Secondary anionic phospholipid binding site and gating mechanism in Kir2.1 inward rectifier channels

Inwardly rectifying potassium (Kir) channels regulate multiple tissues. All Kir channels require interaction of phosphatidyl-4,5-bisphosphate (PIP$_2$) at a crystallographically identified binding site, but an additional nonspecific secondary anionic phospholipid (PL(−)) is required to generate high PIP$_2$ sensitivity of Kir2 channel gating. The PL(−)-binding site and mechanism are yet to be elucidated. Here we report docking simulations that identify a putative PL(−)-binding site, adjacent to the PIP$_2$-binding site, generated by two lysine residues from neighbouring subunits. When either lysine is mutated to cysteine (K64C and K219C), channel activity is significantly decreased in cells and in reconstituted liposomes. Directly tethering K64C to the membrane by modification with decyl-MTS generates high PIP$_2$ sensitivity in liposomes, even in the complete absence of PL(−)s. The results provide a coherent molecular mechanism whereby PL(−) interaction with a discrete binding site results in a conformational change that stabilizes the high-affinity PIP$_2$ activatory site.
The eukaryotic inwardly rectifying potassium (Kir) channel family includes seven subfamilies expressed in tissues throughout the body and implicated in diverse physiological functions, such that mutations are causally linked to a number of metabolic and genetic diseases including diabetes, heart diseases, Andersen–Tawil syndrome and Epilepsy, ataxia, sensorineural deafness, tubulopathy syndrome1–3. Kir channels are regulated by diverse modulators including phosphorylation by protein kinase A, auxiliary proteins (sulfonylurea receptors, G protein βγ subunits), Na⁺, ATP, pore blockers (Mg²⁺, polyamine)1,2 and ethanol6. However, and in common with many other channels and membrane proteins, all Kir channels require the binding of phosphatidyl-4,5-bisphosphate (PI(4,5)P₂, PIP₂) for activation7–9.

The study of PIP₂ effects on ion channels has been facilitated by its low natural abundance (0.4 ± 1.5% of anionic phospholipids)10 and the availability of methods to manipulate PIP₂ concentrations in the membrane during electrophysiological experiments, either through enzymatic reactions or charge screening by specific antibodies or polyelectrolytes11,12. Other anionic lipid species, such as phosphatidylethanolamine (PE), phosphatic acid (PA), phosphatidylserine (PS), phosphatidylinositol (PI) and cardiolipin are also present at variable, and generally much higher, concentrations in native membranes3,13,14. Membrane lipid composition can vary under different physiological and pathological conditions3–5,17, and has been invoked causally in various diseases conditions8–20. However, progress in understanding the role of specific lipids in controlling of membrane protein function has been limited21 because they cannot be readily manipulated in native membranes. Reconstitution of purified proteins in synthetic membranes makes it possible to study function in defined systems of known lipid and protein composition, and the availability of purified eukaryotic Kir2 channel protein, reconstituted into liposomes of different lipid compositions. As shown in Fig. 1a, PPA did not activate the fraction of the lipid (here and in all further liposome experiments). As shown in Fig. 1a, PPA did not activate the channel under these conditions, suggesting that PPA cannot act as a functional substitute for PIP₂. We then tested whether PPA could act as a PL(−) and sufficient for the secondary anionic lipid effect in the presence of 0.1% PIP₂. Although increasing POPG augmented channel activity as shown before (Fig. 1b), PPA significantly decreased channel activity at all concentrations and channel activity was completely inhibited at >10% PPA. To further confirm the inhibitory effect of PPA, channel activity was measured in the presence of 0.1% PIP₂, 10% POPG with or without additional 10% of either POPG or PPA (Fig. 1c).

Docking simulations identify a potential PL(−)–binding site. We previously used docking simulations to identify the crystallographic PIP₂–binding site (‘primary’ site)23 as a main binding site for PIP₂ as well as an additional (‘secondary’) site, located at the interface between two adjacent subunits near the end of the slide helix and the apex of the βC–βD loop24. To determine potential docking sites for other anionic lipids, we have now performed extensive docking simulations of various lipids to Kir2.1, in both the absence (‘Apo-docking’) and presence (‘PIP₂-bound-docking’) of PIP₂ at the crystallographic site. The searched space is shown in Fig. 2a,b; the PIP₂ head group as located in the crystal structure (PDB 3SPI) was removed in the Apo-docking and presence (‘PIP₂-bound-docking’) of PIP₂ at the crystallographic site. The searched space is shown in Fig. 2a,b; the PIP₂ head group as located in the crystal structure (PDB 3SPI) was removed in the Apo-docking and presence (‘PIP₂-bound-docking’) of PIP₂ at the crystallographic site. The searched space is shown in Fig. 2a,b; the PIP₂ head group as located in the crystal structure (PDB 3SPI) was removed in the Apo-docking and presence (‘PIP₂-bound-docking’) of PIP₂ at the crystallographic site.

