONENTS OF K_{ACh} REGULATION

In order to examine the molecular regulators of I_{KACh}, a brief discussion of guanosine triphosphate (GTP)-binding proteins is necessary. Work from Earl Sutherland in the 1950s had demonstrated that several hormones lead to stimulation of adenylyl cyclase and the production of cyclic adenosine monophosphate (cAMP) within cells where it went on to act as a soluble second messenger.[4] At first the GTP dependence of this process was not known, because the process of purifying adenosine triphosphate used as a substrate for the generation of cAMP was imperfect and

allowed for contamination by GTP (reviewed in [59]). Rodbell and colleagues showed that when supplying low concentrations of ATP, application of hormone or agonist became insufficient to stimulate cAMP production unless GTP was supplied.[72] This identified a GTP-dependent step in the signaling process, leading to stimulation of cAMP production, but its identity was not known.

Gill and Meren showed that cholera toxin leads to sustained elevation of cAMP levels by ADP-ribosylation of some unidentified cellular protein component.[23] Haga and colleagues in Al Gilman's laboratory generated a cell line, which failed to elevate cAMP levels in response to known agonists.[25] Assessment of direct adenylyl cyclase function showed that the activity of the cAMP-producing enzyme was intact in these cells, and thus, the deficiency arose from a component in the transduction mechanism. Using cholera toxin and a radioactive ADP substrate, they demonstrated radioisotope incorporation into a 45-kilodalton (kDa) polypeptide occurred in normal cells but not in their deficient cell line. These advances eventually allowed Gilman's laboratory to purify the 45 kDa polypeptide identified as the alpha subunit of the adenylyl cyclase–stimulating GTP-binding protein (Gs).[66] This polypeptide copurified with 35 kDa and 8–10 kDa proteins identified respectively as the beta (β) and gamma (γ) subunits of heterotrimeric GTP-binding proteins (G-proteins).

Today, the G-protein family has been extended to include three more classes of Gα subunits: Gq/11, G12/13, and the pertussis toxin-sensitive Gi/o. Various isoforms of twenty known Gα subunits heteromerize with one of five known Gβ isoforms and one of twelve known Gγ subunits to form the heterotimeric G-protein. Their coupling is promiscuous, but not every combination of isoforms can be found physiologically (reviewed in [59]). The heterotrimeric G-proteins couple to transmembrane G-protein-coupled receptors (GPCRs) and act as molecular switches, which help transduce extracellular signals to downstream effector proteins (Figure 34.3).

Several lines of evidence implicated G-proteins in the signal transduction mechanism that allowed ACh to activate K_{ACh}. Soejima and Noma showed in 1984[77] that the mechanism of K_{ACh} activation is membrane-delimited. Bath application of ACh did not activate K_{ACh} current in a cell-attached patch, but inclusion of ACh within the patch pipette solution did activate the channels.[77] Thus, it was concluded that ACh must be applied directly to the patch of membrane being recorded and no freely diffusible intracellular signaling mechanism could be responsible for activating the channel (discussed further below as part of Figure 34.5). Evidence from two groups implicated guanine nucleotide–binding proteins (G-proteins) in the signaling mechanism. Breitwieser and Szabo used intracellular application of nonhydrolyzable GTP analogs to maximally activate I_{KACh} such that it no longer responded to extracellular application of ACh[5]. This implied the channel was a distinct entity from the ACh receptor and that the activation mechanism likely involved G-proteins. Pfaffinger and colleagues demonstrated the sensitivity of the current to pertussis toxin and thus implicated G-proteins and specifically, the Gi/o family.[69] Logothetis et al demonstrated that it was the Gβγ subunits of heterotrimeric G-proteins that were responsible for channel activation.[53] Purified Gβγ but not Gα protein could be applied to the intracellular surface of an excised membrane patch to activate the channels (Figure 34.4). Although activation of I_{KACh} was the

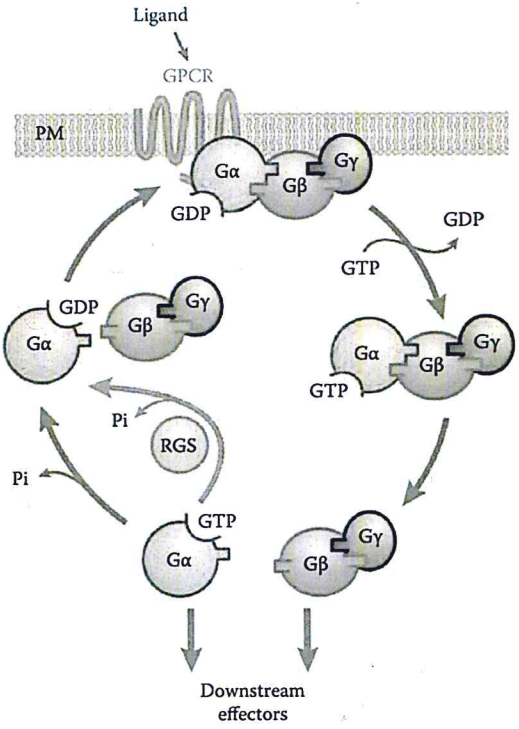

Figure 34.3 The G-protein cycle. In the inactive state, G-proteins exist as heterotrimers. Gα is bound to GDP and thus stabilized in a conformation with high affinity for Gβγ. Agonist binding to the GPCR elicits a conformational change in the receptor, allowing it to catalyze the exchange of GTP for GDP on the Gα subunit. Binding of GTP to Gα reduces its affinity for Gβγ, but complete dissociation of the two may or may not occur.[45] This represents the active form of both G-protein subunits and each of Gα and Gβγ may interact with various downstream effector proteins to modulate their activity. Gα has intrinsic GTPase activity, which causes it to hydrolyze the GTP back into GDP, releasing a pyrophosphate. Alternately, association of a regulator of G-protein signaling (RGS) molecule with Gα may accelerate its GTPase activity. The conversion of GTP to GDP returns alpha to a conformation with high affinity for Gβγ and the inactive heterotrimer is re-formed. (Adapted with permission from Li, L. et al., *Annu. Rev. Microbiol.*, 61(1), 423, 2007.)

first example of direct Gβγ signaling, many Gβγ effectors are now known and their numbers rival Gα effectors (reviewed in [7]).

