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Letter

Entropy in the Molecular Recognition of Membrane Protein−Lipid Interactions

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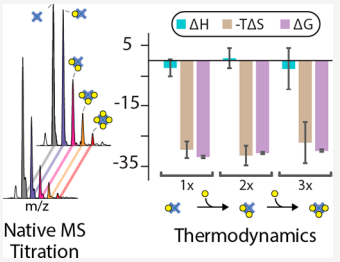
ABSTRACT: Understanding the molecular driving forces that underlie membrane protein−lipid interactions requires the characterization of their binding thermodynamics. Here, we employ variable-temperature native mass spectrometry to determine the thermodynamics of lipid binding events to the human G-protein-gated inward rectifier potassium channel, Kir3.2. The channel displays distinct thermodynamic strategies to engage phosphatidylinositol (PI) and phosphorylated forms thereof. The addition of a 4′-phosphate to PI results in an increase in favorable entropy. PI with two or more phosphates exhibits more complex binding, where lipids appear to bind two nonidentical sites on Kir3.2. Remarkably, the interaction of 4,5-bisphosphate PI with Kir3.2 is solely driven by a large, favorable change in entropy. Installment of a 3′-phosphate to $PI(4,5)P_2$ results in an altered thermodynamic strategy. The acyl chain of the lipid has a marked impact on binding thermodynamics and, in some cases, enthalpy becomes favorable.



Inward rectifier potassium (Kir) channels are expressed in tissues throughout the body where they play central roles in many physiological processes, such as parasympathetic slowing of the heart,[1] pain perception,[2] and pancreatic insulin secretion.[3−5] Some mutations in these channels result in improper trafficking associated with Andersen syndrome.[6] In particular, phosphatidylinositol 4,5-bisphosphate ($PI(4,5)P_2$), a minor component of the cytoplasmic leaflet,[7] is required for activation of all Kir channels.[8−10] Kir channels are also regulated by many other molecules including phosphorylation by kinases, sodium, pore blockers (polyamine, Mg^{2+}), and ethanol.[6,11] There are seven subfamilies of Kir channels: classical Kir channels (Kir2.x) are strong rectifiers that have central roles in cardiac inward rectifier current; Kir3.x channels are unique in that they require $G_{\beta\gamma}$ in addition to PIP_2 for function with some of these channels modulated by sodium ions; ATP-sensitive potassium channels (Kir6.x); and transport potassium channels (Kir1.x, Kir4.x, Kir5.x, and Kir7.x).[5,6,12]

All Kir channels form tetrameric complexes composed of similar or different subunits.[10,13,14] Each subunit encodes two transmembrane domains (TMD), in which the K^+ selectivity filter resides, that is tethered by a short linker to the cytoplasmic domain (CTD).[13,14] Structures have also revealed a $PI(4,5)P_2$ binding pocket located at the interface of the TMD and CTD.[10,14] The binding pocket is a highly conserved region consisting of a set positively charged amino acid residues that engage the phosphorylated headgroup of $PI(4,5)P_2$.[10,14,15] Mutations in residues important for binding the signaling lipid are associated with Bartter and Andersen syndromes, and other diseases.[4,16] The specific lipid binding site within Kir channels has garnered the attention of computational studies to identify and predict lipid-binding sites on ion channels.[17,18]

Kir channels have varied specificity and degrees of channel activation by the different phosphoinositides. All Kir channels are maximally activated by $PI(4,5)P_2$ and, in some cases, $PI(4,5)P_2$ is the only phosphoinositide (PIP) that stimulates activity.[9,19] In contrast, Kir6.x appears to be promiscuously activated by $PI(3,4)P_2$, $PI(4,5)P_2$ and $PI(3,4,5)P_3$.[19] The acyl chain composition has also been shown to be a contributing factor to the level of stimulation, such as Kir3.1/Kir3.4 channels display a preference for $PI(4,5)P_2$ with 18:0−20:4 (SA) acyl chain whereas Kir2.1 shows no preference toward acyl chains.[20] The concentration dependence of $PI(4,5)P_2$ activation of Kir3.2 in a lipid bilayer exhibits positive cooperativity.[21] Similar observations were made by employing a soluble fluorescent lipid binding assay wherein the binding of a fluorophore modified $PI(4,5)P_2$ to Kir3.2 fused to a fluorescent protein is monitored by Förster resonance energy transfer.[22,23]

