Structural insights into GIRK2 channel modulation by cholesterol and PIP2

Graphical abstract

Highlights
- CryoEM structure identifies cholesterol binding site in brain GIRK2 channel
- Mutational and functional studies reveal key residues in cholesterol potentiation
- Elucidating cholesterol binding pockets can aid in developing new modulators

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In brief
Ion channels are important in determining neuronal excitability. Elevated cholesterol levels found in some neurodegenerative diseases can affect the function of ion channels. Mathiharan et al. take a structural and functional approach to identifying physical sites for cholesterol, and they provide details on how cholesterol potentiates ion channel activity.
Structural insights into GIRK2 channel modulation by cholesterol and PIP2

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SUMMARY

G-protein-gated inwardly rectifying potassium (GIRK) channels are important for determining neuronal excitability. In addition to G proteins, GIRK channels are potentiated by membrane cholesterol, which is elevated in the brains of people with neurodegenerative diseases such as Alzheimer’s dementia and Parkinson’s disease. The structural mechanism of cholesterol modulation of GIRK channels is not well understood. In this study, we present cryo-electron microscopy (cryoEM) structures of GIRK2 in the presence and absence of the cholesterol analog cholesteryl hemisuccinate (CHS) and phosphatidylinositol 4,5-bisphosphate (PIP2). The structures reveal that CHS binds near PIP2 in lipid-facing hydrophobic pockets of the transmembrane domain. Our structural analysis suggests that CHS stabilizes PIP2 interaction with the channel and promotes engagement of the cytoplasmic domain onto the transmembrane region. Mutagenesis of one of the CHS binding pockets eliminates cholesterol-dependent potentiation of GIRK2. Elucidating the structural mechanisms underlying cholesterol modulation of GIRK2 channels could facilitate the development of therapeutics for treating neurological diseases.

INTRODUCTION

G-protein-gated inwardly rectifying potassium (GIRK) channels provide a major source of inhibition in the brain and have been implicated in a variety of neurological disorders (Lüscher and Slesinger, 2010). Loss of GIRK channels leads to hyperexcitability and sensitivity to seizures, changes in alcohol consumption, and increases in sensitivity to psychostimulants (Signorini et al., 1997; Hill et al., 2003; Clarke et al., 2011; Munoz and Slesinger, 2014; Rifkin et al., 2018). Activation of G-protein-coupled receptors (GPCRs) that signal through Gαi/o G protein, such as GABAAβ2, D2 dopamine, and m2 muscarinic receptors, leads to liberation of G protein Gβγy subunits that open GIRK channels through direct protein-protein interactions (Logothetis et al., 1987; Reuveny et al., 1994; Wickman et al., 1994).

Interestingly, some GIRK channels are found in lipid rafts enriched in cholesterol (Delling et al., 2002), and GIRK channels have been shown to be potentiated by increases in membrane cholesterol (Bukiya and Rosenhouse-Dantsker, 2017; Glaaser and Slesinger, 2017; Rosenhouse-Dantsker, 2019). Consistent with these findings, purified GIRK2 channels reconstituted into lipid membranes with phosphatidylinositol 4,5-bisphosphate (PIP2) are potentiated by cholesterol, demonstrating that cholesterol, similar to alcohol, can directly activate GIRK channels (Glaaser and Slesinger, 2017). Cholesterol levels in the brain are quite high, achieved mainly through local de novo synthesis, as transport of lipoproteins across the blood-brain barrier is not very efficient (Zhang and Liu, 2015; Jin et al., 2019). Elevated cholesterol in the brain has been recently implicated in people with neurodegenerative diseases, such as Alzheimer’s dementia (AD) and Parkinson’s disease (PD). Postmortem AD brains and patients with AD showed dysregulation of cholesterol metabolism, with presenilin 1 (PS1) mutations associated with elevations in cholesterol (Cho et al., 2019). Elevated levels of cholesterol and homocysteine in blood have been linked with the pathology of Parkinson’s disease (Paul et al., 2018). Hypercholesterolemia induces dopamine neuronal loss, where GIRK2 homotetramers are expressed, leading to reduced DA in the striatum (Paul et al., 2017b). The molecular mechanism underlying cholesterol regulation of these potassium channels is poorly understood. Mutagenesis studies and simulations have indirectly implicated multiple regions of inwardly rectifying potassium channels in cholesterol modulation, including the cytoplasmic domain (CTD), the PIP2 binding site at the inner leaflet of the membrane, and the transmembrane domain (TMD) (Epshtein et al., 2009; Rosenhouse-Dantsker et al., 2010, 2013; Bukiya et al., 2017). The precise physical location of the cholesterol sites in GIRK channels remains to be determined.
To better understand the structural basis for cholesterol modulation of GIRK channels, we employed cryo-electron microscopy (cryoEM) to visualize GIRK2 under different conditions. We present structures of the GIRK2 channel in the presence and absence of the cholesterol derivative cholesteryl hemisuccinate (CHS) and PIP2, revealing their effects on mechanistic aspects of GIRK2 gating. In addition, we provide evidence with site-directed mutagenesis and functional expression of GIRK2 channels that link the structural site for CHS with cholesterol potentiation of GIRK2 channels.

RESULTS

CHS potentiates GIRK2 channel activity

Previously, we showed that GIRK2 channels are potentiated by cholesterol in the absence of G proteins (Glaaser and Slesinger, 2017). To better understand the mechanism of cholesterol modulation, we sought to determine the site of interaction of a cholesterol analog, CHS, in GIRK2 channels. To verify the functional effects of CHS, we expressed Mus musculus GIRK2 in Pichia pastoris and extracted from membranes with n-dodecyl b-D-maltoside (DDM) detergent in the presence or absence of CHS (Figures S1A). Purified GIRK2 tetrameric channels were reconstituted into liposomes containing 1% brain PIP2 alone, which is required for channel function (Huang et al., 1998; Glaaser and Slesinger, 2017), and either 2.5% CHS or 5% cholesterol. We measured K+ permeation through GIRK2 channels using a flux assay (Figures 1A), as described previously (Whorton and MacKinnon, 2013; Glaaser and Slesinger, 2017). Upon addition of carbonyl cyanide m-chlorophenyl hydrazone (CCCP), the fluorescence emitted from 9-amino-6-chloro-2-methoxyacridine (ACMA) preloaded into proteoliposomes containing GIRK2 channels and 1% brain PIP2 (GIRK2PIP2) quenches to F/F0 under basal conditions (Figures 1A and 1B) and quenches to F/F0 with proteoliposomes also containing CHS (GIRK2PIP2/CHS), indicating CHS-dependent potentiation of GIRK2 channels. Empty liposomes with CHS contain little K+ flux (black circles/white bar) There was no statistical difference between HE and chol/HE conditions.

See also Figure S1.
of GIRK2 channel activity (Figures 1A and 1B). To ensure that the effect of CHS was via specific interactions with GIRK2, and not due to indirect effects via changes in membrane permeability, we reconstituted liposomes containing 1% PIP₂ with and without 2.5% CHS (empty liposomes) and measured flux as with liposomes containing GIRK2. Representative traces of empty liposomes with and without 2.5% CHS (Figure 1C) show virtually no quenching, with the normalized relative K⁺ flux of CHS containing empty liposomes (~7.4% ± 1.3%; n = 4) (Figure 1F). The GIRK2 channel-specific inhibitor 2-hydroxyethyl methanethiosulfonate (MTS-HE) (Glaaser and Slesinger, 2017) reduced the extent of quenching, indicating inhibition of the K⁺ conductance. We used the inhibition with MTS-HE to quantify changes in quenching under different conditions and converted this to a percentage of relative K⁺ flux. Under basal conditions with proteoliposomes containing GIRK2PIP₂, the relative K⁺ flux was 44.0% ± 2.3% (n = 18) (Figures 1D–1F). Both CHS and cholesterol significantly increased the relative K⁺ flux to 65.2% ± 0.4% (n = 10) and 69.4% ± 0.3% (n = 17), respectively, compared to basal conditions (Figures 1D–1F). Taken together, these results demonstrate that CHS, similar to cholesterol, potentiates basal PIP₂-dependent GIRK2 channel activity (Glaaser and Slesinger, 2017).