Molecular cloning of the first component underlying I_{KACh}[16,43] led to the identification of the atrial heterotetrameric K_{ACh} channel comprised of GIRK1 and GIRK4 subunits.[41] A homotetramer of GIRK4 has also been reported in atrial myocytes, implying an unidentified role for homomeric GIRK channel in cardiac physiology.[15] Heterologous expression of GIRK1 alone yields no currents, and GIRK4 alone yields very small currents compared to the heterotetramer. Thus, functional GIRK1-containing channels can only exist in heterotetrameric form. Other known GIRK subunits are the neuronal GIRK2 and GIRK3 (reviewed in [28] and discussed in the following). Introduction of single point mutations in the reentrant pore helix region of these channels yields the GIRK1* (GIRK1-F137S), GIRK2* (GIRK2-E152D), and GIRK4* (GIRK4-S143T) channels, which yield robust currents and allow for the study of functional homomeric channels.[10,83,94] GIRK channels have now been found to be expressed in endocrine tissues, besides heart and brain, such as in pancreas and thyrotrophs of the rat pituitary gland (reviewed in [28]).

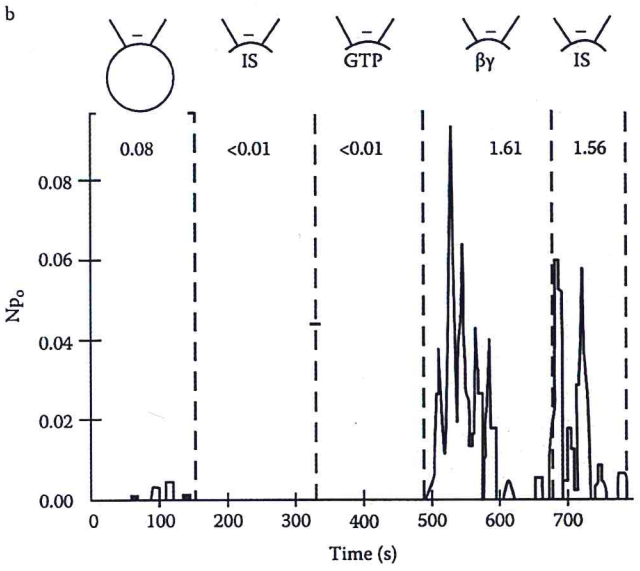

Figure 34.4 Np_o is plotted for observed potassium channel activity in a recording from cell-attached (first section) and inside-out patch (remaining sections) configurations of chick atrial myocytes. Application of purified Gβγ protein to the intracellular surface of the patch causes robust activation even in the absence of ACh in the pipette (–). The perfused Gβγ protein is not readily washed out. (Adapted with permission from Logothetis, D.E. et al., *Nature*, 325(6102), 321, 1987.)

Although it has been shown that Gβγ activates GIRK channels, there is some evidence that Gα plays a modulatory role. Receptor activation of native GIRK currents is selective to Gi-coupled receptors. Because a variety of Gβγ subunit combinations can stimulate GIRK currents,[88] the reason for this specificity is not known. It has been suggested that such specificity is achieved by colocalization of signaling components into preformed complexes. Chimeric analysis of different Gα subunits suggested that domains of Gαi may play a role in conferring this specificity.[73] Several studies have shown biochemically that Gα can bind to various domains of GIRK.[12,14,31,73] Fluorescent techniques also suggest that there is a basal interaction between Gα and GIRK channels even in the absence of receptor activation.[3,71] Reconstitution studies of pure components of a GIRK signaling system, namely of a GIRK1 chimera[64] with $G\beta_1\gamma_2$ and/or $G\alpha i_1$ subunits, but in the absence of GPCRs, suggested an active and required role of activated Gα subunits for Gβγ stimulation of channel activity.[47] Whether these results also hold true for GIRK1 channels whose origin is from only mammalian sources remains to be examined. In contrast, Gα subunits were not found to be required for Gβγ stimulation of GIRK2 channels both in a purified liposome assay as well as in lipid bilayers.[85,87] These results suggest that there may be differences in the way different GIRK isoforms couple to the G-protein signaling system.

Like all members of the Kir inward rectifying potassium channel family, GIRK channels require the presence of phosphatidylinositol 4,5-bisphosphate (PIP_2) for function.[32,80,97] PIP_2 is necessary for channel activation but is not sufficient.[97] An additional gating molecule such as Gβγ is required. Another gating molecule, which can activate GIRK2 and GIRK4 (but not GIRK1) channels, is intracellular Na^+. Both Gβγ and Na^+ appear to stabilize channel PIP_2 interactions.[32,97] Other activators include alcohols such as ethanol[2] and strongly reducing intracellular environments.[96] The multiple modulators of GIRK activity have been summarized in past comprehensive reviews.[28,79]

As mentioned earlier, in cell-attached recordings, Gi signaling stimulated outside the patch (agonist applied in the bath rather than in the patch pipette) does not result in channel, activity within the patch.[77] In contrast, Gq signaling does not appear to be restricted by the patch pipette. Stimulation of Gq signaling outside the patch consistently inhibits GIRK activity recorded from a cell-attached patch via a mechanism involving hydrolysis of PIP_2 in an intramembrane-diffusible (or membrane delimited) manner[96] (Figure 34.5). Thus isolation of the patch, as in the cell-attached mode of the patch-clamp technique, does prevent