Historically it has been difficult to dissect and interrogate individual lipid binding events to membrane proteins, which is necessary to fully characterize binding thermodynamics. Recently, native mass spectrometry (MS) has emerged as an indispensable biophysical technique for characterizing membrane proteins and their interactions with lipids and other molecules, such as regulatory proteins.[24] In contrast to other

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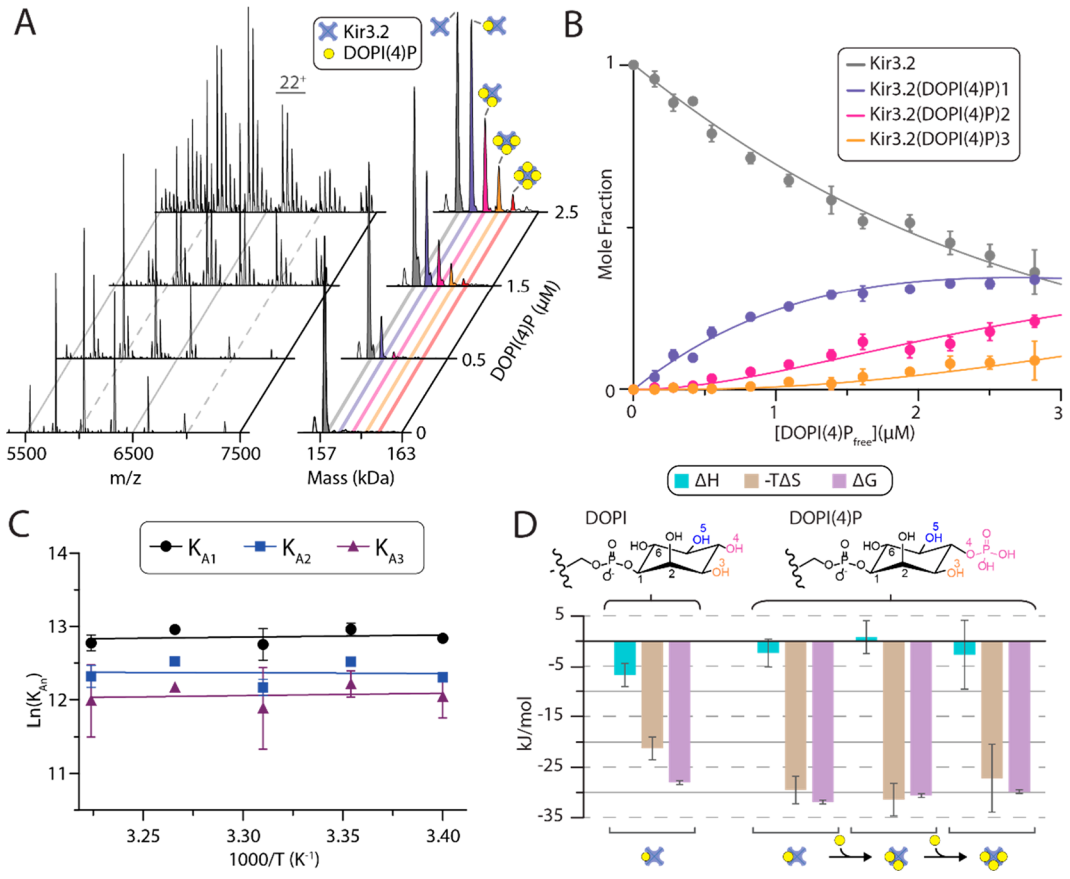