**Structure of GIRK2 in the presence of CHS and PIP₂**

To gain insights into CHS binding in GIRK2, we proceeded with optimizing the sample for cryoEM studies. The sample quality was assessed by size-exclusion chromatography (Figure S1A) and further evaluated by negative stain electron microscopy (Figure S1B) to identify conditions with predominantly well-formed GIRK2 tetramers (Peisley and Skiniotis, 2015). To ensure the presence of CHS and PIP₂ (GIRK2PIP₂/CHS), the sample was purified in the presence of CHS and incubated with 2 mM diC₈-PIP₂ prior to cryoEM grid preparation.

We subsequently employed cryoEM and determined the structure of GIRK2PIP₂/CHS at a global indicated resolution of 3.5 Å (Figure S2). The structure revealed an overall architecture similar to those solved by X-ray crystallography (Whorton and MacKinnon, 2011, 2013), with two distinct domains, the TMD and CTD, and the pore along the four-fold axis with a well-ordered inner-helix bundle crossing and G-loop gates (Figures 2A and 2B). PIP₂ is bound at its canonical site near inter-domain linkers, as also observed in other inwardly rectifying potassium channels (Hansen et al., 2011; Whorton and MacKinnon, 2011, 2013) (Figure 2B).

Interestingly, we observed a planar density in “site A” on the opposite side of the PIP₂-channel interface that is consistent with CHS. Initial modeling of CHS in the EM density was performed with the GemSpot pipeline (Robertson et al., 2020), followed by manual refinement in Coot (Emsley and Cowtan, 2004). This approach yielded largely identical poses for CHS in good agreement to the EM map and chemically plausible interactions with GIRK2 transmembrane helices that had subtle differences in the orientation of the isooctyl tail (Figures 2B and 2C; Figure S3A). The head group of CHS is in position to form a salt bridge with R92, while its sterane rings and isooctyl tail are stacked against the TMD, surrounded by hydrophobic residues F93, L95, L96, and V99 of the M2 helix from one subunit, and I175, V178, and L179 of the M2 helix from the adjacent subunit (Figures 2B and 2C; Figure S3B). Another CHS molecule could be modeled in “site B” near the N terminus surrounded by hydrophobic residues that include V72, L79, I82, L86, L89, I97, V101, and F186 (Figure 2C; Figure S3C). We cannot rule out other putative CHS sites, as additional densities are observed near the TMD-CTD interface, but they are poorly resolved (Figures S3D–S3F). We note, however, that the positions of CHS in site A and site B in the cryoEM GIRK2 structures are different from those regions identified with computational molecular docking (cf. Bukiya et al., 2017).

**Structures of GIRK2PIP₂ in the absence of CHS**

To assess whether CHS alters the interaction of PIP₂, we next determined the structure of GIRK2⁻PIP₂ in the absence of CHS during the purification. Interestingly, cryoEM analysis resolved
several different conformers of GIRK2<sub>PIP2</sub> (Figure S4). The predominant conformer, referred to as GIRK2<sub>PIP2*</sub>, accounted for ~39% of well-defined particles and its structure was obtained at a global resolution of 3.2 Å (Figures S4C–S4F). This structure is comparable to GIRK2<sub>PIP2/CHS</sub> (Figure 3), with four PIP<sub>2</sub> molecules bound at equivalent sites, but importantly lacked densities for CHS in site A and site B (Figures S3D–S3F). A second conformer, GIRK2<sub>PIP2**</sub>, accounting for ~27% of projections, was determined at 4.8 Å global resolution (Figures S4C and S4G). Comparison of this conformation with GIRK2<sub>PIP2**</sub> revealed that the CTD is rotated from 8° to 12° around the four-fold axis and is partially disengaged from the TMD by 0–1 Å (as evaluated by the distance between Ca atoms of T80 and L229), with a stretched tether helix (residues 197–203, Figures S5A–S5C).

The disengagement of the CTD is more pronounced in two additional conformers, GIRK2<sub>PIP2***</sub> and GIRK2<sub>PIP2****</sub>, as the distance between Ca atoms of T80 and L229 is increased by 1–8 Å, stretching further the tether helix (Figures S5A and S5B). These conformers appear very similar and were obtained at low overall resolution (~7.7 Å), primarily due to the relative flexibility between TMDs and CTDs. Nevertheless, rigid body docking of TMDs and CTDs was sufficient in revealing the overall flexibility between TMDs and CTDs. Nevertheless, rigid body docking of TMDs and CTDs was sufficient in revealing the overall flexibility between TMDs and CTDs. Nevertheless, rigid body docking of TMDs and CTDs was sufficient in revealing the overall flexibility between TMDs and CTDs.

In both the GIRK2<sub>PIP2**</sub> and GIRK2<sub>PIP2/CHS</sub> maps, we observe densities that are compatible with Na<sup>+</sup> and K<sup>+</sup> ions. A Na<sup>+</sup> ion is coordinated by D228, as shown previously in X-ray crystal structures (Whorton and MacKinnon, 2011). Similarly, densities for K<sup>+</sup> ions are found near the selectivity filter, proximal to Y266, M319, G318, and E236 as previously determined (Whorton and MacKinnon, 2011, 2013), but also observed at positions near G158, F192, and M313. We also note the presence of additional, less well-resolved densities along the pore near N184, V188, E236, and M319. The pore diameter at the inner helix (F192) and G-loop (M313) gates is ~6.5 Å and 4 Å, respectively, which is too small for hydrated K<sup>+</sup> (~8 Å) to permeate (Figure 3B). However, previous studies utilizing cross-linked KirBac3.1 channels (Black et al., 2020) and MD simulations of GIRK channels (Bernsteiner et al., 2019) suggest this extent of opening at the inner-helix gate may be sufficient to pass partially hydrated or transiently non-hydrated K<sup>+</sup>.

**GIRK2 apo structure (no PIP<sub>2</sub>)**

Lastly, we examined the structure of GIRK2 apo, which lacks both PIP<sub>2</sub> and CHS. CryoEM maps of GIRK2 apo determined at an overall 4.8 Å resolution revealed that the CTD is detached from the TMD (Figures 4 and 5). This configuration of the CTD is quite different from the GIRK2 apo structure determined by X-ray crystallography, which showed the CTD engaged with the TMD (Whorton and MacKinnon, 2011) (Figure SSD). Inter-domain linkers consisting of tether helix and N-terminal residues 67–78 appear mostly disordered, suggesting that this interface is unstable in the absence of PIP<sub>2</sub> and CHS (Figure 5; Figure S6; and Videos S1). The CTD has moved toward the TMD by about 2–8 Å to be engaged in the GIRK2<sub>PIP2*</sub> structure (Figure 4). Interestingly, the detached CTD appears tilted below the membrane,
GIRK2\textit{PIP2*} structure with the tether helix (residues 197–203) and N-terminal formations; the CTD is disengaged from membrane in apo compared to the 

eliminated cholesterol potentiation, whereas decreasing side

at the L96 and V99 position (i.e., L96W, L96F, V99W, and V99I)

CHS pocket, we found that increasing the side-chain volume

and four residues in site B (Figures 6D and 6E). In the site A

in the absence of PIP2 and CHS. Our classification showed addi-

reflecting a “wobble” in this structure and an overall asymmetry

in the absence of PIP2 and CHS. Our classification showed addi-
tional 3D classes with different orientations of detached CTD,
and we used 3D variability analysis in cryoSPARC (Punjani and
Fleet, 2021) to further visualize the CTD wobbling (Video S1).