Results
PPA is a competitive inhibitor of Kir2.1 channels. Recently resolved crystallographic structures of chicken Kir2.2 (ref. 23) reveal a specific PIP₂–binding site at the interface of the M2 helix and the cytoplasmic domain. These structures also show that an anionic lipid with a PPA head group (PPA) can bind at essentially the same site. This raises the possibility that the secondary requirement for anionic lipids to activate Kir2 channels might be satisfied through binding to this site, perhaps if PIP₂ was only required at a subset of the subunits. Alternatively, PPA may act as either a competitive analogue or competitive antagonist of PIP₂ interaction and as a secondary activator PL(−). To resolve these possibilities, the functional role of PPA was first determined. Purified wild-type (WT) Kir2.1 protein was reconstituted into liposomes of different lipid compositions. First, to test whether PPA could act as a PIP₂ analogue and activate channels in the absence of PIP₂, liposomes were formed with PPA (zero, 0.1% or 1%), plus 10% 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoglycerol (POPG) (to supply the secondary non-specific lipid requirement), and 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphatidylethanolamine (POPE) for the remaining fraction of the lipid (here and in all further liposome experiments). As shown in Fig. 1a, PPA did not activate the channel under these conditions, suggesting that PPA cannot act as a functional substitute for PIP₂. We then tested whether PPA could act as a PL(−) and sufficient for the secondary anionic lipid effect in the presence of 0.1% PIP₂. Although increasing POPG augmented channel activity as shown before (Fig. 1b), PPA significantly decreased channel activity at all concentrations and channel activity was completely inhibited at >10% PPA. To further confirm the inhibitory effect of PPA, channel activity was measured in the presence of 0.1% PIP₂, 10% POPG with or without additional 10% of either POPG or PPA (Fig. 1c). Although additional 10% POPG (that is, 20% total POPG) further enhanced activity (P < 0.05, one-way analysis of variance, Turkey’s honestly significant difference post hoc test), 10% PPA again completely abolished the channel activity. These results are reminiscent of competitive inhibition of PIP₂-activated channel activity by other PIPs22,25 and clearly show that PPA acts as a competitive inhibitor of PIP₂ and not as an activatory PL(−).

Docking simulations identify a potential PL(−)–binding site. We previously used docking simulations to identify the crystallographic PIP₂–binding site (‘primary’ site)23 as a main binding site for PIP₂ as well as an additional (‘secondary’) site, located at the interface between two adjacent subunits near the end of the slide helix and the apex of the βC–βD loop24. To determine potential docking sites for other anionic lipids, we have now performed extensive docking simulations of various lipids to Kir2.1, in both the absence (‘Apo-docking’) and presence (‘PIP₂-bound-docking’) of PIP₂ at the crystallographic site. The searched space is shown in Fig. 2a,b; the PIP₂ head group as located in the crystal structure (PDB 3SPI) was removed in the Apo-docking but present in PIP2-bound-docking below. Tested lipids included negatively charged PPA, PA, PG, PS as well as neutral phosphatidylethanolamine (PE) and phosphatidylcholine (PC). Lipid head group structures and partial charges used for docking simulations are shown in Supplementary Fig. S1. Accepted poses, based on appropriate orientation relative to the bilayer normal and proximity to the membrane (see Methods),
were clustered using a K-clustering algorithm\textsuperscript{27}. For each tested lipid, optimal clustering efficiency was achieved by division into two clusters (see Supplementary Fig. S2 and Methods), corresponding to the previously suggested primary and secondary phospholipid sites. As an illustrative example, every accepted (3,727 at primary and 758 at secondary out of 10,000) docked poses of PG is shown in Fig. 2c.

In Apo-docking, all tested lipids, except PS, docked to the primary site with higher frequency and greater binding affinity compared with the secondary site (Fig. 2d, top panel; Tables 1 and 2). The inconsistent behaviour of PS may originate from its unique zwitterionic head group structure with a positively charged amine and negatively charged carboxylic group. The relative distribution of docking events between the two sites was in reasonable agreement with the Boltzmann distribution, based on predicted binding free energies (Fig. 2d, inset). Predicted binding at the primary site was much stronger for PIP\textsubscript{2} and PPA than for other lipids that at the primary site (Fig. 2d), but binding free energy at the secondary site was similar for all lipid species (maximum difference: 3.36 kcal mol\textsuperscript{-1}, mean ± s.d.: −3.37 ± 1.24 kcal mol\textsuperscript{-1}), consistent with the relatively non-specific and redundant character of the secondary PL(−) activating effect\textsuperscript{22}.