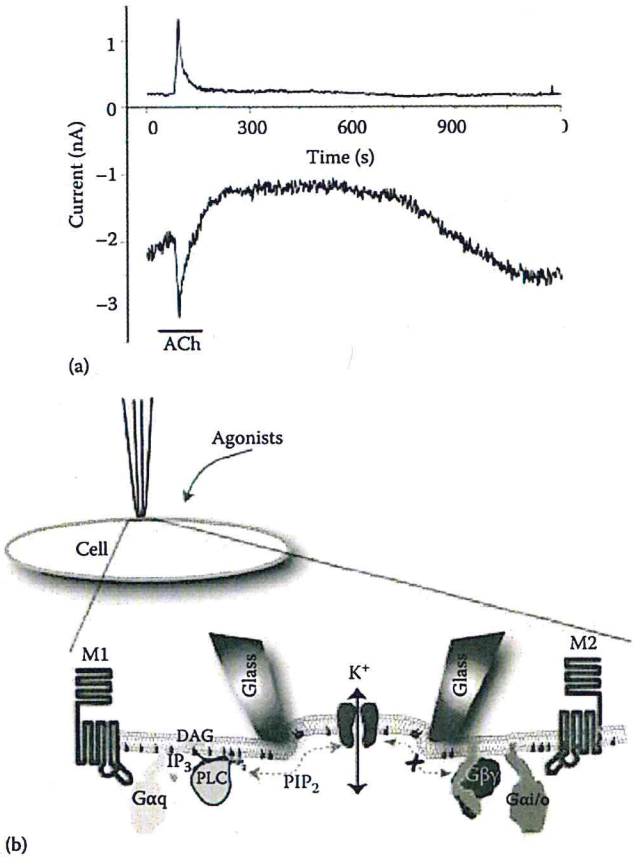

Figure 34.5 Signaling through a membrane-delimited diffusible second messenger. (a) Bath application of ACh outside the patch pipette activates M1 receptor and inhibits the active homomeric GIRK4(S143T) channel currents[83] recorded in a cell-attached patch from a *Xenopus* oocyte. M1 receptor activation also stimulates endogenous calcium-activated Cl^- currents (outward and inward spikes during ACh application) that are elicited by the increase in intracellular Ca^{2+} released by IP_3 receptors in the endoplasmic reticulum (IP_3 is generated by the M1-mediated activation of PLCβ1 and hydrolysis of PIP_2 to DAG and IP_3). Symmetrical high-K^+ solutions were used in the pipette as well as in the bath, 5 μM ACh was applied to the cell via the bathing solution. Representative record is from three similar experiments. (b) Cartoon depicting the experimental setup[77] showing that diffusion of Gβγ subunits across the patch is not possible, unlike diffusion of PIP_2. (Adapted with permission from Zhang, H. et al., *Neuron*, 37(6), 963, March 27, 2003.)

G-protein-signaling from outside the patch (Gi-mediated activation) but does not prevent the diffusion of phosphoinositides out of (Gq-mediated inhibition) and into (recovery via PIP_2 resynthesis) the patch. This restriction of G-protein diffusion is consistent with the idea of a G-protein macromolecular complex (receptor/G-protein subunits/channel) remaining intact within and outside the isolated patch.

34.3 NEURONAL GIRK CHANNELS

All four mammalian GIRK channel subunits are expressed in the nervous system. Neuronal GIRK channels play important roles in neuronal function including pain perception, reward-related behavior, mood, cognition, and memory modulation. Malfunctions in GIRK-mediated signaling in the brain have been linked to epilepsy, Down syndrome, Parkinson's disease, and drug addiction.[54,55] Knockout studies of neuronal GIRK channels have revealed their critical involvement in the formation of inhibitory postsynaptic potentials (IPSPs) in hippocampal and cerebral neurons.[55,75] GIRK knockout mice develop a number of defects that have been summarized in multiple comprehensive reviews.[28,54,55]

GIRK1–3 proteins are expressed throughout the brain, while GIRK4 is found in specific areas.[24,33,35,40,49,90] GIRK channels are involved in mediating mainly IPSPs but also presynaptic modulation of neuronal activity. There are four alternatively spliced isoforms of GIRK2 expressed as homomers (e.g., in dopaminergic neurons of the substantia nigra) or heteromers with GIRK1, GIRK3, or GIRK4 [reviewed in 28]. Some of the GIRK2 isoforms contain a PDZ domain that can interact with PDZ-binding proteins. Some GIRK2 isoforms may associate with proteins enriched in lipid rafts, such as the neural cell adhesion molecule. Such complexes can regulate the localization and function of neuronal GIRK channels.[54,55]

34.4 STRUCTURAL INSIGHTS

The first crystal structure of any potassium ion channel confirmed many predicted features of the potassium channel structure.[17] Among these were the presence of pore constrictions comprising the selectivity filter at the extracellular end of the pore and the helix bundle crossing (HBC) gate (also referred in the literature as *inner helix gate*) toward the intracellular end. The first structures of GIRK channels consisted of a fusion construct of the intracellular N- and C-termini of the channel. The transmembrane regions were deleted, and the termini were connected with a linker region.[63,68] These structures reveal the presence of a third putative gate at the apex of the intracellular region, the G-loop gate. A subsequent GIRK structure of Kir3.1 was a chimera between a mammalian and prokaryotic channel.[64] Substitution of the top three-fourths of the transmembrane region of GIRK1 with prokaryotic residues allowed the crystallization of a more complete channel, which places the intracellular termini in the proper context of a transmembrane region. The intracellular regions are organized through the cascading arrangement of secondary structure elements (Figure 34.6). Beginning most centrally near the pore is the G-loop, which comprises a gate in the channel structure. Moving outward and downward are the CD loop, the N-terminus of the adjacent subunit, the LM loop, and the DE loop of the adjacent subunit. Furthermore, this crystal structure captures the G-loop gate in two distinct conformations such that it is dilated *open* in one and constricted or *closed* in the other. The secondary structure elements also show some reorganization between the two conformations.

A previous paper reported molecular dynamics simulations of these conformations of the Kir3.1-chimera structure in the absence and presence of PIP_2 to study the interactions of the channel which allow for PIP_2 stabilization of the *open* conformation.[58] The conclusions of the detailed channel motions observed in this study can be summarized in terms of movements of the secondary structure elements. Transition to the *open* conformation of the channel stabilized by PIP_2 saw a dramatic upward movement of the LM loop, causing it to interact strongly with the N-terminus. The LM loop thus moves up and acts as a *sink* for the N-terminus so that the N-terminus switches its interactions from the CD to the LM loop. This frees the CD loop to interact with G-loop to stabilize its open state. PIP_2 interacts

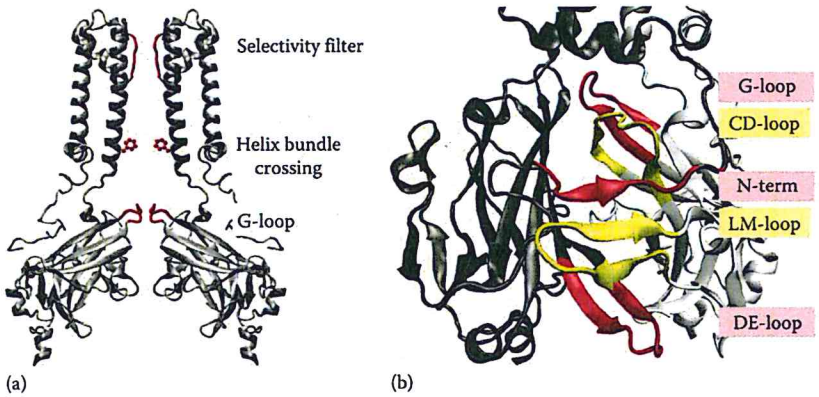

Figure 34.6 Summary of key structural features of GIRK1channels. The structure depicted is a Kir3.1 chimera.[64] (a) shows a cartoon depiction of two opposite subunits of the channel. Putative gates along the potassium permeation pathway are highlighted in red and labeled. (b) shows a close-up view of a cartoon depiction of the intracellular region of two adjacent subunits of the channel. Secondary structure elements that play important roles in gating are highlighted alternately in red and yellow and labeled. The configuration depicted corresponds to the *open* conformation of the crystal structure and thus shows the LM loop in the *raised* conformation interacting closely with the N-terminus, while the CD loop interacts closely with the G-loop.

directly with the CD loop and parts of the N-terminus to stabilize a conformation containing these interactions.