Functional effects of mutations in the CHS pockets on cholesterol potentiation of GIRK2

We used the GIRK2\textit{PIP2*CHS*} cryoEM structure with CHS modeled in site A and site B for mutagenesis, and then studied the effect on cholesterol potentiation of native GIRK2 channels expressed in HEK293 cells using electrophysiology. Whole-cell patch-clamp currents were recorded from HEK293 cells transiently transfected with GIRK2 cDNA that were exposed to either wa-

ter-soluble cholesterol with methyl-\(\beta\)-cyclodextrin (M\(\beta\)CD
(+cholesterol) or M\(\beta\)CD alone (control) (Figure 6A). The chole-
sterol enrichment resulted in a significant (\(\approx\)2-fold) increase in the Ba\textsuperscript{2+}-sensitive basal current for GIRK2 wild-type (WT) chan-
nels, with no change in inward rectification (Figures 6B and 6C) (cf. Bukiya et al., 2017).

We next conducted an Ala/Trp scan of five residues in site A and four residues in site B (Figures 6D and 6E). In the site A CHS pocket, we found that increasing the side-chain volume at the L96 and V99 position (i.e., L96W, L96F, V99W, and V99I) eliminated cholesterol potentiation, whereas decreasing side

chain volume (L96A, V99A) had little effect (Figures 6F–6H). Both L95A and L95W, alternatively, had no effect on cholesterol potentiation. Interestingly, both F93A and F93W disrupted cholesterol potentiation (Figure 6h). To determine whether the phenyl group was important, we examined the effect of F93Y mutation and observed normal cholesterol potentiation (Figure 6h). The effect of Ala/Trp mutations at L79, I82, L86, and V101 in site B did not eliminate cholesterol potentiation, or this effect created a non-functional channel (NC, Figure 6i). Taken together, these data provide evidence that the cholesterol inter-
action with site A is functionally relevant for potentiation of natively expressed GIRK2 channels.

DISCUSSION

Cholesterol binding sites on membrane proteins

Cholesterol binding sites on membrane proteins were initially characterized using a variety of techniques, including electron spin resonance, photoaffinity labeling, molecular modeling, and site-directed mutagenesis, which led to the identification of amino acid recognition motifs (Middlemas and Raftery, 1987; Jones and McNamee, 1988; Dreger et al., 1997; Hanson et al., 2008; Rosenhouse-Dantsker, 2019; Duncan et al., 2020a). Structures of membrane proteins (e.g., GPCRs and ion channels) determined in the presence of cholesterol or CHS have revealed interactions with “greasy hollows,” which refer to pockets in the transmembrane portion lined with hydrophobic residues (Lee, 2019; Duncan et al., 2020b). Previous work with GIRK2 has also supported the idea of a direct effect of cholesterol on the channel protein, as opposed to possible indirect effects on the bilayer (Bukiya et al., 2017; Glaaser and Slesinger, 2017). Furthermore, we now provide a structural view of a CHS/cholesterol pocket (site A) in GIRK2. The sterane rings and isooctyl tail of CHS are stacked against the TMD, between two adjacent subunits, and surrounded by hydrophobic resi-
dues. Generally, bulky substitutions at F93, L96, and V99 in site A eliminated cholesterol potentiation. One position (F93) was sensitive to both Ala/Trp mutations but not a Tyr mutation. This finding suggests that cholesterol binding may be favored by the phenyl group in this position. Some features of cholesterol binding in this pocket may be slightly different from CHS, since the head group in CHS is different from that of cholesterol (i.e., the CHS hemisuccinate is larger and more polar). For example, a salt bridge appears to form between the polar head group of CHS and R92, which also interacts with PIP2 (Tang et al., 2015). It is possible that cholesterol may interact with R92 as well. Interestingly, a R92Y mutation in GIRK2 eliminated cholesterol potentiation and greatly increased the Ba\textsuperscript{2+}-sensitive basal current (data not shown). The putative interaction between R92, PIP2, and cholesterol requires further investigation to better understand the role of R92 in GIRK2 function.

Mutation of amino acids in the site B pocket, alternatively, appeared to have little effect on cholesterol potentiation. One possible explanation for this is that CHS is partially exposed to the intracellular side, and thus potentially less stable in that po-

sition and less sensitive to mutagenesis. Alternatively, site B may not be a cholesterol potentiation site, and instead another type of lipid may occupy this space. The higher percentage of
non-functional channels could also indicate that this region of the channel is important for tetramer assembly.

Computational docking studies with cholesterol and a homology model of GIRK2 revealed two putative cholesterol-binding regions located at the center of the TMDs, near the TMD-CTD interface (Bukiya et al., 2017). These two regions are near to, but different from, the site A and site B pockets identified in the cryoEM GIRK2 structure (see Figure S8). Nonetheless, some of the amino acids previously identified using docking (e.g., V99 and V101) do overlap with site A and site B (Bukiya et al., 2017). It is possible that cholesterol may bind to several different regions of the channel in other lipid membrane environments, but it is more stable in site A under our experimental conditions.

Mechanism of PIP2 and cholesterol gating of GIRK2 channels

One of the main findings of our cryoEM studies is that the CTD is detached from the TMD in both the GIRK2apo and a significant population of GIRK2\textsubscript{PIP2}\textsuperscript{CHS} particles. While the CTD appears detached, it is actually connected via a stretched tethered linker that is unresolved. In contrast, the CTD is engaged with the TMD in the structure of GIRK2apo solved by X-ray crystallography (Whorton and MacKinnon, 2011). One possibility for this difference could be due to lattice packing in X-ray crystal structures. Indeed, a recent cryoEM study of GIRK2 from MacKinnon and colleagues also demonstrated that the CTD is detached (i.e., extended) in the GIRK2apo structure determined with cryoEM (Niu et al., 2020). A detached CTD in the absence of PIP2 and other auxiliary proteins (e.g., G proteins, SUR) may be a common feature of inward rectifiers (Whorton and MacKinnon, 2013; Martin et al., 2019), since the CTD is also detached in the Kir2.2apo X-ray crystal structure (Hansen et al., 2011).

Based on the observation of several different conformations of GIRK2 in the absence and presence of PIP2, we suggest a plausible model for gating transitions from closed to open in GIRK2 channels. First, without PIP2, the CTD is uncoupled (i.e., detached) from the TMD and the channel cannot open. This is consistent with the functional studies showing that PIP2 is necessary for channel activation (Huang et al., 1998; Sui et al., 1998; Glaaser and Slesinger, 2017). Second, the inter-domain region and a wobbling CTD are stabilized by the binding of four PIP2 molecules at the TMD-CTD interface (see Video S1), thus repositioning the stretched tether helix into a helical conformation, rotating the CTD by \( \sim 31^\circ -38^\circ \) clockwise (when viewed top-down), and reducing the distance between Cz atoms of T80 and L229 by 2-8 Å (Figure 4). Similarly, the CTD rotates \( \sim 35^\circ \) from GIRK2apo to GIRK2-PIP2 cryoEM structures (Niu et al., 2020). In contrast, the CTD rotates in the opposite direction by \( \sim 4^\circ \) with G\( \beta \)Y in the X-ray structure of GIRK2-G\( \beta \)Y-PIP2 (Whorton and MacKinnon, 2013). Taken together, these studies suggest that the PIP2 binding induces a major conformational change in GIRK2, involving a “twisting engagement” of the CTD with the TMD, and then a “locking rotation” of the CTD with the TMD after engagement that is required for the two gates to open. This observation potentially explains why GIRK2 channels are not gated open by G\( \beta \)Y, ethanol, or cholesterol in the absence of PIP2.