We then carried out a series of docking simulations of the same lipids with PIP\textsubscript{2} pre-bound at the primary site (‘PIP\textsubscript{2}-bound-docking’; Fig. 2e). In this situation, PIP\textsubscript{2} precluded additional binding of all but one of the lipids at the primary site and shifted binding almost exclusively to the secondary site, without substantially altering binding free energies at that site (Fig. 2e). PE was one exception in that it still docked to the primary site, essentially next to the PIP\textsubscript{2} head group, presumably facilitated by the small head group size and high charge density in the amine group. These simulations, using a rigid backbone model, therefore indicate that when PIP\textsubscript{2} is pre-docked at the primary site, the

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**Figure 1** | PPA is a competitive inhibitor of PIP\textsubscript{2} (left) \textsuperscript{86}Rb\textsuperscript{+} uptake (normalized to maximum activity of WT Kir2.1 in 0.1% PIP\textsubscript{2}, 10% POPG). (Right) Maximum activity for 20 min. (a) Ten percent POPG was present in each case, and PPA or PIP(4,5)P\textsubscript{2} as indicated. \( n = 3 \), mean ± s.e.m.; ***, \( P < 0.001 \) compared to the activity at PIP\textsubscript{2} 0.1 (\( P = 0.0002 \), one-way analysis of variance (ANOVA), Tukey’s honestly significant difference (HSD) post hoc test). (b) PIP\textsubscript{2} (0.1%) was present in each case, and increasing POPG (pink) or PPA (blue) were added as indicated. Control experiment with no POPG or PPA is shown in black box. \( n = 3 \), mean ± s.e.m.; *, \( P < 0.05 \) and **, \( P < 0.001 \) compared to the activity at PL(−) 0 (\( P = 0.0084 \) and \( P = 0.0042 \), one-way ANOVA, Tukey’s HSD post hoc test). (c) PIP\textsubscript{2} (0.1%) and 10% POPG were present in each case, with an additional 10% PIPG (total 20%) or 10% PPA, as indicated. \( n = 3 \), mean ± s.e.m. **, \( P < 0.01 \) compared to the activity at PG 10 (\( P = 0.0004 \), one-way ANOVA, Tukey’s HSD post hoc test).
Two lysines are the main determinants of PL(−) interaction. To identify residues that directly interact with lipids at the secondary site, hydrogen bonding between lipids and neighboring residues was assessed (Supplementary Table S1). Figure 3a shows residues that have more than 10% hydrogen bond (H-bond) frequency out of all H-bonds detected between the accepted poses combined from Apo-docking (left) and PIP2-bound-docking (right) experiments. The H-bond frequency results were qualitatively similar for both cases, with two features of note. First, K64, at the N-terminal end of the slide helix of one subunit and K219 at the apex of the BC–BD loop of the adjacent subunit are the two key residues that make direct contacts with the docked lipids (Fig. 3). In typical docked structures, these two positive side chains contributed to coordination of the negative phosphate group (Fig. 3b,c). Functional tests (below) demonstrate that these two residues are necessary for PL(−) activation and that chemical tethering of residue 64 to the membrane generates PL(−)-independent, high sensitivity to PIP2 activation. Second, the residues found in the secondary site do not overlap with the residues forming the primary site (Fig. 3b). This observation...
demonstrates that the secondary site is indeed a unique and separate site from the primary PIP₂-binding site.

**Mutant channel activity is reduced in cell membranes.** To experimentally test the functional relevance of the secondary site

| Table 1 | Number of accepted poses. |
|---------|---------------------------|
|         | Apo-docking               | PIP₂-bound docking |
|         | Primary | Secondary | Primary | Secondary |
| PIP₂    | 5,568   | 157       | 0       | 4,030      |
| PPA     | 6,448   | 147       | 0       | 4,853      |
| PA      | 9,373   | 364       | 0       | 7,169      |
| PS      | 1,005   | 1,525     | 0       | 5,482      |
| PG      | 3,727   | 758       | 0       | 5,448      |
| PE      | 6,652   | 104       | 395     | 891        |
| PC      | 4,574   | 45        | 0       | 3,335      |

PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphophatidylethanolamine; PG, phosphatidylglycerol; PIP₂, phosphatidyl-4,5-bisphosphate; PPA, pyrophosphatidic acid; PS, phosphatidylserine.

Table 2 | Binding free energy of accepted poses. |
|---------|-------------------------------|
|         | Apo-docking                   | PIP₂-bound docking |
|         | Primary | Secondary | Primary | Secondary | ΔΔG (PIP₂-Apo) | P-value |
| PIP₂    | 7.133±1.301  | –4.266±0.588 | –2.922±0.883 | 1.344±1.061  | <0.0001 |
| PPA     | –7.702±1.042 | –5.467±0.090 | –4.516±0.564 | 0.950±0.571  | <0.0001 |
| PA      | –4.381±0.665 | –3.517±0.278 | –2.863±0.432 | 0.654±0.514  | <0.0001 |
| PS      | –1.861±0.736 | –2.099±0.607 | –2.886±0.519 | –0.787±0.799 | <0.0001 |
| PG      | –3.370±0.939 | –2.572±0.757 | –2.064±0.802 | 0.507±1.103  | <0.0001 |
| PE      | –3.054±0.662 | –2.302±0.386 | –2.405±0.503 | –2.262±0.330 | 0.040±0.508 | 0.251  |
| PC      | –3.063±0.508 | –2.281±0.159 | –2.131±0.326 | 0.150±0.363  | 0.002   |

PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphophatidylethanolamine; PG, phosphatidylglycerol; PIP₂, phosphatidyl-4,5-bisphosphate; PPA, pyrophosphatidic acid; PS, phosphatidylserine.