Figure 1. Determination of binding thermodynamics for Kir3.2-lipid interactions. (A) Representative native mass spectra and their deconvolution from a titration series of Kir3.2 with dioleoyl (18:1−18:1) phosphatidylinositol 4-phosphate (DOPI(4)P) recorded at 298 K. Kir3.2 and the various lipid bound states of Kir3.2 are labeled. (B) Plot of the mole fraction for Kir3.2 and Kir3.2·DOPI(4)P_{1-3} bound states of Kir3.2 determined from a titration series and resulting fit (R^2 = 0.99) of a sequential lipid binding model (lines). (C) van't Hoff plot for Kir3.2 binding Kir3.2·DOPI(4)P_{1-3} (dots) and regression of linear equations (solid lines) to deduce thermodynamics for each lipid binding event. (D) Thermodynamics of dioleoyl DOPI(4)P and phosphatidylinositol (DOPI) binding Kir3.2 at 298 K. The first, second, and third lipid (labeled as 1x−3x) is shown for DOPI(4)P. Shown above are the headgroup structures with the 3′, 4′, and 5′ positions of the inositol headgroup colored orange, pink, and blue, respectively. ΔG was calculated using K_A values determined at 298 K. Reported are the average and s.e.m. from repeated measurements (n = 3).

biophysical techniques that report on the ensemble, native MS can not only capture a snapshot of solution equilibria but also resolve individual ligand-bound states of membrane protein complexes.[25] Over the past decade, native MS has discovered the role of specific lipids in stabilizing membrane protein complexes,[25,26] allosteric modulation of membrane protein interactions with protein,[27,28] lipids,[29] and drugs,[30,31] and those important for function,[25,31,32] such as $PI(4,5)P_2$ in G-protein-coupled receptor activation and G-protein selectivity.[28] Lipid and toxin binding to Kir3.2 has been interrogated using native MS that has provided insight into the binding preferences for PIPs.[33] Native MS combined with mutational studies has shed light on the contribution of amino acids in the PIP binding site of Kir3.2 and how they impact binding preferences for PIPs.[22] Despite progress in understanding Kir-lipid interactions, the thermodynamics for the association of lipids with Kir3.2 have been enigmatic.

To determine the thermodynamic basis for Kir3.2-lipid interactions, we used native MS coupled with a variable-temperature nanoelectrospray ionization (nESI) apparatus, to determine through van't Hoff analysis, the change in enthalpy (ΔH) and entropy ($-T\Delta S$), components of the change in Gibbs free energy (ΔG).[34,35] The first set of lipids we investigated were dioleoyl (18:1−18:1) phosphatidylinositol (DOPI) and phosphatidylinositol 4-phosphate with DO tails (DOPI(4)P). Here, we focus our initial discussion on DOPI(4)P as up to three bound Kir3.2 whereas only one DOPI bound the channel (Figures S1 and S2). More specifically, Kir3.2 solubilized in $C_{10}E_5$, a detergent that in the nESI process results in charge reduced ions that aids preservation of noncovalent interactions and native-like structure in the mass spectrometer,[25,36] at a concentration of 0.25 μM was titrated with DOPI(4)P up to a final concentration of 3.0 μM. These samples were then incubated online using a variable temperature nESI apparatus[37] for several minutes to reach equilibrium followed by acquiring their native mass spectra (Figures 1A, S3, and S4). The mass spectra from this titration series at a given temperature were deconvoluted using UniDec[38] and the abundance of Kir3.2·DOPI(4)P_{0-3} was used to compute the mole fractions for the different lipid-bound states (Figure 1B). A sequential ligand binding model was fit to the mole fraction data to determine the equilibrium binding constants at a given temperature (Figure 1B). Thermodynamics for the individual DOPI(4)P binding events to Kir3.2 was deduced through van't Hoff analysis (Figure 1C).[34] Interestingly, the binding of one to three DOPI(4)P molecules to Kir3.2 is driven by entropy with the enthalpic term near zero (Figure 1D). This result is in

