Supplementary Information

Title: PIP_2 stabilises active states of GPCRs and enhances the selectivity of G-protein coupling

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Note on the strategy of the protocol to confirm lipid identities

Our methodology is not designed to identify all lipids that co-purify with receptors under different detergent environments. Rather we first identify those lipid classes that bind preferentially by directly detecting protein-lipid interactions by native MS and identifying the bound lipids through tandem MS. In our experiments two lipid classes were identified (PS and PIP) bound to all three receptors. Lipids with low affinity are ejected during collisional activation, during the removal of the detergent micelle in the MS, leaving only lipids with high binding affinity. Subsequently we incubate receptors with known concentrations of the lipids identified (from the PS and PIP classes) and use titration and competition experiments to identify PIP_2, from the PIP class, as the preferred interactor. We then investigate the effects of PIP_2 on the receptor structure and function.

Note on the composition of different membranes

The receptors used here were isolated either from insect cells or *E. coli* membranes raising concerns about the differences in composition between these cell membranes and those in mammalian systems. Several lipidomics studies of insect cell membranes have addressed the similar abundance of common phospholipids identified in mammalian membranes such as PE, PC, PI and cardiolipin[54] (ISSN 0014-

5793). These lipids were also observed in our lipidomics analysis. Sphingolipids and sterols are also present in insect cell membranes although the types and structural features may differ from mammalian membranes. Cholesterol has been identified in several insect cell lines with a lower expression level than in mammalian cells (around 20-40% compared to mammalian cells)[55]. We did not observe any cholesterol bound to receptor or present in our lipidomics study. The impact of cholesterol on modulating GPCRs, especially their conformational stability, has been widely studied and cholesterol is often present in X-ray structures[56-58]. For example, the presence of cholesterol at the surface between TM1 to TM4 of β_2AR increases kinetic, energetic, and mechanical stability of receptor as well as potentially modulates receptor dimerization[56,57,59]. Furthermore, cholesterol increases the stability of NTSR1 in the detergent micelles but shows no effect when the receptor is reconstituted in lipid nanodiscs[60]. MD simulations indicate the highly dynamic nature of cholesterol binding hot-spots, suggesting that these sites have high occupancy rather than specific binding[61]. All these studies highlight the importance of the presence of cholesterol, however the detailed mechanism of its interaction remains inconclusive.

Note on the use CGMD Simulations of Protein-Lipid Interactions

The simulations use coarse-grained (CG) MD simulations with the Martini forcefield. This methodology is well established for the study of membrane proteins and their interactions with lipids[62,63]. The primary strength of the approach is long simulations are possible and so one can fully sample the interactions of membrane proteins with (slowly diffusing) lipids[64], which remains challenging for all atom simulations. Furthermore, the approach has been carefully validated for a number of membrane proteins whose lipid interactions are well understood experimentally (see below). Furthermore, recent studies from our group have suggested that atomistic force-fields may actually *overestimate* the strength of protein-lipid interactions and thus further work is need to refine these models[65]. The primary limitation of the CG method is one does not get full atomic resolution detail for the predicted interactions. However, when needed this can be obtained by a multiscale approach in which the CG structure is converted back to atomic resolution and an all atomic simulation then performed[66].

In terms of lipid specificity, we have previously employed two canonical membrane proteins, whose protein/lipid interactions have been characterised both structurally and functionally, namely: (i) the mitochondrial ATP/ADP transporter ANT1, which has three binding sites specific for cardiolipin seen in the crystal structure (CL); and (ii) inward rectifier potassium (Kir) channels the PIP_2 binding site of which has also been resolved at high resolution by X-ray crystallography. In both of these cases, we have demonstrated the ability of CGMD simulations to distinguish between specific and 'annular' lipid interactions, e.g. comparing the interactions of ANT with CL vs. PS or vs. PC [67] or of Kir channels with PIP_2 vs. PS[68].

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