**Figure 3 | Residue contributions to the secondary site.** (a) Hydrogen bond frequency between all accepted poses and surrounding residues at the secondary site are shown for Apo- (left) and PIP₂-bound-docking (right). All residues having H-bonding frequency >0.1 with any one of the tested lipids are shown. (b) Spatial location of the residues shown in a are depicted. The two residues, K64 and K219, of the highest contact frequency, are shown in red and others in orange. (c) Example structures of different lipids at the secondary site. K64, K219 and individual lipids (PIP₂, PPA, PS and PA) are shown in stick and in atom colours.
inward rectifier currents slowly developed through K219C and especially K64C channels following patch excision, until current amplitudes were comparable to those measured from CLS_Cont patches (Fig. 4b,c). This remarkable behaviour demonstrates that channels are indeed present in the cell membranes but substantially closed when exposed to the intact intracellular milieu. The normal cytoplasmic environment is strongly reducing29, but membrane excision exposes the cytoplasmic face of the membrane to an oxidizing environment. Given the proximity of the two residues within the protein, K64C/K219C double mutants are likely to be disulphide bonded to each other (see Supplementary Fig. S4), but single mutant K64C and K219C will probably be reduced to free cysteines in the intact cell. They will be free to oxidize to surrounding proteins or lipids following patch excision, and we suggest that this oxidation then effectively tethers these residues to the membrane, thereby providing similar conformational energy to that which is normally provided by PL(C0) interaction, to promote channel opening. This hypothesis is strongly supported by experiments below on recombinant channels in which the idea of ‘membrane tethering’ is directly assessed.

**Figure 4 | Mutation of secondary site lysines inhibits channel activity in cell membranes.** (a) Cumulative 86Rb+ efflux from Cosm6 cells versus time for WT (grey) and mutant Kir2.1 channels (CLS_Cont: red, K64C: blue, K219C: green, K64/219C: yellow). Nonspecific background flux was determined from GFP-transfected cells (white). Error bars are smaller than the symbols. Lines are the best fits of the single exponential equation described in the Methods, from which a rate constant (k in min\(^{-1}\)) for each protein is obtained and shown in the figure. (b) Channel activity determined electrophysiologically on-cell (left), 5 min after excision (middle) and then in the presence of 10 μM spermine (right). (c) Current increase on patch excision (off-cell; \(I_{\text{off}}\)) from cell-attached (on-cell; \(I_{\text{on}}\)) configuration. The fold-increase in current is shown for individual patches (white square), and mean and s.e. for each construct (n = 6).
Mutant channel activity is reduced in synthetic membranes. Channel activities were also assessed from recombinant proteins incorporated into liposomes of defined lipid composition and in which nonspecific influence from cellular components is excluded. Control and mutant proteins were expressed in Saccharomyces cerevisiae, and then purified and reconstituted into liposomes with increasing POPG concentrations in the presence of constant 0.1% PIP\(_2\) (Fig. 5a, left). \(^{86}\text{Rb}^+\) influx into liposomes was measured as a function of time to assess channel activity. CLS\_Cont protein showed essentially identical PL(−)−dependent activation (Fig. 5a, left) to that of WT protein\(^{22}\); activity reached maximum at ~10% POPG and there was no significant further activation at 20% POPG. Similar PL(−)−dependent activation was still observed but maximal channel activity was reduced slightly for the K219C mutant, markedly so for K64C, and there was no detectable activity from the K64C/K219C double mutant (Fig. 5a, left).

Membrane tethering at secondary site restores channel activity. Kir2 subfamily channels specifically require PI(4,5)P\(_2\) for activation although all six other PIP species can interact with the PIP\(_2\) site\(^{22,26,30,31}\). On the other hand, multiple different anionic phospholipids can satisfy the synergistic PL(−) effect with similar concentration dependence\(^{22}\). This suggests that the molecular mechanism exerted by PL(−) binding might be through a simple conformational change. We hypothesize this to be a generic pulling of the Kir domain towards the membrane. To directly test this idea, purified cysteine mutant and control proteins were modified in the detergent micelle state by decyl-MTS. This places a very hydrophobic 10-carbon acyl chain on modified cysteines, which will strongly partition into the membrane phase if appropriately located, as with modification of K64C and K219C. PL(−)−dependent activation of modified proteins was then again assessed using the \(^{86}\text{Rb}^+\) influx assay. Decyl modification did not affect CLS\_Cont activation (Fig. 5a, right), indicating that the expected disulphide bond (cystine) generated by the remaining two cysteines on the extracellular site of the protein remains as a disulphide bond. In contrast, decyl modification substantially increased the activity of single mutant K64C channels (Fig. 5a, right) comparable to the activity of maximally active CLS\_Cont channels (that is, in 0.1% PIP\(_2\) plus 10% PG). Even more strikingly, marked channel activity was now present at zero POPG, R64C + decyl becoming almost PL(−) independent (Fig. 5a, right). Decyl modification of K219C decreased maximum channel activity but had no significant effect on PL(−) dependence (Fig. 5a, right).