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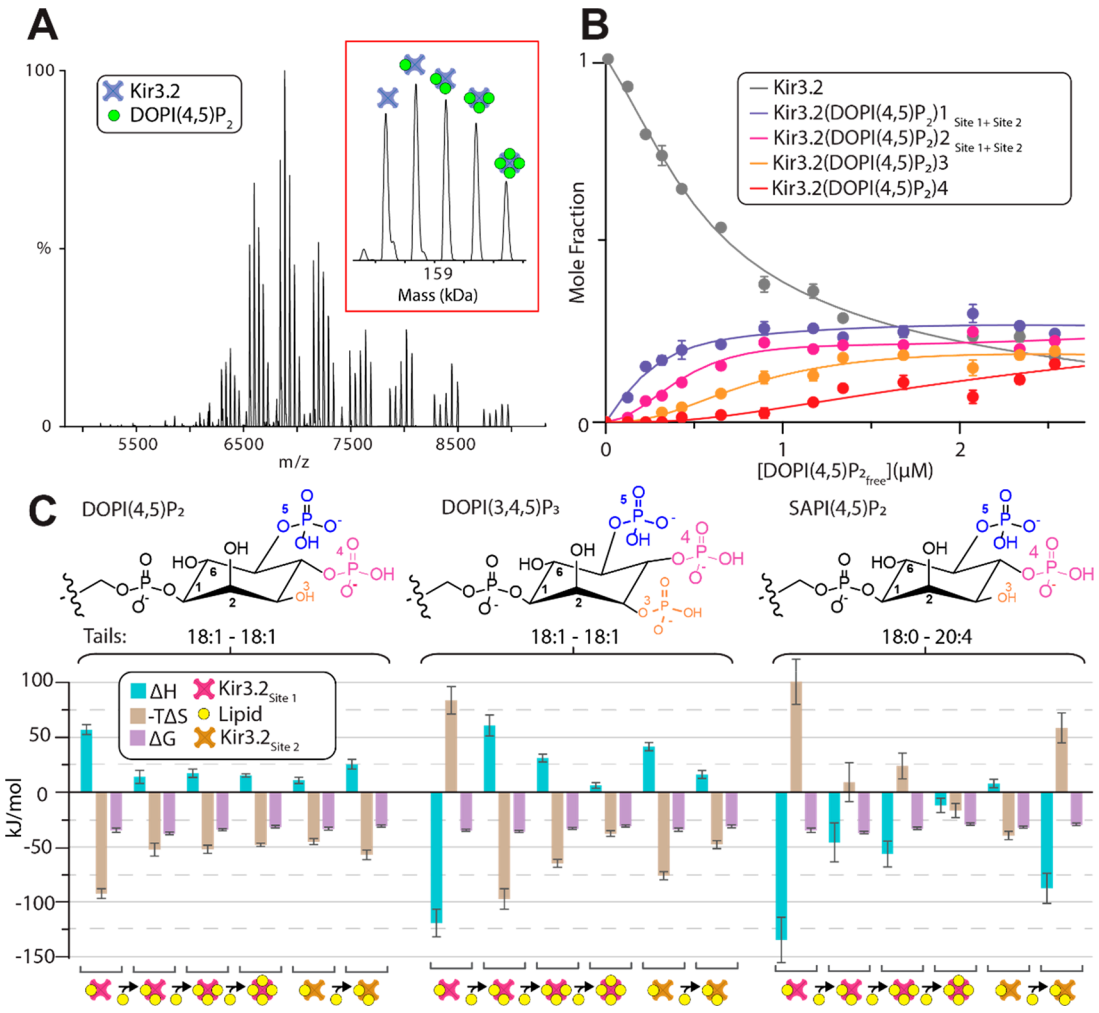