Several lines of evidence suggest that the movements of the putative channel gates are not independent but that the gates likely undergo correlated movements. Clarke and colleagues examined eleven crystal structures of bacterial Kir channels and concluded that changes in intracellular domain orientations were correlated with changes in the selectivity filter gate.[13] Xiao and colleagues examined the state-dependence of accessibility of intracellular cationic modifiers to a cysteine-modified pore residue.[92] They suggest that the HBC gate may not close completely to exclude their cationic modifiers, but its motions are correlated to changes in the selectivity filter gate. Finally, by determining multiple crystal structures of the full-length GIRK2 channel in the presence and absence of PIP_2, Whorton and MacKinnon suggest that PIP_2 acts to couple opening of the G-loop gate to movements of the transmembrane helices to cause opening of the HBC gate.[86]

Unlike channel structures, which have only been achieved within the last decade, G-protein structures have existed since the mid-1990s. Structures of the inactive GDP bound heterotrimer revealed that Gα consists of an upper GTPase domain and a helical domain. Its interaction surface with Gβγ consists of loops in the GTPase domain called the switch regions as well as its long N-terminal helix[46,84] (Figure 34.7). This N-terminal helix is disordered when Gα is not bound to Gβγ [reviewed in 67].

The Gβγ structure consists of a 7-blade beta-propeller structure. It has been crystallized alone[78] or together with regulatory proteins such as beta adrenergic receptor kinase (βARK), phosducin,[81,101] or of course, Gα. Comparisons of the various Gβγ structures do not reveal any major conformational changes, although small changes in interstrand loops and side-chain positions are observable. The exception is the cocrystal of Gβγ with phosducin where a separation of blades 6 and 7 is observed.[50,102] This conformation may be unique to the effect of phosducin. The farnesyl moiety, which normally anchors the C-terminus of $G\gamma_1$ to the membrane, is observed to occupy the cleft created between the propeller blades. Phosducin has the particular ability to dissociate Gβγ from the membrane and cause it to translocate to the cytoplasm. The opening of a cleft in the protein to bind the lipid anchor would be consistent with this function.

Numerous studies have addressed GIRK–Gβγ interactions. Biochemical binding studies employ different strategies for choosing fragments of the channel and testing their ability to bind Gβγ.[14,30,31,34,36,37,41,42,44] Other studies have focused on making and functionally characterizing chimeras between GIRK channels and the closely related but G-protein-insensitive IRK channels.[18,26,27,76] Some studies have used the chimeric analysis to suggest a functionally critical region and then created specific point mutants within the region. Such a previous study identified a residue in the LM loop at position L333 of GIRK1 to play a critical role in activation by Gβγ.[26] Mutation of GIRK1* L333 to the corresponding glutamate residue in IRK1 produced a phenotype such that the mutant channel showed intact basal activity but was not activated by Gβγ coexpression or agonist-induced receptor activation. While by no means exhaustive, results from many of these studies are summarized in Figure 34.8.

Studies have also attempted to identify important regions of Gβγ for channel interaction. Ford et al. performed an alanine scan of all residues, which comprised the Gα binding site on Gβγ.[19] These mutants were tested for their ability to regulate various Gβγ effectors, including their ability to activate GIRK channels upon coexpression. Albsoul-Younes and colleagues preformed a chimeric analysis between mammalian Gβ and a yeast Gβ deficient in activating GIRK channels.[1] They identified blades 1 and 2 as the critical regions for channel interaction.

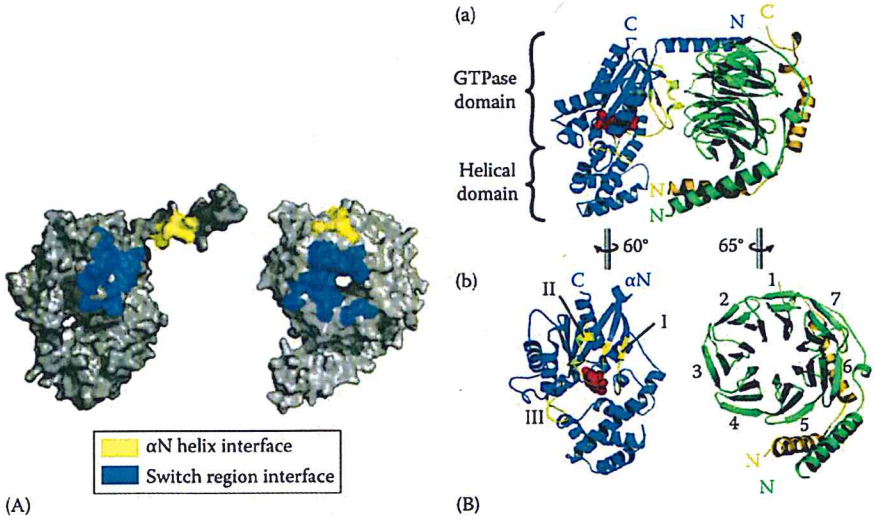

Figure 34.7 (A) Surface depiction of Gα (left) and Gβγ (right). The binding site of Gα on Gβγ can be separated into two regions: the regions contacting Gα switch regions (blue) or the Gα N-terminal helix (yellow). (Adapted with permission from Lambert, N.A., *Sci. Signal.*, 1(25), re5, June 2008.) (B) (a) The inactive heterotrimeric configuration is depicted as a cartoon (Gα, blue; Gβ, green; Gγ, yellow). The domains of Gα are labeled. (b) A separated view of Gα and Gβγ is shown in order to label the individual switch regions of Gα and the individual propeller blades of Gβγ. (Adapted with permission and with minor revisions from Oldham, W.M. and Hamm, H.E., *Q. Rev. Biophys.*, 39(2), 117, 2006.)