The cryoEM structures also reveal subtle differences in the coordination of PIP2 between GIRK2\textsubscript{PIP2/CHS} and GIRK2\textsubscript{PIP2}, and the X-ray crystal structure of GIRK2-PIP2 (PDB: 3SYA). The 1\( ^{st} \) PO4 of PIP2 in the cryoEM structures interacts with the side chain of R92, whereas in the X-ray crystal structure PIP2 is coordinated by the amide backbone of R92 and the side chain is not resolved (Figures 3A; Figure S7C). In addition, the 4\( ^{th} \) PO4 interaction with K64 in the 3SYA structure is not evident in the cryoEM structures. The phosphate atom of 1\( ^{st} \) PO4 of PIP2 is moved from the membrane toward the cytoplasmic side by 3 Å along with an \( \sim 3^\circ \) rotation of the CTD around the 4-fold axis, resulting in a slightly wider pore opening at the inner-helix gate in the GIRK2\textsubscript{PIP2} cryoEM structure compared to the crystal structure of GIRK2 (PDB: 3SYA; Figure S7; Figure 3B). These differences could indicate a possible unique transition state, but additional studies are needed to rule out an effect of experimental conditions, such as the concentration of PIP2.

What is the role of cholesterol/CHS in GIRK2 channel activation? In the presence of PIP2 and CHS, our analysis revealed predominantly a conformation of GIRK2\textsubscript{PIP2/CHS} that is remarkably similar to the GIRK2\textsubscript{PIP2} structure. In the absence of CHS, there is a decrease in the fraction of particles assuming the fully engaged conformation relative to the disengaged states, suggesting that CHS may stabilize the four PIP2-bound GIRK2 with an engaged CTD. By comparison, the presence of cholesterol increases the thermal stability of GPCRs and often lowers the energy barrier for agonist-induced conformational changes (Gimpl, 2016). The binding of CHS in peripheral hydrophobic sites with no highly specific interactions is in agreement with the lack of large conformational changes on GIRK2 as a result of this engagement (i.e., the similar structure of GIRK2\textsubscript{PIP2} and GIRK2\textsubscript{PIP2/CHS}), implying the importance of CHS/cholesterol for primarily enhancing PIP2 binding. The increase in PIP2 affinity,
as promoted by cholesterol (Glaaser and Slesinger, 2017), might increase the probability of the channel entering the open state. Similarly, Niu et al. (2020) found that increasing the PIP2 concentration shifts the equilibrium of GIRK2 between a mix of engaged and disengaged states, toward the engaged form. However, one noticeable difference is that GIRK2 with PIP2 in the absence of Gbg has a wider pore opening at the inner-helix gate and, importantly, is gated open by PIP2 and potentiated further by cholesterol even in the absence of Gbg.

Physiological significance of cholesterol gating of GIRK2 channels

The potential connection between elevated plasma cholesterol and increased risk for cardiac disease is well known (Rosenson et al., 2018). Less well understood is the role of cholesterol in the brain. Nearly all cholesterol in the CNS is derived from de novo synthesis, and it is therefore not influenced by dietary changes in cholesterol (Dietschy and Turley, 2001). Nevertheless, changes in brain cholesterol have been implicated in several neurodegenerative diseases, such as Alzheimer’s disease, Huntington’s disease, and Niemann-Pick disease (Vance, 2012). Hypercholesterolemia causes impairment of dopamine signaling and psychomotor dysfunction in mice (Engel et al., 2016; Paul et al., 2017a, 2018). Increases in cholesterol levels have been shown to elevate β-amyloid precursor protein levels in cholesterol-enriched lipid rafts (Cho et al., 2019) and are associated with increased risk for AD (Zarrouk et al., 2018). Accordingly, there is a growing need to understand how cholesterol affects brain function.

GIRK2 is relatively unique among members of the Kir family because it is potentiated in response to cholesterol rather than inhibited, as occurs with most other members of the Kir family (Rosenhouse-Dantsker, 2019). Acute elevation of brain cholesterol in hippocampal neurons increases both basal and GPCR-dependent activity of GIRK channels (Bukiya et al., 2017), suggesting that changes in brain cholesterol in vivo could affect

Figure 6. Effect of mutations in CHS pockets on cholesterol potentiation of GIRK2 channels

(A) Cartoon shows protocol used for cholesterol potentiation of GIRK2 channels expressed in HEK293 cells.

(B) The inward current (−120 mV) is plotted as a function of time, showing a large Ba2+-sensitive current in cholesterol-enriched HEK293 cells, as compared to control. Dashed line indicates zero current.

(C) Current-voltage plots show the mean Ba2+-sensitive current densities for control (black) and cholesterol-enriched (green) conditions for GIRK2 WT (n = 10 control; n = 10 +chol). A Student’s unpaired two-tailed t test was used for evaluation at −120 mV (p < 0.05). SEM is shown in lighter color.

(D and E) Five residues surrounding the CHS in site A and four residues surrounding the CHS in site B were mutated and tested for cholesterol potentiation.

(F and G) Current-voltage plots show the mean Ba2+-sensitive current densities for control (black) and cholesterol-enriched (green) conditions for mutant F93W (F) (n = 8 control; n = 10 +chol) and V99W (G) (n = 7 control; n = 7 +chol) channels. NS, not significant.

(H and I) Bar graphs show the normalized % currents for GIRK2 WT compared to GIRK2 with a mutation in site A (H) or site B (I). For each, the Ba2+-sensitive basal current density for cholesterol enrichment (green bar) is normalized to each control condition (black bar). A Student’s unpaired two-tailed t test was used for evaluation at −120 mV (p < 0.05, *p < 0.01, **p < 0.001, control versus cholesterol treatment). N is indicated on the graph. Dashed line indicates 100% (i.e., no potentiation). NC, no current. Error bars represent SEM.
both the basal and neurotransmitter-activated GIRK currents. Our cryoEM study of GIRK2 provides important information on the structural mechanism of cholesterol potentiation and reveals a cholesterol pocket that may be suitable for targeting with drugs. The structure of the alcohol pocket in GIRK2 was recently used in a virtual screen for GIRK modulators and identified a GIRK1-activating compound, GiGA1 (Zhao et al., 2020). Now, a similar screening can be conducted using the structure of the CHS site A in GIRK2, which may lead to the discovery of a new family of GIRK modulators.

**STAR METHODS**

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**SUPPLEMENTAL INFORMATION**

Supplemental information can be found online at https://doi.org/10.1016/j.celrep.2021.109619. A video abstract is available at https://doi.org/10.1016/j.celrep.2021.109619#mmc4.

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**AUTHOR CONTRIBUTIONS**

Y.K.M., I.W.G., G.S., and P.A.S. were responsible for the initial design and execution of the experiments; I.W.G. and Y.Z. prepared protein; I.W.G. conducted functional flux assays; Y.K.M. prepared vitrified samples, collected and processed cryoEM data, performed modeling, and analyzed EM data under the supervision of G.S.; M.J.R. implemented GemSpot; Y.Z. conducted and analyzed electrophysiological recordings; Y.K.M., I.W.G., G.S., and P.A.S. interpreted the results and contributed to writing the manuscript with input from Y.Z.

**DECLARATION OF INTERESTS**

The authors declare no competing interests.

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## STAR★METHODS

### KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| **Antibodies**      |        |            |
| GFP Mouse Anti-tag, clone: GF28R | Invitrogen | Cat #: MA515256 |
| **Biological samples** |        |            |
| Yeast strain *Pichia Pastoris* SMD 1163H | Invitrogen | SMD1163H |
| **Chemicals, peptides, and recombinant proteins** |        |            |
| 1-Propanol          | Sigma-Aldrich | Catalog #: 402893 |
| Barium Chloride     | Sigma-Aldrich | Catalog #: 342920 |
| Cholesterol-methyl-β-cyclodextrin | Sigma-Aldrich | Catalog #: C4951 |
| Salts for electrophysiology | Sigma-Aldrich |        |
| ACMA (9-Amino-6-Chloro-2-Methoxyacidine) | Life Technologies | Catalog #: A1324 |
| CCCP (Carbonyl cyanide m-chlorophenyl hydrazone) | Sigma-Aldrich | Catalog #: C2759 |
| Valinomycin         | Life Technologies | Catalog #: V1644 |
| MTS-HE (2-hydroxyethyl methanethiosulfonate) | Toronto Research Chemicals | Catalog #: H942250 |
| Cholesterol, ovine wool | Avanti Polar Lipids | Catalog #: 700000 |
| CHS (Cholesteryl Hemi succinate Tris Salt) | Anatrace | Catalog #: CH210 |
| Lipids-PE (1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine) | Avanti Polar Lipids | Catalog #: 850757 |
| Lipids- PG (1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-(1'-rac-glycerol) | Avanti Polar Lipids | Catalog #: 840457 |
| PIP2 (P(4,5)P2-diC8) | Echelon | Cat #: P-4508 |
| DDM (n-Dodecyl-β-D-Maltopyranoside) | Anatrace | Catalog #: D310 |
| GIRK2 recombinant protein | This study | N/A |
| **Critical commercial assays** |        |            |
| QuikChange II XL | Agilent Technology | N/A |
| **Deposited data** |        |            |
| GIRK2*PIP2/CHS* map | EMDB | EMD-22154 |
| GIRK2*PIP2+ map | EMDB | EMD-22150 |
| GIRK2*PIP2+ map | EMDB | EMD-22151 |
| GIRK2*PIP2++ map | EMDB | EMD-22152 |
| GIRK2*PIP2+++ map | EMDB | EMD-22153 |
| GIRK2*Apo map | EMDB | EMD-22155 |
| GIRK2*PIP2/CHS* model | PDB | 6XEV |
| GIRK2*PIP* model | PDB | 6XEU |