Figure 2. Thermodynamic signatures of specific phosphoinositides binding to different states of Kir3.2. (A) Representative native mass spectrum of the Kir3.2 (0.25 μM) mixed with 2.4 μM DOPI(4,5)P_2 in the $C_{10}E_5$ detergent. The inset is the deconvoluted mass spectrum. (B) Plot of the mole fraction of Kir3.2 and the channel bound to PI(4,5)P_2 with DO tails (DOPI(4,5)P_2). Resulting fit (solid lines, R^2 = 0.99) of lipid binding model where the lipid can bind to either site 1 or site 2 of Kir3.2. (C) Binding thermodynamics for DOPI(4,5)P_2, phosphatidylinositol 3,4,5-trisphosphate with DO tails (DOPI(3,4,5)P_3), and PI(4,5)P_2 with 1-stearoyl-2-arachidonoyl (18:0−20:4) tails (SAPI(4,5)P_2) to Kir3.2 determined through van't Hoff analysis for binding to site 1 and site 2 at 298 K. Reported are the average and s.e.m. from repeated measurements (n = 3).

complete contrast to our previous study of the bacterial ammonia channel (AmtB) binding phospholipids, where the binding was driven by enthalpy and, in most cases, entropy was unfavorable.[35] The thermodynamic parameters for each DOPI(4)P binding event are statistically indistinguishable. Thermodynamics for the binding of Kir3.2 to one DOPI (up to 5 μM) was determined in a similar fashion as done for DOPI(4)P. The binding of this lipid is also largely driven by entropy with a marginal favorable contribution from entropy (Figures S11 and S14).

To better understand the molecular forces that underlie their molecular recognition, we next focused on other phosphorylated forms of phosphatidylinositol known to activate Kir3.2 and other channels. These lipids include 4,5-bisphosphate PI with DO (DOPI(4,5)P_2, up to 3.0 μM) and 1-stearoyl-2-arachidonoyl (SAPI(4,5)P_2, up to 3.5 μM) tails and 3,4,5-trisphosphate PI with DO (DOPI(3,4,5)P_3, up to 5.0 μM). In a similar fashion as described above, we first titrated Kir3.2 with DOPI(4,5)P_2 and recorded their native mass spectra at different temperatures (Figures S5 and S6). Regardless of the temperature, the application of a sequential lipid binding model resulted in poor fits, especially for the first and second lipid bound states of Kir3.2 (Figure S12). Specifically, the trend in the mole fraction data for the first and second binding event does not follow a smooth curve but is asymptotic at higher lipid concentrations suggestive of two underlying binding distributions. This result along with the fact more than four PIP binding events to the channel are observed at higher concentrations of lipid (Figure S15) indicates that the lipid can bind to different nonidentical sites. Modification of the lipid binding model such that the lipid can bind to two nonidentical sites with the one site (site 1) binds up to four whereas the second site (site 2) can bind up to two (see Materials and Methods) resulted in substantially improved fits (Figures 2B and S12).

Application of lipid binding model to two nonidentical states of Kir3.2 was used to determine the K_D for each lipid binding event at a given temperature, followed by van't Hoff analysis (Figures 2C and S14). The K_D values for one to four DOPI(4,5)P_2 binding events to site 1 of Kir3.2 displayed positive cooperativity where the K_d for binding the second was lower than that for binding the first (Table S2). Surprisingly,

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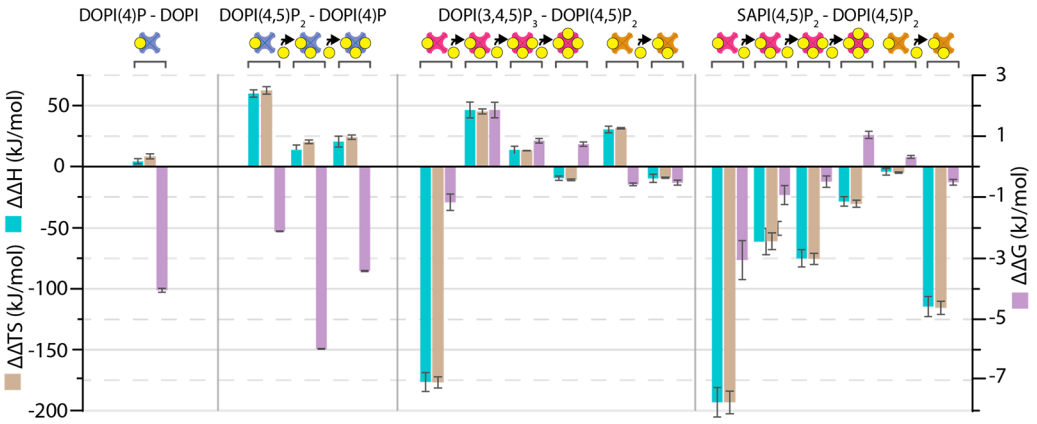