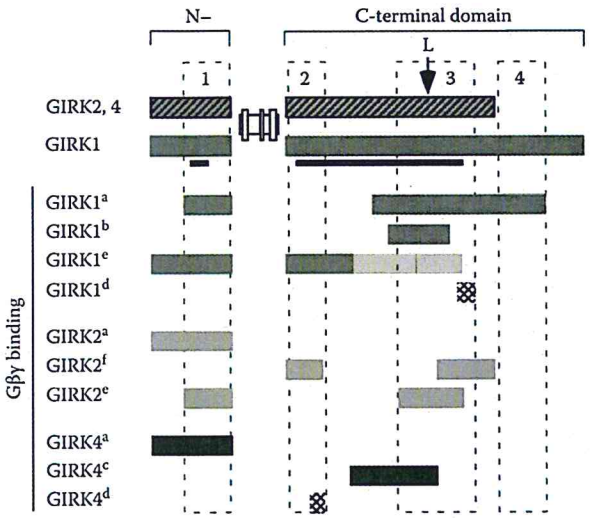

Figure 34.8 Critical regions of the GIRK channel for Gβγ binding as determined by eight different studies (designated by a–f superscript labels) of biochemical binding of fragments of channel protein to Gβγ. The position marked L at the top represents the critical leucine reside (position 333 in GIRK1) discovered by He et al. [26]. This residue was shown to be critical for both channel activation by Gβγ coexpression and by agonist-induced stimulation. While no obvious consensus region emerges from the data, the LM loop area, which contains the highlighted leucine residue overlaps with the critical regions identified by four of the six studies. (Studies depicted are in the following references: a[30,31], b[44], c[26,27], d[42], e[36], f[18]). (Adapted with permission from Finley, M. et al., *J. Physiol.*, 555(3), 643, 2004.)

Several other studies also identified mutants with reduced ability to activate GIRK channels within Gb blades 1 and 2.[60,61,99]

In 2013, two independent studies reported the long awaited complexes of Gβγ with GIRK1 [a computational study[56]] and GIRK2 [a crystallographic study[87]]. The two studies identified Gβγ to interact between two subunits making contacts within a cleft generated by the LM loop and the DE loop of two adjacent channel subunits. Other interaction contacts between Gβγ and the two channel subunits involved the βN strand and the βK-L loop. There was excellent agreement between the two complex structures with some interesting differences that will be discussed below.

Using computational modeling, Mahajan and colleagues generated models of the complex between the βγ subunits of G-proteins and the GIRK1 channel. The predictions of the models were tested experimentally using electrophysiological and biochemical techniques, providing a compelling picture of how Gβγ interacts with GIRK1 channels to stimulate their activity.[56] A multistage docking strategy was adopted (Figure 34.9). Two models stood out, the best scoring model (BSM) was found within a steep energy well, while the largest cluster model (LCM) represented a large group of favorable models that localized nearby in conformational space. A largely hydrophobic contact surface (~1800 Å) was seen between GIRK1 and Gβ in the BSM (Figure 34.10). The footprint of Gβγ onto the GIRK1 cytosolic domain predicted by these models was in excellent agreement with NMR data.[95] Gβ blades 7, 1, and 2 of Gβγ interacted with two adjacent subunits of the channel. Interestingly, several residues shown previously to be important[19,26,27,60,99,100] were identified in these structural studies.[56,86] In particular, key residues (GIRK1-L333,[26] and the Gα-interacting Gβ-L55, Gβ-K89[19]) in Gβγ stimulation of GIRK activity were found to interact with residues near the DE-LM cleft between adjacent channel subunits and stabilize the cleft in a *raised* conformation (the LM loop apart from the DE loop) (Figure 34.11). Electrophysiological evidence of paired interactions as predicted by the BSM of the GIRK1–Gβγ complex in *Xenopus laevis* oocytes showed that (1) steric defects in protein-protein interactions caused by mutations in one protein could be rescued by compensating mutations in the interacting protein; (2) introduced electrostatic repulsion between the LM and DE loops could stabilize the LM loop in the raised conformation; (3) disulfide cross-linking of Gβ(L55C) with GIRK1(L333C) caused channel activation; and (4) salt bridge stabilization of the LM loop (E334) could be achieved with the Gβ(K89)γ interaction.

These studies have given rise to a model for how the Gβγ–channel interactions comprising this binding mode promote the open state of the channel: by stabilizing the *raised* conformation of the LM loop to allow it to interact strongly with the N-terminus (Figure 34.6). The work by Mahajan and colleagues[56] extends the gating mechanism proposed in Meng et al. to include the role of the DE loop and the LM–DE loop cleft.[58] Meng and colleagues had proposed that in the closed state, the CD loop and N-terminus are closely interacting, while the LM loop has moved down and away sharply and the G-loop has shifted to its closed configuration (Figure 34.12). Introduction of PIP_2 stabilizes a different conformation of the secondary structure elements such that each element switches its close interactions from one adjacent element to its other adjacent element. Rather than constricting the pore, the G-loop interacts with the adjacent CD loop. CD loop interactions with N-terminus are in turn weakened, and the N-terminus switches to interacting with the adjacent LM loop.

The work by Mahajan and colleagues proposes that this cascade of switching adjacent element interactions continues down to the DE loop. In the closed state, the downward moved LM loop closely interacts with the adjacent DE loop, but this interaction switches in the open state, so the LM loop instead interacts closely with the adjacent N-terminus. Thus, this model proposes that PIP_2 and Gβγ modulate the same cascade of switching interactions, but their sites of action are distinct. PIP_2 acts close to the pore and gates by directly interacting with residues of the CD loop and N-terminus. Gβγ acts at the level of the LM and DE loops. Ethanol, which activates the channel, also interacts with the channel at the LM–DE loop cleft,[2] supporting the importance of this cleft in channel activation.

Furthermore, the Gβγ residues implicated in channel activation by the BSM of Mahajan and colleagues are part of the Gα-binding site on Gβγ. Specifically, these residues including L55 and K89 are among the residues that interact with the N-terminal helix of Gα.[19,84] Thus, we may speculate that although this model and experiments provide no information about the interactions of Gα, agonist-induced activation involves the unbinding of the Gα-N-terminal helix to reveal these important residues to allow them to interact with the channel. As it is known that the N-terminal helix adopts a disordered conformation upon Gβγ unbinding from Gα,[67] even a partial unbinding of the two proteins may be enough to remove the Gα N-terminal helix from these residues.