**Experimental models: Cell lines**

| HEK293T cells | ATCC | Catalog #: CRL-3216 |

**Oligonucleotides**

| DNA primers for mutagenesis | IDT DNA technologies | N/A |
| GIRK2 L79A F GAG ACG TAC CGA TAC GCG ACG GAC ATC TTC ACC | IDT DNA technologies | N/A |
| GIRK2 L79W F GAG ACG TAC CGA TAC TGG ACG GAC ATC TTC ACC | IDT DNA technologies | N/A |

(Continued on next page)
| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| GIRK2 I82A F CGA T AC TCTG ACG GAC GCC TTC ACC ACC CTG G | IDT DNA technologies | N/A |
| GIRK2 I82W F CGG ATA CCT GAC GGA CTG GTT CAC CTT GCT GGT | IDT DNA technologies | N/A |
| GIRK2 L86A F GAC ATC TCT ACC ACC GCC GTG GAC CTG AAG TGG | IDT DNA technologies | N/A |
| GIRK2 L86C F GAC ATC TCT ACC ACC TGC GTG GAC CTG AAG TGG | IDT DNA technologies | N/A |
| GIRK2 L86W F GAC ATC TCT ACC ACC TGG GTG GAC CTG AAG TGG | IDT DNA technologies | N/A |
| GIRK2 F93A F GTG GAC CTG AAG TGG AGA GCC AAC CTG TTG ATC TTT G | IDT DNA technologies | N/A |
| GIRK2 F93Y F GTG GAC CTG AAG TGG AGA TAC AAC CTG TTG ATC TTT G | IDT DNA technologies | N/A |
| GIRK2 F93W F GAC ATC TTC ACC ACC GAC ATG GAA CCT GTC ATC TTT G | IDT DNA technologies | N/A |
| GIRK2 L95A F CCT GAA GTG GAG ATT CAA CGC GTT GAT TGT CAT GTC | IDT DNA technologies | N/A |
| GIRK2 L95W F CCT GAA GTG GAG ATT CAA CGC GTT GAT TGT CAT GTC | IDT DNA technologies | N/A |
| GIRK2 L96A F CTG AAG TGG AGA TTC AAC CTG GCG ATC TTT GTC ATG GTC | IDT DNA technologies | N/A |
| GIRK2 L96F F CTG AAG TGG AGA TTC AAC CTG GCT ATC TTT GTC ATG GTC | IDT DNA technologies | N/A |
| GIRK2 L96W F CTG AAG TGG AGA TTC AAC CTG TGG ATC TTT GTC ATG GTC | IDT DNA technologies | N/A |
| GIRK2 V99A F CAA CCT GTT GAT CTT TGC CAT GGT CTA CAC AGT GAC G | IDT DNA technologies | N/A |
| GIRK2 V99I F CAA CCT GTT GAT CTT TAT CAT GGT CTA CAC AGT G | IDT DNA technologies | N/A |
| GIRK2 V99S F GAT TCA ACC TGT TGA TGT TTT GCA TGG TCT ACA CAG TCA | IDT DNA technologies | N/A |
| GIRK2 V101A F GAT CTT TGT CAT GGC CTA CAC AGT GAC GTG | IDT DNA technologies | N/A |
| GIRK2 V101T F GAT CTT CTC CAT GAC GTA CAC AGT GAC GTG GC | IDT DNA technologies | N/A |

**Recombinant DNA**

- mouse GIRK2c cDNA Horvath et al., 2018 N/A

**Software and algorithms**

| Name | Version | Website |
|------|---------|---------|
| SerialEM | Mastronarde, 2005 | [https://bio3d.colorado.edu/SerialEM/](https://bio3d.colorado.edu/SerialEM/) |
| MotionCor2 | Zheng et al., 2017 | [https://emcore.ucsf.edu/ucsf-software](https://emcore.ucsf.edu/ucsf-software) |
| CTFFIND4 | Rohou and Grigorieff, 2015 | [https://grigoriefflab.janelia.org/ctffind4](https://grigoriefflab.janelia.org/ctffind4) |
| Relion | Scheres, 2012, 2015, 2016; Zivanov et al., 2018 | [https://www3.mrc-lmb.cam.ac.uk/relion/index.php?title=Download_%26_install](https://www3.mrc-lmb.cam.ac.uk/relion/index.php?title=Download_%26_install) |
| Phenix | Afonine et al., 2018 | [https://phenix-online.org/](https://phenix-online.org/) |

(Continued on next page)
RESOURCE AVAILABILITY

Lead contact
Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Paul Slesinger (paul.slesinger@mssm.edu).

Materials availability
Plasmids generated in this study are available from the lead contact upon request.

Data and code availability

- CryoEM maps of GIRK2<sup>PIP2/CHS</sup>, GIRK2<sup>PIP2*</sup>, GIRK2<sup>PIP2**</sup>, GIRK2<sup>PIP2***</sup>, GIRK2<sup>PIP2****</sup> and GIRK2 apo are deposited in Electron Microscopy Data Bank under accession codes EMD-22154, EMD-22150, EMD-22151, EMD-22152, EMD-22153 and EMD-22155, respectively. The coordinates of GIRK2<sup>PIP2/CHS</sup> and GIRK2<sup>PIP2*</sup> models are deposited in Protein Data Bank under accession codes 6XEV and 6XEU, respectively.

- This paper does not report original code.

- Any additional information required to reanalyze the data reported in this work is available from the Lead Contact upon request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Molecular biology and cell culture
A truncated Mus musculus Girk2 (Kcnj6) cDNA (containing amino acids 52–380) linked in-frame with an HRV 3C protease site, green fluorescent protein (GFP) and a decahistidine (HIS10) tag (a generous gift from R. MacKinnon, The Rockefeller University, New York, NY) in pPICZ (ThermoFisher) was transformed into Pichia pastoris using electroporation for protein expression (according to manufacturer protocols). Transformants were screened based upon Zeocin resistance (> 1 mg/ml) and GFP emission. Highest expressing clones were selected for large-scale purification.

Mutations were introduced to mouse GIRK2c using site-directed mutagenesis (QuickChange II XL, Agilent Technology) and confirmed by DNA sequencing. Human Embryonic Kidney 293T (HEK293T) cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% (v/v) Fetal Bovine Serum (FBS), 100 U/ml penicillin, 100 mg/ml streptomycin and 1X Glutamax (ThermoFisher) in a humidified 37°C incubator with 5% CO2. Cells were plated 12-well plates and transiently transfected with cDNA (GIRK2c WT or mutant: 0.5 μg; eYFP: 0.02 μg, to identify transfected cells) using Lipofectamine 2000 (ThermoFisher). 48h after transfection, cells were plated onto poly-D-lysine (100 mg/ml; Sigma-Aldrich) coated 12-mm glass coverslips in 24-well plates for whole-cell patch-clamp recording.