The double mutant (K64/K219C) was inactive before modification, but there was some low level of activity of decyl-MTS-

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**Figure 5** Channel activation by decyl modification of secondary site residues. (a) Anionic lipid (PL(−)−)−dependent activation of CLS\_Cont, K64C, K219C and K64/219C was determined without (left, blue colours) or with (right, red colours) modification by decyl-MTS by measurement of \(^{86}\text{Rb}^+\) uptake into liposomes. PIP\(_2\) (0.1%) was present in all cases, and POPG varied as indicated. \(^{86}\text{Rb}^+\) uptake was normalized to the valinomycin count of the same sample. (b) Maximum channel activities with or without modification are plotted. n = 6 (unmodified), 6 (modified) for CLS\_Cont, n = 6, 5 for K64C, n = 6, 5 for K219C and n = 6, 5 for K64/K219C. Error bars, s.e.m. *, P < 0.05 and ***, P < 0.001 compared to unmodified protein (two-way analysis of variance).

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modified channels (Fig. 5a, right). We suggest that inactivity of the unmodified channels may be a result of disulphide bond formation between the two introduced cysteines. The low level of activity following decyl-MTS treatment was probably attributable to subsequent modification of a low level of reduced cysteines in the presence of dithiothreitol (<50 μM). Disulphide bond formation between the two introduced cysteines is supported by the presence of dithiothreitol-sensitive dimers of the double-mutant protein, but not CLS_Cont protein, in western blots (Supplementary Fig. S4).

Membrane tethering at secondary site enhances PIP2 sensitivity. In the absence of POPG or other PL(−), WT Kir2.1 channels are very weakly activated by supra-physiological levels of PIP₂, whereas 25% PL(−) left-shifts PIP₂ sensitivity of human Kir2.1 channels by ~100-fold, making the channel fully active at only ~0.1% PIP₂ (ref. 22). If membrane tethering by decyl modification of K64C is functionally equivalent to the PL(−) interaction, then it should also cause left-shift in PIP₂ sensitivity of the modified channel in the absence of PL(−). However, in striking agreement with the above prediction, and K219C was unaffected by decyl-MTS treatment (Fig. 6a–c, right). However, in striking agreement with the above prediction, the POPG-dependent shift was completely absent in decyl-modified K64C (Fig. 6b, right), with the channels exhibiting identical high PIP₂ sensitivity in 0 or 10% POPG. This dramatic result not only suggests that K64 is the key residue for POPG interaction but also indicates that decyl modification of this residue can replace POPG interaction with this site to provide the membrane tethering that energetically sensitizes the channel to PIP₂ activation.

Discussion
It is becoming increasingly clear that the lipid components of the membrane are major regulators of the activity of ion channels. Interaction with PIP₂ or related PIPs is essential for activation of many channels, including all Kir subfamily members, and recent crystal structures have demonstrated a discrete binding site in Kir2.2 channels that underlies this effect, and provides significant insight to the molecular mechanism. More generally mechanistic details of channel-lipid interactions are far from clear. The realization of obligate synergistic PL(−) regulation of PIP₂-mediated Kir2.1 and Kir2.2 channel activation indicates a previously unrecognized complexity, requiring the simultaneous binding of both lipids, a mechanistic scenario that is currently without explanation. Moreover, the relatively low affinity of PL(−) for this action suggests that, although the interaction is key for physiological channel activity, crystallographic resolution of the interaction may not be forthcoming. To gain further insight to the molecular basis of the PL(−) action, we therefore turned to the powerful approaches of molecular simulation, followed by experimental analysis using biochemical manipulation of channel structure. Using these approaches, we have successfully defined a discrete binding site for PL(−) that is both necessary and sufficient for PL(−) action, and a mechanism by which the interaction leads to PIP₂ sensitivity at physiological PIP₂ levels (Fig. 7a).

Our docking simulations identify two sites to which anionic lipids could bind (Fig. 2). The first site (primary) at which the majority of docking events occurs corresponds to the PIP₂ site identified in X-ray crystal structures. This agreement supports our Kir2.1 homology model and the parameters for ligand and docking simulations. When both this and an additional distinct PL(−)-binding (secondary) site are available, almost all lipids dock preferentially to the primary site (Fig. 2d), but if the primary site is occupied by PIP₂, the secondary site becomes the most frequently docked site (Fig. 2e). Conversely, if the secondary site is pre-occupied by PIP₂, 100% of PIP₂ docking is to the primary site, indicating that the secondary site is a spatially separate site to which anionic lipids can bind, such that even two PIP₂ molecules can bind to the two sites simultaneously (Fig. 2e).