Figure 3. Alterations in thermodynamic signatures for the stepwise transition from DOPI to DOPI(3,4,5)P_3 and acyl chain chemistry of PI(4,5)P_2. Values were computed using a temperature of 298 K. Shown as described in Figure 2. Reported are the average and s.e.m. (n = 3).

the thermodynamics for DOPI(4,5)P_2 associating with Kir3.2 reveal that the molecular driving force is solely entropic, outweighing an enthalpic penalty, regardless of binding to either site (Figure 2C). In the case of binding to site, which we presume is binding to the specific PIP binding state based on structures,[14,15] unfavorable enthalpy was largest for the first binding event and for subsequent binding events the thermodynamic parameters were similar. In contrast, the enthalpy for the binding event to site 2 increased. The additional 3′-phosphate on DOPI(3,4,5)P_3 resulted in distinct thermodynamic signatures in comparison to DOPI(4,5)P_2. Excluding the first binding event to site 1 where it is driven by entropy and enthalpically unfavorable, all the other binding events showed enthalpy−entropy compensation whereby entropy and enthalpy were altered in opposing directions for each subsequent binding event (Figure 2C). Altering acyl chain chemistry of PI(4,5)P_2 to contain SA tails, resulting in a new subset of thermodynamic values with the majority of binding events driven by enthalpy. For binding to site 1, favorable enthalpy was greatest for binding the first lipid whereas the second and third were similar but larger compared to the fourth. The fourth SAPI(4,5)P_2 binding event is unique among the PIPs investigated in that both entropy and enthalpy are favorable. Remarkably, these results demonstrate that specific phosphorylated forms of PI selectively engage the different states of Kir3.2.

It is striking when comparing the impact of stepwise progression from DOPI to DOPI(3,4,5)P_3 and different acyl chains on binding thermodynamics (Figure 3). The addition of a 4-phosphate to DOPI pushes the reaction to be driven by entropy. Going from DOPI(4)P to DOPI(4,5)P_2 results in ∼60 kJ/mol contributing to both enthalpy and entropy but in opposing directions for the first lipid binding. This is consistent for the other binding events but to half the extent. DOPI(3,4,5)P_3 with three phosphates displays a strong enthalpy−entropy dependence. A remarkable 175 kJ/mol alteration in thermodynamic parameters but in the opposing direction is observed for the first binding event of DOPI-(3,4,5)P_3 to site 1. Compared to DOPI(4,5)P_2, the binding of two or more DOPI(3,4,5)P_3 to Kir3.2 are accompanied by compensatory gains in favorable entropy and unfavorable enthalpy spanning 50 kJ/mol. The replacement of DOPI-(4,5)P_2 with SA tails displayed marked gains in favorable enthalpy and unfavorable entropy, and the first SAPI(4,5)P_2 binding event had a remarkable change of nearly 200 kJ/mol. Moreover, the change in Gibbs free energy (ΔG) collected for DOPI(4,5)P_2 (∼−33 ± 2 kJ/mol) are close to those computed (∼−42 kJ/mol) by coarse-grained molecular dynamics free-energy perturbation (CG-FEP) calculations.[18] The difference in ΔG between DOPI(4)P and DOPI(4,5)P_2 is −6 kJ/mol, nearly identical with that computed by CF-FEP (−7 kJ/mol).[18] These results illustrate the marked impact on binding thermodynamics that can be observed with changing the chemistry of the lipid and similar values to those computed from CG-FEP.