METHOD DETAILS

Protein purification and reconstitution
All GIRK channels were expressed and purified in P. pastoris as described previously (Glaaser and Slesinger, 2017). Briefly, the highest-expressing clone was grown in BMGY medium and induced in BMM medium containing 1% methanol. Cells were harvested, resuspended in buffer (50 mM HEPES, pH 7.4; 150 mM KCl; 1 mM TCEP; 1 mM AEBSF and Complete EDTA-free protease inhibitor tablets (Roche)), flash frozen in liquid nitrogen, and stored at −80°C. Frozen cells were lysed in a Mixer Mill (Retsch) 5-times for 3 minutes at 25 Hz and stored as powder at −80°C until needed. The cell powder was solubilized in buffer containing 50 mM HEPES, pH 7.35; 150 mM KCl; 1 mM TCEP; 1 mM AEBSF; Complete ULTRA EDTA-free protease inhibitor tablets (Roche) and either 2% (w/v) n-Dodecyl-β-D-maltoside (DDM; Anatrace) or 2% (w/v) DDM supplemented with 0.2% (w/v) Cholesteryl Hemisuccinate Tris Salt (Anatrace) with gentle stirring at 4°C. Unsolubilized material was separated by centrifugation at 40,000 × g for 40 min at 4°C.
and filtered. The supernatant was incubated with HISPur Cobalt charged resin (ThermoFisher) equilibrated in wash buffer (50 mM HEPES, pH 7.0; 150 mM KCl; 0.2% DDM or 0.2%DDM/0.02% CHS; 20 mM imidazole). The resin was subsequently washed in 10 column volumes (CV) of wash buffer, 5 CV containing 40 mM Imidazole, and eluted in buffer containing 300 mM imidazole. The eluate was pooled, exchanged into imidazole-free buffer and digested overnight at 4 °C with HRV 3 C protease, purified as described (Shaya et al., 2011) (a generous gift of D. Minor, UCSF, San Francisco, CA). The protein was subsequently concentrated and run on a Superdex-200 gel filtration column in buffer containing 20 mM TRIS-HCl pH 7.5, 150 mM KCl, 2 mM DTT, 3 mM TCEP, and 1 mM EDTA, 0.025% (w/v) DDM (anagrade) alone or with 0.0025% CHS. Fractions eluting at a volume consistent with the GIRK channel tetramer were pooled, concentrated and examined by SDS-PAGE and Coomassie blue staining.

Purified GIRK2 channels were reconstituted into lipid vesicles as described previously (Glaaser and Slesinger, 2017). Briefly, a lipid mixture containing 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine (POPE), 1-palmitoyl-2-oleoyl-sn-glycero-3-phospho(1’-rac-glycerol) (POPG), and L-α-phosphatidylinositol-4,5-bisphosphate (Brain, PI(4,5)P2 (Porcine)) at mass ratios of 3:1:0.04 (POPE:POPG:PIP2) or 3:1 (POPE:POPG) was prepared, reconstituted in vesicle buffer (20 mM K-HEPES, pH 7.4; 150 mM KCl; 0.5 mM EDTA containing 35 mM CHAPS) and incubated with protein in detergent at a 1:200 protein: lipid ratio unless otherwise indicated. Where indicated, cholesterol (Ovine wool; Anatrace) was added to vesicles at a mole percentage of 5% or Cholesteryl Hemisuccinate (CHS; Anatrace) was incorporated at 2.5% mol percentage. Detergent was removed through sequential addition of Bio-beads SM-2 (Bio-rad). All phospholipids, cholesterol, and Brain PIP2 were purchased from Avanti Polar Lipids, Inc. Water-soluble PIP2 (diC8-PIP2) was purchased from Echelon Biosciences.

Flux assay
The fluorescence-based flux assay for GIRK channel activity was performed as described previously (Whorton and MacKinnon, 2013; Glaaser and Slesinger, 2017). Briefly, Liposomes were diluted 1:20 into flux buffer (20 mM Na-HEPES, pH 7.4; 150 mM NaCl; 0.5 mM EDTA) containing 5 μM of the H+ sensitive dye 9-Amino-6-chloro-2-methoxyacridine (ACMA)(Invitrogen). Fluorescence was measured using a Flexstation 3 microplate reader (Molecular Devices) with the following parameters: 410 nm excitation, 480 nm emission, 455 nm cutoff, medium PMT sensitivity, and sampling at 2 seconds. After a stable baseline fluorescence (150 s) was obtained, the H+ ionophore m-chlorophenyl hydrazone (CCCP)(Sigma) was automatically added (1 μM final), then a second addition of vehicle or methanethiosulfonate hydroxyethyl (100 μM final; MTS-HE, Toronto Research Chemicals) was added 150 s later, followed 900 s later by a third addition with the K+ ionophore Valinomycin (100 nM final; Invitrogen), to determine the maximal K+ flux. GIRK2 channels are likely arranged in both orientations in the liposomes. However, we expect the channels oriented inside-out to support high K+ flux because of high Na+ in the flux buffer and high K+ in the liposome (Glaaser and Slesinger, 2017). The percentage relative K+ flux (or relative fluorescence intensity) was calculated by measuring the extent of quenching 10 s before VM addition (F), and dividing by the total quenching capacity of the liposomes normalized to the relative fluorescence units (RFU) 10 s before the addition of vehicle or MTS-HE (F0). Liposome flux assay illustration created with Biorender.com

CryoEM sample preparation, data collection, and processing
The quality of purified samples was initially screened by negative stain EM using established protocols (Peisley and Skiniotis, 2015). For cryoEM, 3.5 μl of GIRK2 at a concentration of 7-10 mg/ml was applied to glow-discharged Quantifoil Au1.2/1.3, 200 mesh grids, blotted and then plunge-frozen in liquid ethane using FEI Vitrobot. All cryoEM data were collected at 300 kV on a Titan Krios equipped with a Gatan K3 direct detection camera. Raw images were collected as movies, recorded at a magnification of 58,824 X corresponding to 0.85 pixel per Å at the specimen level. Each movie was recorded for 3 s at 0.05 s/frame with a total dose of 60 electrons/pixel and defocus values ranging from −0.8 to −2.2 μm.

The movies were motion-corrected and dose-weighted using Motioncor2 (Zheng et al., 2017) and defocus values were determined by CTFIND4 (Rohou and Grigorieff, 2015). Template-based particle picking, 2D/3D classification, and 3D refinements were performed using the Relion pipeline (Scheres, 2012, 2015, 2016; Zivanov et al., 2018). The 3D classes with both domains distinguishable were refined with C4 or C1 symmetry as shown in Figures S2, S4, and S6, post processed with a mask encompassing both the domains, corrected for modulation transfer function (MTF) of K3 direct detection camera at 300 kV and sharpened with suitable B factor (Tables S1 and S2). The resolution of the maps reported here was estimated according to the 0.143 “gold-standard” Fourier Shell Correlation (FSC) criterion. The resolution of GIRK2PIP2, GIRK2PIP2*, GIRK2PIP2**, GIRK2PIP2***, and GIRK2 apo was indicated globally as 3.5, 3.2, 4.8, 7.7, 7.7 and 4.8 Å, respectively. Local resolution was estimated using Relion. In GIRK2 apo, two 3D class with significant disengagement and similar relative orientation of domains were combined for the final 3D refinement (Figure S6). The input particle stack for 3D classification was also used for cryoSPARC 3D variability analysis (Punjani and Fleet, 2021) (Video S1).