Two important experimental features of the synergistic PL(−) effect further support this argument. First, the calculated binding free energies at the secondary site vary little between test lipids (maximum difference: 3.37 kcal mol⁻¹, mean ± s.d.: −3.21 ± 1.24 kcal mol⁻¹), much less than the variability of binding free energies at the primary site (maximum difference: 5.84 kcal mol⁻¹, mean ± s.d.: −4.37 ± 2.22 kcal mol⁻¹; Table 2), consistent with the experimental finding that the secondary PL(−) effect is nonspecific and that the efficacy of different lipids are similar. Second, high-affinity PIP₂ sensitivity is specific, no other phospholipids can substitute. In Apo-docking experiments the binding affinity of PIP₂ and of the competitive antagonist PPA were both much higher at the primary site than that of other anionic phospholipids (Fig. 2d). Interestingly, the pre-occupancy of PIP₂ at the primary site actually increases the efficacy of PL(−) binding at the secondary site. PIP₂ at the primary site may create a negative electric potential that prevents the binding of
negative lipid to other locations of weaker binding free energy, thereby restricting binding to the secondary site, that is, the next available high-affinity site. It also seems likely that the strong negative charges of PIP₂ and PPA are most sensitive to the negative electric potential generated by PIP₂ at the primary site; the binding free energy of most lipids at the secondary site was diminished but the reduction was greatest for PIP₂ and PPA, which brought the binding free energies of all tested lipids at the secondary site to within 3.37 kcal mol⁻¹ of each other (Fig. 2e, inset). This may explain why PIP₂ is not more efficient than other PL(−) at this action; 10% PIP₂ is required to activate channels as much in zero POPG⁵, as 10% POPG activates channels in 0.1% PIP₂ (ref. 22) (Fig. 6a). Similar binding energies for both lipids at the secondary site provide a simple interpretation of their nonspecific and similar efficacy.

The secondary PL(−)-binding site overlaps with likely sites for allosteric regulation by Na⁺ (refs 24,33), cholesterol³⁴,³⁵ and Gβγ³⁷ that have been identified in other Kir channels. Na⁺ increases the PIP₂ sensitivity of Kir3.2 and Kir3.4 channels, binding in an analogous pocket generated by carbonyl oxygen atoms of the βC-βD loop and the D226 side chain (see Fig. 7b). The side chain of L222 in Kir2.1 (shown in green in Fig. 7b), located at the βC-βD loop, points towards the interior of the large β sheet of the Kir domain and is spatially close to where Na⁺ binds in Kir3.2. Although cholesterol inhibits WT Kir2.1 channels³⁴,³⁶, mutation of this particular residue (L222I) abolishes cholesterol sensitivity³⁵, and mutation of the equivalent residue (I229) of Kir3.4 to leucine results in constitutively active channels in the absence of Gβγ (ref. 8). Other residues in the βC-βD loop are recognized to be involved in ligand regulation of other subfamily members. The same region (cyan Fig. 7b) is implicated in a common binding site for alcohol⁶, as well as Gβγ (ref. 37) and Gα₅(β) (ref. 38) for allosteric regulation of Kir3 channels. Convergence of allosteric sites is very consistent with the idea that they all regulate sensitization to PIP₂ activation.

The proposed secondary site was experimentally tested through single or double mutation of the two lysine residues. In intact cells, both single and double mutations of K64 and K219 significantly decreased channel activity, although channels are evidently in the membrane and can spontaneously activate following patch excision (Fig. 4). In purified proteins, the effect of the individual mutations is predominantly to reduce lipid sensitivity, in a manner consistent with loss of PL(−) binding and loss of allosteric stabilization of the PIP₂ interaction. Modification with decyl-MTS, which covalently attaches a 10-carbon acyl chain to the cysteine sulfhydryl group, dramatically enhances channel activity of the K64C mutant and effectively maximally enhances PIP₂ sensitivity even in the complete absence of PL(−), thus indicating that decyl modification of K64C is functionally equivalent to PL(−) interaction (Fig. 5). These equivalent effects of decyl-MTS modification and PL(−) interaction at the secondary site on PIP₂ sensitivity support the hypothesis that PL(−) interactions nonspecifically act to tether

Figure 7 | Proposed model for secondary PL(−) regulation. (a) Hypothetical model: (i) in the absence of PL(−)s in the membrane, or when PL(−) interaction is lost by neutralization of the two positive charges at the secondary site, the cytoplasmic Kir domain is displaced from the membrane surface. (ii) The presence of PL(−)s in the membrane—or decyl modification of one of these—residues pulls the Kir domain closer to the membrane, which in turn induces conformational change in the primary site to generate high-affinity PIP₂ interaction. (iii) PIP₂ can then fully activate channels at low concentrations (0.05%). (b) The surface of Kir2.1 is shown with the same colour codes in Fig. 2a. Residues (H53, R78, R80, K182, K185, K187 and K188) forming the primary site are shown in blue, and the secondary site (K64 and K219) in red. Residues implicated in interaction with other Kir channel modulators are also shown: L222 for cholesterol and Gβγ regulation are shown in green; the residues (F47, H42, L232, L245, P244, Y242, Y337 and L330) for ethanol and Gβγ are shown in cyan; N216 for Na⁺ binding is shown in orange. (c) Structural models of Kir2.1 based on Kir2.2 bound to PIP₂ (3SPI, orange) and Kir3.2 additionally bound to Gβγ (4KFM, blue) to indicate potential motions associated with PL(−) interaction.
the cytoplasmic Kir domain, specifically the N-terminal slide helix, to the membrane and that it is this conformational effect that serves to increase PIP$_2$ affinity and stabilize the open state, as other allosteric regulators do$^{39,40}$. Direct support for this idea comes from Kir2.2 crystal structures generated either in the absence$^4$ or presence$^2$ of anionic lipids (PIP$_2$ and PPA). These show marked differences in the location of the Kir domain relative to the potential membrane surface. In PIP$_2$-bound structures, the C-linker adopts an α-helical conformation and the Kir domain is pulled towards the potential membrane surface by −6 Å, relative to the apo structure$^2$. Comparison of recent Kir2.2 and Kir3.2 channel structures shows that the additional binding of the allosteric regulator Gβγ to Kir3.2 further pulls the slide helix towards the membrane (4RFM)$^{14}$, relative to the PIP$_2$-bound Kir2.2 structure (3SP)$^{25}$. Figure 7C shows Kir2.1 homology models based on these two structures to illustrate potential motions coupled to PL(−) interaction. Rotation of the cytoplasmic domain with respect to the membrane as a result of Gβγ binding is suggested to be part of the gating mechanism$^{44,45}$ and may well occur with PL(−) binding to Kir2 channels, although our experiments cannot address this possibility.