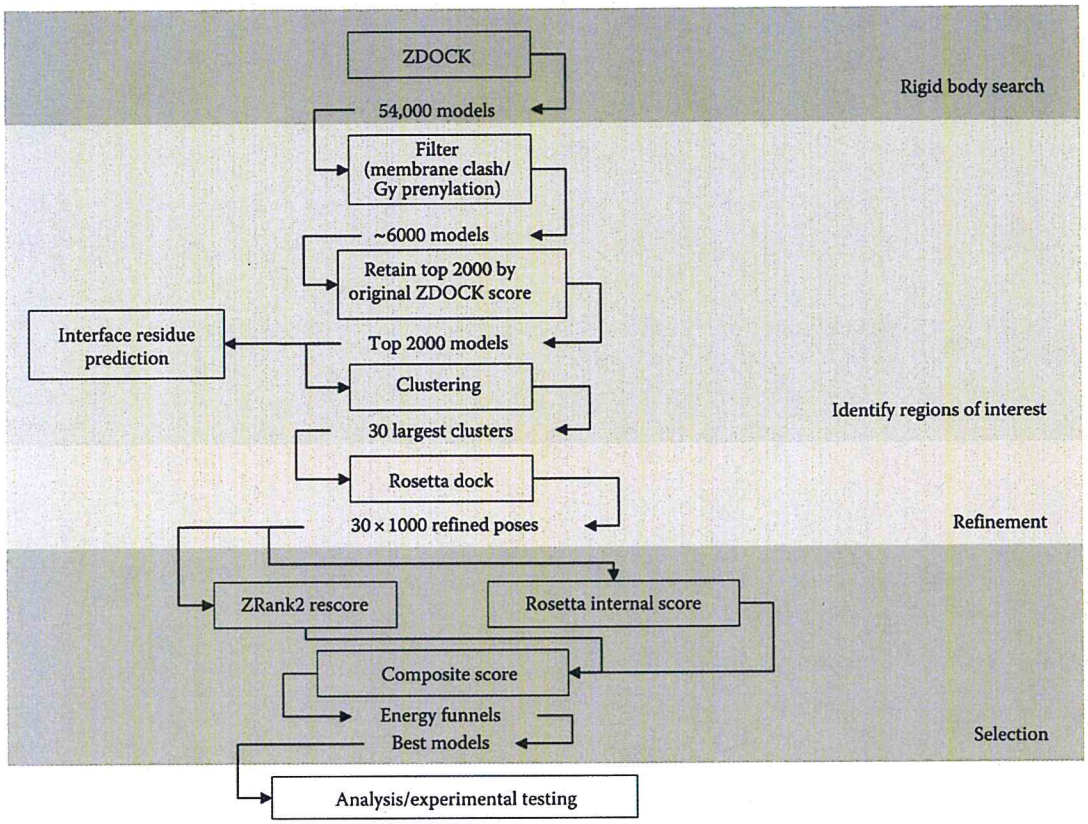

Figure 34.9 Summary of docking protocol used to predict the channel-Gβγ binding mode. ZDock was used as the global rigid-body docking program. Only the intracellular N and C termini of two adjacent subunits of the channel were included in the docking. 54,000 poses were retained, and these were then subject to a filter, which excluded any poses containing any Gβγ atoms protruding more than 8 Å above the expected plane of the membrane at the interfacial helix of the channel. Similarly, the filter also excluded any poses where the C-terminus of Gγ was more than 30 Å from the expected plane of the membrane. The first constraint reflects exclusion of the protein by the lipid bilayer and the second constraint reflects the expected prenylation (geranyl-geranylation) of the Gγ2 C-terminus, which would anchor it to the lipid bilayer. The top 2000 scored poses, which passed the filter, were subjected to the Cluspro 1.0 algorithm to sample the energy landscape and look for broad energy minima by simple hierarchical clustering. Clustering was done based on interface root mean square deviation (RMSD) and we employed the 9 Å clustering radius recommended for average. Structures representing the centers of the 30 largest clusters were retained for further analysis. Refinement via flexible docking was performed for each of these 30 structures using the local refinement module of RosettaDock. One thousand models were calculated to sample the energy landscape around each of the 30 starting structures. The scoring function used for selection employed a combination of the rigid and flexible docking algorithms and is detailed in Ref. [56].

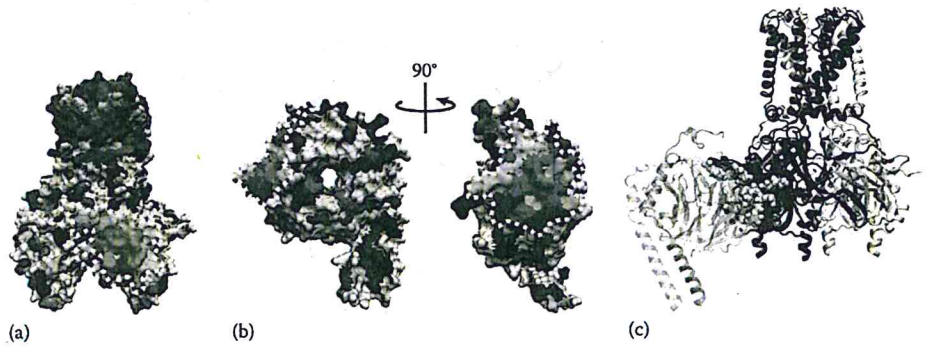

Figure 34.10 Surface representations of the channel and Gβγ (a and b respectively) are colored by residue hydrophobicity[91]: blue is most hydrophobic, white is intermediate, and red is least hydrophobic. Interface regions found in the BSM are highlighted in yellow. (c) Cartoon illustration of the two proteins together: two adjacent subunits of the channel are highlighted in red and gray, while the Gβ1 is yellow (transparent), Gγ2 is tan (transparent). Interface residues of the channel (red/gray) and the corresponding residues of Gβγ (yellow) are illustrated as spheres.