Modeling of protein and lipids in the cryoEM maps
The GIRK2 backbone and PIP2 at the TMD-CTD interface as determined in the X-ray crystal structure of GIRK2 (PDB: 3SYA) was initially fit as rigid-body into the EM maps using UCSF Chimera (Pettersen et al., 2004) and subsequently interactively adjusted using COOT (Emsley and Cowtan, 2004). The GemSpot pipeline (Robertson et al., 2020) was used for modeling CHS into EM map followed by refinement in COOT (Emsley and Cowtan, 2004). The models in the target EM maps were refined further using Phenix real-space
refinement (Afonine et al., 2018). Pore dimension in the model was analyzed using the HOLE program (Smart et al., 1996). The figures in the manuscript were prepared using UCSF ChimeraX (Goddard et al., 2018).

Electrophysiological studies
Mutant and wild-type GIRK2 channels were transiently expressed in HEK293T cells and whole-cell patch-clamp recordings were made as described previously (Zhao et al., 2020). All mutants were confirmed by DNA sequencing. Borosilicate glass electrodes (Warner Instruments) of 3-6 MΩ were filled with an intracellular solution containing 130 mM KCl, 20 mM NaCl, 5 mM EGTA, 5.46 mM MgCl₂, 2.56 mM K₂ATP, 0.3 mM Li₂GTP and 10 mM HEPES (pH 7.4, ~320 mOsm). The extracellular ‘20K’ solution contained 20 mM KCl, 140 mM NaCl, 0.5 mM CaCl₂, 2 mM MgCl₂, and 10 mM HEPES (pH 7.4; ~310 mOsm). Currents were elicited at 0.5 Hz with a voltage step to −120 mV from a holding potential of −40 mV, followed by a ramp voltage protocol (−120 mV to +50 mV, EK = −50 mV with 20 mM Kout). K⁺ currents were adjusted for series resistance, and measured at −120 mV with either 20K solution or 20K + Ba²⁺ (1 mM) solution. The basal current was defined as the Ba²⁺-sensitive current. Cholesterol-mediated potentiation of the basal current was normalized as a percentage of the control (vehicle) basal current (Normalized Current %). All currents are expressed as current density (current / cell capacitance; pA/pF).

Cholesterol enrichment
We used water-soluble cholesterol with methyl-β-cyclodextrin (MβCD) (40mg cholesterol per gram, Sigma-Aldrich; C4951) for cholesterol enrichment in HEK293T cells. MβCD is a well-known cholesterol donor (Zidovetzki and Levitan, 2007). On the day of the recording, transfected HEK293T cells were incubated in DMEM containing 0.625 mM cholesterol modified from previously described on HEK293 cells (Wu et al., 2013). We kept the molar ratio of cholesterol: MβCD ~1:8. Controls were incubated with serum-free DMEM. After 1 h of incubation, which was shown to potentiate GIRK currents in hippocampal pyramidal neurons (Bukiya et al., 2017), HEK293T cells were washed once with FBS, and subjected to whole-cell patch-clamp recording. The data that support the findings of this study are available from the corresponding author upon reasonable request.

QUANTIFICATION AND STATISTICAL ANALYSIS
For three or more groups, a one-way ANOVA followed with a Dunnett’s multiple comparisons post hoc test was used for evaluating significant differences (*p < 0.05, **p < 0.01, **** p < 0.001) (Figure 1). For groups of two, a Student’s unpaired two-tailed t test was used for evaluating significant differences (*p < 0.05, **p < 0.01, **** p < 0.001) (Figure 6). All statistical tests were conducted with Prism 9 (Graphpad). The mean ± SEM are shown. The specific statistical details of experiments can be found in the figure legends.
Supplemental information