Pulling of the Kir domain of bacterial Kir channels (KirBac1.1) through membrane tethering also affects channel activity$^{46}$. In this case, membrane tethering by decyl-MS2 modification of introduced cysteines in the N-terminus or C-terminus ends of the slide helix resulted in opposite effects on KirBac1.1 activity, with tethering of the N terminus (R49C, that is, away from the pore axis) augmenting the channel activity, and tethering of the C terminus (L56C, close to the pore axis) reducing channel activity$^{46}$. It is noteworthy that R49 of KirBac1.1 is structurally quite close to K64 of human Kir2.1, although these residues do not align exactly (Supplementary Fig. S5). Moreover, recent analysis of sequence elements affecting PL(−) activation of KcsA indicates residues in exactly the same N-terminal slide helix region$^4$. These results suggest that cytoplasmic domain tethering to the membrane through PL(−) binding at the secondary site may be a broadly conserved gating mechanism shared between pro- and eukaryotic $K^+$ channels.

The K64 and K219 residues are loosely conserved among the seven eukaryotic Kir channel subfamilies (Supplementary Fig. S5). Kir2 and Kir4 channels contain both lysines, Kir1 and Kir6, and Kir7 subfamily members have one or other of the two basic residues and Kir3 and Kir5 channels contain neither. Interestingly, physiologically, Kir5 subunits only appear to form functional channels as heterotetramers with Kir4 subunits$^{48}$ and PL(−) regulation might then be conferred on the channels by the Kir4 subunits. As discussed above, Kir3 channels exhibit allosteric regulation of PIP$_2$ activation by other modulators, including Gβγ and Na$^+$. It seems possible that these other modulators may convey the same effect on GIRQ channels that PL(−) has on Kir2 channels, consistent with the cytoplasmic domain, and in particular the N-terminal slide helix, being pulled towards the membrane in the Gβγ-bound crystal structure$^{44}$ (c.f. Fig. 7c).

### Methods

**Human Kir2.1 protein purification.** WT and mutant Kir2.1 proteins, tagged by Flag-His$^8$ peptide at the C terminus, were expressed in and purified from the FGY217 strain of *S. cerevisiae*.$^{49}$ Expression levels and purification yields were similar for WT and mutant proteins. Mutagenesis was performed using QuickChange II site-directed mutagenesis kits (Stratagene Cloning Systems, CA) and verified by sequencing. Cysteine-less mutant Kir2.1 (C154, Cont) was generated by introduction of a series of mutations: C435S, C545V, C676V, C891, C1011L, C149F, C169V, C209S, C311A, C356S and C375S. The two remaining cysteines (C122 and C154) are known to form a very stable intrasubunit disulphide bond$^{50}$ and were not substituted to maintain functional channels.

$^{86}$Rb$^+$ **uptake assay.** Channel activity was assessed by $^{86}$Rb$^+$ uptake into proteoliposomes$^{52}$. POPE and POPG lipids were dissolved at 10 mg ml$^{-1}$ concentration in buffer A (450 mM KCl, 10 mM HEPES, 4 mM MgCl$_2$, pH 7.4) with 35 mM CHAPS. PIP$_2$ was solubilized at 1 mg ml$^{-1}$ concentration in 10 mg ml$^{-1}$ POPE solution. One milligram of lipid mixture in 100 μl was incubated at room temperature for 2 h, and 5 μg of protein was added and incubated for another 20 min. A lipid-protein mixture was run through partly dehydrated G-50 beads secured by a buffer with 1 mg ml$^{-1}$ POPE. The putative 600 ng of the corresponding Kir2.1 plasmid using Fugene6 and used for electro-