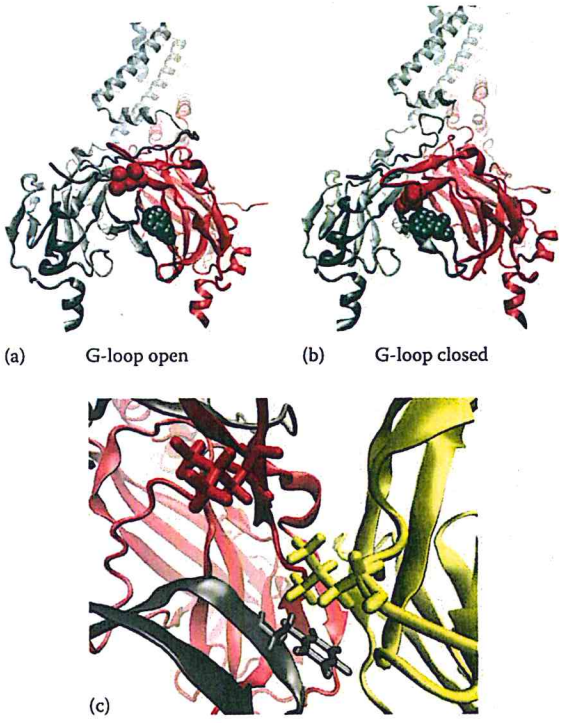

Figure 34.11 Cartoon depictions (a) and (b) of two adjacent subunits of the channel in the G-loop open and closed conformations respectively. GIRK1 residues F243 (gray) and L333 (red) are depicted as spheres in both panels. (c) Close-up view of the cleft between LM and DE loops in the BSM. Cartoon depictions of two adjacent channel subunits are in red and gray, while the $G\beta_F$ is yellow. Specific residues are highlighted in stick representation: GIRK1 L333 (red), GIRK1 F243 (gray), $G\beta_1$ L55 (yellow).

Whorton and MacKinnon[87] combined individually purified GIRK2 and Gβγ with diC8-PIP_2 (a soluble form of PIP_2 that has been used extensively in electrophysiological dose–response studies[98]) and incubated them at room temperature before crystal trials that were conducted in a high salt solution containing Na^+. Crystals of the complex diffracted at 3.5 Å resolution capturing the channel in a conformation that may represent an intermediate between the closed conformation and a partially open conformation previously determined by the same group.[86] The complex included a PIP_2 molecule and a Na^+ ion, revealing a smaller contact surface (~700 Å) (Figure 34.13) than that seen in the BSM of GIRK1 or other Gβγ effectors for which complex structures have been determined.[70] The GIRK2 secondary structure elements involved in contact with Gβ were the βK, βL, βM, and βN from one subunit with the βD and βE from an adjacent subunit. The Gβ secondary structure elements involved in contact with GIRK2 were the β-sheet elements forming blades 1 and 7 on one edge of the propeller. The GIRK2-binding site on Gβγ overlapped the Gα-binding site, consistent with the notion that Gβγ can interact with either the channel or Gα utilizing this interaction surface. Comparisons of the complex structure with prior structures in the absence of Gβγ suggested a 4° clockwise rotation (viewed from the inside) of the cytoplasmic domains along the central axis of the channel relative to the transmembrane domains. The F192 side chains were partially disordered (6–7 Å apart), although not enough to conduct hydrated K^+ ions (minimum of 10 Å apart). The authors concluded that the conformation captured by the crystal structure represents a *pre-open* state, consistent with the low open probability and burst kinetic behavior of unitary GIRK currents that show rapid flickering between open and closed (or pre-open) conformations.

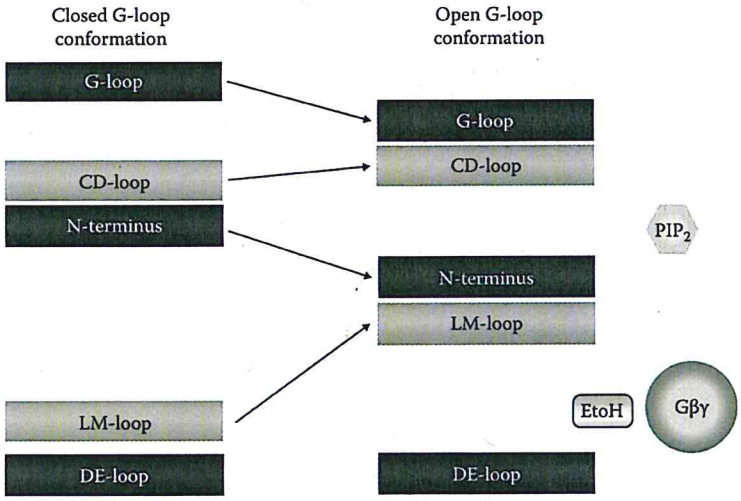

Figure 34.12 Summary of the major results of Meng et al. and their extension to include the DE loop and LM–DE loop cleft.[52] Transitioning from the closed to open, the secondary structure elements switch their close interactions from one adjacent element to the other. PIP_2 stabilizes the conformation on the right by direct interactions with the CD loop and N-terminus. Mahajan and colleagues have proposed that Gβγ works through a similar mechanism by stabilizing the same overall conformation but by direct interactions with a different part of the channel. Its proposed site of action at the DE–LM loop cleft is shared with the site of ethanol (EtOH) action. (Adapted with permission from Mahajan, R. et al., *Sci. Signal.*, 6(288), ra69, 2013.)