Structural insights into GIRK2 channel modulation by cholesterol and PIP₂

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Figure S1: Purification and assessment of sample quality by negative staining electron microscopy. Related to Figures 1, 2 and 4. (a) Size exclusion chromatogram and Coomassie staining of purified GIRK2. The chromatogram (left panel) shows purification of mouse GIRK2 from *Pichia pastoris*. The absorbance is plotted as a function of elution volume. The major peak elutes at a volume consistent with the size of a ΔGIRK2 tetramer (Glaaser and Slesinger, 2017). Peak fractions (PF) utilized in both functional and structural studies are indicated by dashed vertical lines. Coomassie blue staining (right panel) of pooled and concentrated peak fractions (PF) on a gradient SDS-PAGE protein gel shows three bands, one for tetramer (T), one for dimer (D), and one for monomer (M). Protein ladder shows molecular weights in kDa. (b) Micrograph of negative stained GIRK2. The image shows predominantly homogenous GIRK2 tetramers in detergent micelle. The scale bar is 20 nm in the negative stain image.
Figure S2: CryoEM data processing of GIRK2 in presence of modulators PIP2 and cholesteryl hemisuccinate. Related to Figures 2 and 5. (a) and (b) are representative cryoEM image and 2D averages. The scale bars are 20 nm in the cryoEM image and 6 nm in the 2D averages. (c) Data processing flow chart. (d) FSC curve, the dotted line indicating FSC at 0.143. (e) Local resolution estimate by Relion. (f) Fit of the transmembrane helices M1 and M2 into the 3.5 Å GIRK2_{PIP2/CHS} cryoEM map.
Figure S3: Modeling of cholesteryl hemisuccinate (CHS) in the cryoEM map. Related to Figure 2. (a) Modelling of CHS at Site A (green) in GIRK2^{PIP2/CHS} cryoEM map suggests we can model poses with subtle differences in the orientation of iso-octyl tail. In the final deposited PDB, we have modeled CHS at Site A as in pose1, the density is contoured at a threshold level of 0.004 in ChimeraX. (b) Illustration of residues around CHS at Site A colored based on hydrophobicity (dark cyan – most hydrophilic, yellow – most hydrophobic) in ChimeraX, it is bound in a hydrophobic environment. (c) CHS modelled at Site B (green) is also surrounded by hydrophobic residues including F186 (pink), V72, L79, I82, L86, L89, I97 and V101 (purple), the density is contoured at a threshold level of 0.0073 in ChimeraX. (d) Comparison of GIRK2^{PIP2/CHS} (contoured at a threshold level of 0.0102 in ChimeraX) and GIRK2^{PIP2*} (contoured at a threshold level of 0.009 in ChimeraX) maps. The densities for CHS, PIP2 and micelle are shown in green, red and orange, respectively with micelle noise hidden in both the maps (dust below the size of 4 in ChimeraX); protein is in grey and position of CHS (green) and PIP2 (red) are as in GIRK2^{PIP2/CHS} model. Densities observed in GIRK2^{PIP2/CHS} map at Sites A and Site B (densities colored in green) are absent in GIRK2^{PIP2*} map. Other poorly resolved densities (colored in orange) near PIP2 consistent in both maps (highlighted one of them in black box) might correspond to other putative CHS or phospholipid binding sites. (e) Zoom-in view of the densities at Site A and Site B in the respective GIRK2^{PIP2/CHS} and GIRK2^{PIP2*} maps, residues Y78, F93, L96 and I97 approximately show the region of interest. (f) Zoom-in view (same as e) of the densities at Site A and Site B for unsharpened GIRK2^{PIP2/CHS} (contoured at a threshold level of 0.0075 in ChimeraX) and GIRK2^{PIP2*} (contoured at a threshold level of 0.008 in ChimeraX) maps without any post processing, the micelle noise is hidden (dust below the size of 4 in ChimeraX).
Figure S4: CryoEM data processing of GIRK2 in presence of PIP2. Related to Figures 3, 4 and 5. (a) and (b) are representative cryoEM image and 2D averages. The scale bars are 20 nm in the cryoEM image and 6 nm in the 2D averages. (c) Data processing flow chart. (d) FSC curve of GIRK2\textsuperscript{PIP2*}, the dotted line indicating FSC at 0.143. (e) Local resolution estimate of GIRK2\textsuperscript{PIP2*} by Relion. (f) Fit of the transmembrane helices M1 and M2 into the 3.2 Å GIRK2\textsuperscript{PIP2*} cryoEM map. (g) FSC curve of GIRK2\textsuperscript{PIP2**}, the dotted line indicating FSC at 0.143. (h) FSC curve of GIRK2\textsuperscript{PIP2***}, the dotted line indicating FSC at 0.143. (i) FSC curve of GIRK2\textsuperscript{PIP2****}, the dotted line indicating FSC at 0.143.
Figure S5: Evaluation of CTD detachment and twist in different structures. Related to Figure 5. (a) CryoEM maps of GIRK2\textsuperscript{PIP2*}, GIRK2\textsuperscript{PIP2**}, GIRK2\textsuperscript{PIP2***} and GIRK2\textsuperscript{PIP2****} with varying conformation of CTD with respect to TMD, one of the subunits is highlighted in cyan. (b) In these structures, CTD detachment is assessed by evaluating the distance between C\alpha atoms of residues T80 (denoted by red sphere) and L229 (denoted by blue sphere). The values shown are the respective CTD upward shift between the disengaged conformers and engaged GIRK2\textsuperscript{PIP2*}. (c) Comparison of CTD twist of GIRK2\textsuperscript{PIP2**} (green) with GIRK2\textsuperscript{PIP2*} (purple) assessed by the angle of rotation of C\alpha atom of G347 (represented in sphere) around the four-fold axis after aligning the TMD near selectivity filter. (d) GIRK2 apo X-ray crystal structure (PDBID: 3SYO; green) is docked into GIRK2 apo cryoEM map (orange). In contrast to the apo cryoEM map, the CTD in the crystal structure is engaged to the membrane.
Figure S6: CryoEM data processing of apo GIRK2. Related to Figures 4 and 5. (a) and (b) are representative cryoEM image and 2D averages. The scale bars are 20 nm in the cryoEM image and 6 nm in the 2D averages. (c) Data processing flow chart. (d) FSC curve, the dotted line indicating FSC at 0.143.
Figure S7: Comparison of CTD and PIP$_2$ pocket in cryoEM and X-ray crystal structures. Related to Figure 3. (a) Comparison of CTD in GIRK2$^{PIP_2^*}$ (PDBID:6XEU, violet) with crystal structure bound with only PIP$_2$ (PDBID:3SYA, pink), crystal structure with Gβγ/PIP$_2$ (PDBID:4KFM, orange), and cryoEM structure PDBID:6XIT (green) with PIP$_2$ after aligning the TMD near selectivity filter. There are subtle differences in the position of PIP$_2$, CTD and inner-helix gate residue F192. Compared to crystal structure with only PIP$_2$ (PDBID:3SYA), the position of phosphate atom of 1' PO$_4$ of PIP$_2$ in GIRK2$^{PIP_2^*}$ cryoEM structure has moved away from the membrane towards the cytoplasmic side by ~3Å, and accompanied with ~3° rotation of CTD around the four-fold axis (b) Comparison of the PIP$_2$ pocket (region highlighted in box in panel a) in GIRK2$^{PIP_2^*}$ (PDBID:6XEU, violet) with crystal structure bound with only PIP$_2$ (PDBID:3SYA, pink), crystal structure with Gβγ/PIP$_2$ (PDBID:4KFM, orange), and cryoEM structure PDBID:6XIT (green) with PIP$_2$ after aligning the TMD near selectivity filter. (c) PIP$_2$ coordination in the crystal structure PDBID:3SYA.
Figure S8: Comparison of cholesterol/CHS sites determined from GIRK2\textsuperscript{PIP2/CHS} cryoEM structure with docking simulations. Related to Figure 2. The left panel shows the GIRK2 homotetramer (blue) with one subunit highlighted in cyan, CHS at Site A and B (green) and PIP\(_2\) (red) are shown at TMD-CTD interface. The right panels show the comparison of the Site A and B with the regions 1 (orange shaded area and residues binding to cholesterol in this region are colored in orange) and 2 (pink shaded area and residues binding to cholesterol in this region are colored in pink) predicted based a docking study and electrophysiology with a modified GIRK2 (Bukiya et al., 2017), respectively. This suggests though similar residues are involved in both, there are differences in the orientation and position.
|                                | GIRK2<sub>PIP2/CHS</sub> | Apo    |
|--------------------------------|---------------------------|--------|
| Voltage (kV)                   | 300                       | 300    |
| Magnification                  | 58,824                    | 58,824 |
| Defocus (µm)                   | -1.5 to -2.2              | -0.8 to -2.2 |
| Pixel size (Å)                 | 0.85                      | 0.85   |
| Total electron dose            | 60                        | 60     |
| Exposure time (s)              | 3                         | 3      |
| No. of images                  | 642                       | 8,310  |
| No. of frames per image        | 60                        | 60     |
| Initial particle number        | 297,174                   | 2,532,601 |
| Particle number for 3D          | 84,912                    | 473,059 |
| classification                 |                           |        |
| Final particle number          | 44,990                    | 102,680 |
| Resolution (Å)                 | 3.5                       | 4.8    |
| Symmetry imposed               | C4                        | C1     |
| B-factor (Å<sup>2</sup>)       | 100                       | 201    |

**Model Refinement**

|                                | GIRK2<sub>PIP2/CHS</sub> | Apo    |
|--------------------------------|---------------------------|--------|
| Non-hydrogen atoms             | 10,545                    |        |
| R.M.S Deviations               |                           |        |
| Bond length (Å)                | 0.005                     |        |
| Bond angle (°)                 | 1.075                     |        |
| Ramachandran plot              |                           |        |
| Favored (%)                    | 91.7                      |        |
| Allowed (%)                    | 8.3                       |        |
| Outlier (%)                    | 0.0                       |        |
| Clashescore                    | 11.04                     |        |

**Table S1:** Data collection and processing statistics of GIRK2<sub>PIP2/CHS</sub> and apo structures. Related to Figures 2, 4 and 5.
|                      | GIRK2<sup>PIP2</sup>* | GIRK2<sup>PIP2</sup>** | GIRK2<sup>PIP2</sup>*** | GIRK2<sup>PIP2</sup>**** |
|----------------------|------------------------|-------------------------|--------------------------|---------------------------|
| Voltage (kV)         | 300                    | 300                     | 300                      | 300                       |
| Magnification        | 58,824                 | 58,824                  | 58,824                   | 58,824                    |
| Defocus (µm)         | -0.8 to -2.2           | -0.8 to -2.2            | -0.8 to -2.2             | -0.8 to -2.2              |
| Pixel size (Å)       | 0.85                   | 0.85                    | 0.85                     | 0.85                      |
| Total electron dose  | 60                     | 60                      | 60                       | 60                        |
| (electrons/pixel)    |                        |                         |                          |                           |
| Exposure time (s)    | 3                      | 3                       | 3                        | 3                         |
| No. of images        | 6480                   | 6480                    | 6480                     | 6480                      |
| No. of frames per image | 60                 | 60                      | 60                       | 60                        |
| Initial particle number | 2,058,148          | 2,058,148              | 2,058,148                | 2,058,148                |
| Particle number for 3D classification | 400,342             | 400,342                 | 400,342                  | 400,342                   |
| Final particle number | 154,071               | 109,405                 | 40,463                   | 40,798                    |
| Resolution (Å)       | 3.2                    | 4.8                     | 7.7                      | 7.7                       |
| Symmetry imposed     | C4                     | C1                      | C1                       | C1                        |
| B-factor (Å<sup>2</sup>) | 60                  | 100                     | 60                       | 60                        |
| Model Refinement     |                        |                         |                          |                           |
| Non-hydrogen atoms   | 10,544                 |                         |                          |                           |
| R.M.S Deviations     |                        |                         |                          |                           |
| Bond length (Å)      | 0.008                  |                         |                          |                           |
| Bond angle (°)       | 1.09                   |                         |                          |                           |
| Ramachandran plot    |                        |                         |                          |                           |
| Favored (%)          | 88.7                   |                         |                          |                           |
| Allowed (%)          | 11.3                   |                         |                          |                           |
| Outlier (%)          | 0.0                    |                         |                          |                           |
| Clashscore           | 2.7                    |                         |                          |                           |

**Table S2**: Data collection and processing statistics of GIRK2<sup>PIP2</sup> structures. Related to Figures 4 and 5.