### Electrophysiology.** COSm6 cells between passages 10 and 30 were grown on glass cover slips in 40-mm six-well plates and transiently transfected after reaching 30–50% confluency. Each well of cells was transfected with 200 ng of GFP and 800 ng of the corresponding Kir2.1 plasmid using Fugene6 and used for electrophysiological experiments 24–48 h thereafter. Current responses were recorded with electrodes filled with plain glass capillaries with a resistance of 1–4 MΩ and then washed twice with Ringger’s solution composed of (in mM) 118 NaCl, 2.5 CaCl$_2$, 1.2 KH$_2$PO$_4$, 4.7 KCl, 25 NaHCO$_3$, 1.2 MgSO$_4$ and 10 HEPES (pH 7.4). Ringer’s solution was repeatedly added and removed to collect released $^{86}$Rb$^+$ from cells at 2.5, 5.0, 7.5, 15, 25 and 40 min time points (t). Finally, cells were lysed by 2% SDS solution to collect the remaining $^{86}$Rb$^+$ within the cells. Radial efflux (J) relative to total $^{86}$Rb$^+$ counts was computed to determine macroscopic channel activity. The channel-specific rate constant (k$_s$) of transfected channel proteins was determined by fitting the data using MATLAB (Mathworks, Natick, MA) with the following equation, as described before$^{31}$:

$$J = 1 - e^{-(kt})$$

where rate constant k$_s$, obtained from cells transfected with GFP alone, reflects endogenous background channel activity, and t is time.

**Homology modelling of human Kir2.1 channels.** Homology models of human Kir2.1 were built based on the chicken Kir2.2 (PDB 3SP1) crystal structure$^{23}$. Human Kir2.1 is 76% and ~90% conserved relative to chicken Kir2.2. Sequence alignment was performed using the ClustalW webserver$^{2}$ and is shown in Supplementary Fig. S5. One hundred homology models were generated through random seeding using the MODELLER 9.10 program$^{2}$. Because of high sequence homology, modelled structures had identical backbone structures but with various rotamer orientations. PDBQT structure files, containing charge and atom type information, were generated from the PDB file of every homology model beginning with protonation, assigning Gasteiger charge$^{4}$, merging of nonpolar hydrogen to the bonded carbon atom, and assigning atom type using Autodock Tool (ADT) 1.5 (ref. 53), and then the ion channel was aligned to the bacterial crystal structure using MacPyMOL programs (Schrödinger, New York, NY) to ensure that the docking search area was consistent among models.
Docking simulations. The head groups are critical determinants for lipid interactions with the Kir protein, and to facilitate simulations, especially only the head group was modelled, including up to the first carbon atom of the glycolipid moiety. Atom charges for PI(4,5)P2 were obtained as published26 and the charges for other anionic lipids were taken from the CHARMM force field27 shown in Supplementary Fig. S1. The atom charge of pyrophosphatic acid (PPA) was kindly provided by Wosqip Im (University of Kansas). ADT 1.5.4 was used to assign the atom type and torsion tree of each ligand.

The searched region fully covered the slide helix, the lower end of the TM1 helix, the N-terminal β-strand of subunit B (yellow) and the adjacent part of neighbouring subunits A (green) and C (silver), which was necessary for identification of potential interactions at the subunit interface as shown in Fig. 2b. The parameters for the grid map generation and docking simulations were the same as used previously28 using AutoDock-4.2.3 (ref. 58). Each docking simulation was run for ~8 h on a single processor of the local IBM x3650-m2. For each template, 10,000 poses were generated for each ligand. Poses were accepted based on their orientation and relative location with respect to the potential membrane. First, a pose was accepted if the angle between the molecular vector that is directed for lipid (Supplementary Fig. S1) and the bilayer normal was less than 90°, that is, as will occur when the head group is either pointing away from the membrane or less than parallel to the membrane surface. Structural analysis was performed using in-house codes for MATLAB. The potential membrane surface was approximated by the centre of the slide helix based on studies of the binding of melittin (an amphipathic α-helix) to membranes59,60. If the primary phosphate that is directly connected to the lipid tail was located deeper than 4.5 Å into the membrane, or more than 8 Å from the surface of the membrane (defined by the centre of the slide helix), the pose was rejected. To elucidate representative binding sites, accepted poses were clustered into subgroups. A K-mean clustering algorithm27 was adopted using the root mean squared deviation of the phosphorus atom position as a metric. The number of clusters for each ligand was determined to minimize the performance index (∏j oi ti ni(P expl(P))−Pi j o ti ni(P obs(P))2)61. The smaller the value, the greater the inter-subgroup variance and the smaller the intra-subgroup variance, which indicates better clustering performance (see Supplementary Fig. S2 and text). To determine the residues involved in ligand binding, hydrogen bonds were postulated based on the following criteria62: first, the distance between the donor and acceptor atoms being shorter than or equal to 3.4 Å and second, the angular orientation being smaller than or equal to 30° between the two unit vectors. One joins hydrogen bond donor atom and hydrogen atom and the other joins hydrogen bond donor and acceptor atoms. Contact frequency was determined by counting the number of observed hydrogen bonds and dividing this by the total number of poses in each cluster.

Statistical analysis. Statistical significance was analysed using an unpaired t-test and either one-way or two-way analysis of variance as appropriate. Statistical significance of P<0.05, P<0.01 and P<0.001 is indicated by single, double and triple asterisks, respectively.

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