The thermodynamics of Kir3.2-lipid interactions provide rich chemical insight into the molecular forces underlying specific Kir3.2-lipid interactions. The majority of phosphorylated forms of DOPI binding events are driven by entropy and, in most cases, there is an unfavorable change in enthalpy. It is important to note these lipids possess DO tails and therefore the entropic contribution from desolvation of the acyl chains plays a minor role in the large entropies observed here. While solvent reorganization of the phosphorylated headgroup or PIP binding pocket of Kir3.2 can contribute in a positive way to entropy,[39] the largely favorable entropy accompanied by unfavorable enthalpy observed here is reminiscent of soluble protein−ligand interactions that are driven by large conformational entropy originating in enhanced protein motions.[40,41] The first binding events of DOPI(4,5)P_2, DOPI(3,4,5)P_3, and SAPI(4,5)P_2 indicate significant structural changes upon binding. The thermodynamics for DOPI(4,5)P_2 suggests a significant enhancement in protein dynamics whereas for the two other lipids there is significant structuring of Kir3.2. We have previously observed enthalpy−entropy compensation for AmtB-lipid interactions.[35] However, the enthalpy−entropy compensation is more pronounced for Kir3.2-DOPI(3,4,5)P_3 in comparison to that observed for AmtB-lipid interactions and likely due to significant structuring of the channel at the cost of a reduction in disorder. The enthalpically driven binding of SAPI(4,5)P_2 to Kir3.2 suggests the SA tails interact more favorably with Kir3.2 in comparison to the lipid with DO tails. This result suggests that the binding site for PIPs is specific for SA where the tails actively engage the channel. It is also interesting to note that PI with SA tails is the most abundant form in mammals[42] and binding of this lipid in the membrane could be strongly driven by enthalpy.

Although first described for Kir2.2 (Figure S16),[10] recent structures of Kir3.2 have shown it also populates two distinct conformations where the CTD is docked, forming contacts

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with the cytoplasmic face of the TMD, or in an extended state, CTD displaced from the transmembrane domain.[43] For some of the PIPs, a lipid binding model where the lipid binds to two nonidentical sites on the channel resulted in substantially improved fits. It is plausible the two nonidentical sites arise from binding to the docked and extended conformations of Kir3.2 (Figure S13). Moreover, the K_D values for up to four PI(4,5)P2 and PI(3,4,5)P3 binding to site 1 display positive cooperativity, which we presume represents binding to the specific PIP binding site.[10,14] The origin of positive cooperativity could be described by the "induced-fit" model,[44] in which a lipid binds weakly to the protein subunit and conversion to a tightly bound form. Alternatively, the Monod−Wyman−Changeux and "conformational selection" models, where the lipid binds to pre-existing protein conformations and lipid binding promotes a population shift, redistributing the conformational states.[45] It is challenging from the lipid binding data here to determine if Kir3.2 follows the conformational selection or induced-fit binding pathway. However, recent structural studies show a population of Kir3.2 in docked and extended states (pre-existing populations) and in the presence of increasing concentrations of $PI(4,5)P_2$ a population shift to the docked state.[43]

In summary, the thermodynamics of lipids associating with Kir3.2 reveal different thermodynamic strategies. The changes observed for binding thermodynamics upon phosphorylation of the inositol headgroup or altering the acyl chains are remarkable. Moreover, the association of Kir3.2 with specific PIPs can be driven by a large change in favorable entropy. This entropy is likely the result of considerable solvent reorganization,as well as enhanced protein dynamics, which has been shown to underlie soluble protein−ligand interactions.[40] High conformational entropy has recently been observed for two monomeric membrane proteins, independent of the membrane mimetic.[46] Here, we provide evidence that entropy can indeed greatly influence membrane protein−lipid interactions, and the acyl chain chemistry can dramatically impact the thermodynamic strategy for binding Kir3.2.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.jpclett.1c03750.

Materials and methods, supporting figures,and tables (PDF)

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Author Contributions

P.Q. and A.L. designed research; P.Q., J.L., S.S., T.Z., and Y.Z. performed research; P.Q. and A.L. analyzed data; and P.Q. and A.L. wrote the paper with input from all authors.

Notes

All study data are included in the Letter and Supporting Information.

The authors declare no competing financial interest.

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