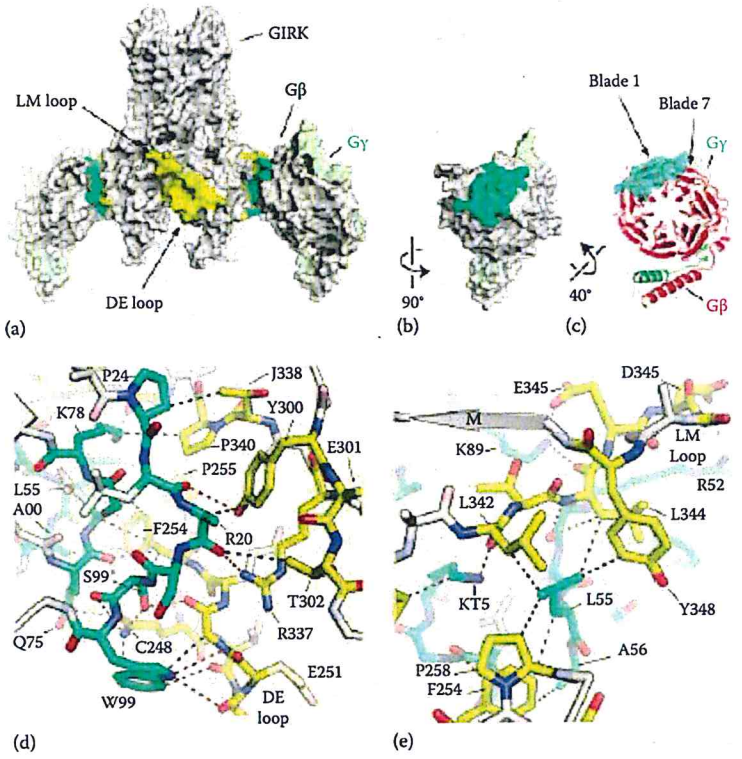

Figure 34.13 The GIRK–Gβγ binding interface. (a) Surface representation of the GIRK–Gβγ complex. The binding site on GIRK is colored yellow and the binding site on Gβγ is colored cyan. The front Gβγ dimer is removed for clarity. The overall orientation in a–c is similar as in Figure 34.10a and b. (b) A 90°-rotated view of a Gβγ dimer from panel a to more clearly show the binding interface. (c) The Gβγ dimer is rotated upward to orient the central axis of the β-propeller orthogonal to the page. (d, e) A close-up of the GIRK–Gβγ interaction, focused on the DE loop, βK and βN region (d) or the LM loop region (e) of GIRK2. Selected hydrogen bond and van der Waals interactions are shown as dashed lines as a visual aid. (Adapted with permission from Whorton, M.R. and MacKinnon, R., *Nature*, 498(7453), 190, 2013.)

The footprint of Gβγ on the GIRK1 and GIRK2 involved the same channel regions. The mGIRK2 shows only a 55.4% identity with the hGIRK1, and the specific interactions of the two channels with Gβγ show many similarities but also several interesting differences.

Comparison of the crystal structure model of the GIRK2–Gβγ complex with the two computational models showed that 64% of the GIRK2–Gβ interactions were the same in the LCM GIRK1–Gβ complex, while only 30% of the GIRK2–Gβγ interactions were the same in the BSM GIRK1–Gβγ complex. Gβγ in the LCM GIRK1 and GIRK2 structures is slightly rotated to include in its interaction surface residues absent from the BSM interaction surface, such as W99 and F335, on the *front* of the molecule closer to the center of the Gβγ propeller structure. In the BSM of GIRK1, Gβγ rotates slightly to instead uniquely engage residues in the *back* of the molecule, such as N88, E130, and N132.[56] The Gβ residues mentioned above (W99, F335, N88, E130, N132) reside in regions implicated by previous mutagenesis studies to be important in GIRK activation;[19,60,97] Binding site residue: in the front of the Gβ molecule are shared with Gα[19] and it is possible that interaction surfaces involving more of these residues underlie agonist-induced currents,[26,60,61] while residues towards the back of the molecule, not shared with Gα, may participate in stimulating basal (or agonist-independent) currents.[27,60] The pattern of interactions between Gβγ and either GIRK1 or GIRK2 suggests that common interactions between Gβγ with GIRK2 and GIRK1 may serve a fundamental role by which Gβγ activates these two channels, while differences could underlie distinct functional effects on the two channel subunits. For example, the unique C-terminus of the GIRK1 subunit confers robust receptor-dependent activity to GIRK heteromers.[11] It is possible that both the crystal structure of the GIRK2/Gβγ complex and the LCM of the GIRK1/Gβγ complex that show the largest similarity reside at a broad energy minimum (a pre-open state) near the final energy well, thus increasing the likelihood that the protein will *find* the most stable conformation represented by the BSM, which resides in a steep energy well representing one of the smaller clusters.

34.5 FUTURE QUESTIONS

The structural insights afforded by complexes of Gβγ with GIRK1 and GIRK2 have stimulated a number of questions, answers to which are likely not only to illuminate our understanding of homomeric vs. heteromeric GIRK channel gating by Gβγ but also to generally provide a structural understanding of G-protein signaling to effector proteins.

- How do the structures of different GIRK homomeric and heteromeric subunits with Gβγ underlie their functional differences?

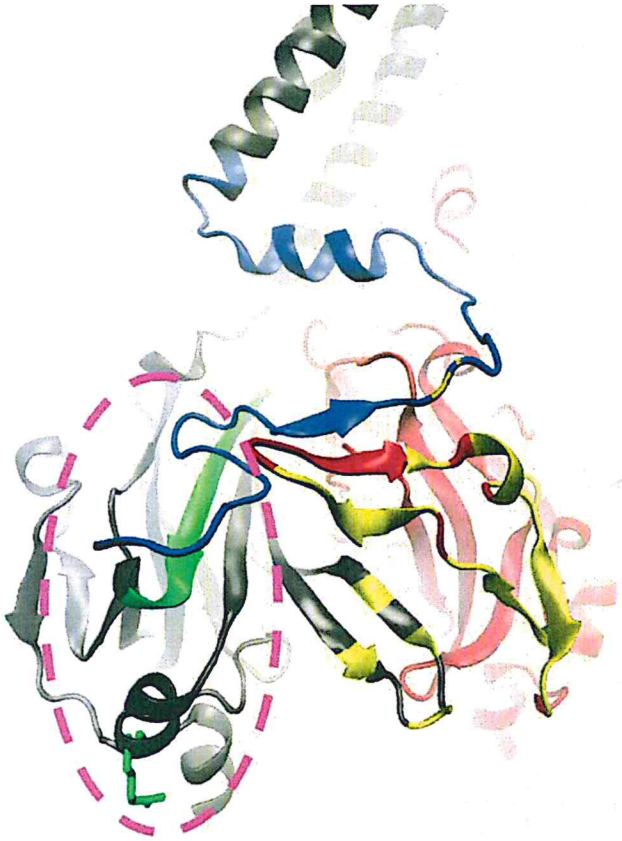

Figure 34.14 Regions of GIRK channels implicated in heterotrimer association. Cartoon depiction of two adjacent subunits of the channel IS shown in gray and red. Green represents the region implicated in Clancy and colleagues.[12] Blue represents the N-terminus implicated by multiple studies.[14,37] Green sticks represent the GIRK1* R286 residue that preliminary results have implicated as Gα-interacting.[57] Yellow highlights the predicted Gβγ interface from Mahajan and colleagues.[56] Dashed pink circle highlights a possible consensus region for heterotrimer association.

- How do Gα-GDP or Gα-GTP structures with different GIRK homomeric and heteromeric subunits explain inhibitory effects on Gβγ stimulation of activity or possibly permit stimulation of GIRK1 activity by Gβγ?
- How does the heterotrimeric G-protein (Gαβγ) interact with the GIRK channels to inhibit their function? Figure 34.14 suggests sites of interaction of Gβγ and Gα on the GIRK1 channel.
- How do structures of the entire macromolecular complex of GPCR/G-proteins/channel fit into producing agonist-independent and agonist-dependent channel activity?

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