Supplementary Information for
Design Principles of PI(4,5)P₂ Clustering Under Protein-Free Conditions: Specific Cation Effects and Calcium-Potassium Synergy

Kyungreem Han, Soon Ho Kim, Richard M. Venable, and Richard W. Pastor

Richard W. Pastor
Email: pastorr@nhlbi.nih.gov

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Supplementary Information Text

Details on MD simulations:
All systems (S1 – S4 as listed in Table 1 in the main text) were minimized using the steepest descent algorithm and heated to 293.15 K over 40 ps, and then simulated under constant number, pressure, and temperature (293.15 K) using the Nosé–Hoover thermostat (1) for production. The integration time step equals 1 fs, and coordinate sets were saved every 5 ps. Electrostatics were evaluated using particle–mesh Ewald (PME) with ca. one grid point per angstrom (Å), a sixth-order spline interpolation for the complementary error function, a $\kappa$ value of 0.32, and a 12 Å real space cutoff. The van der Waals term used a standard 6–12 LJ form, with force–switched truncation over the range 8–12 Å. The SHAKE constraint method (2) was applied to all covalent bonds to hydrogen, with the default tolerance ($1.0 \times 10^{-10}$ Å). As already noted in the main text, the CHARMM C36 lipid force field (3) with the newly developed pair-specific LJ parameters (NBFIX) of Ca$^{2+}$ with Cl$^-$ and with the carbonyl oxygen of the phosphate group were used (4). The NBFIX parameters yielded good agreement with experimental osmotic pressure over a wide range of concentrations for solutions of Ca$^{2+}$, Cl$^-$, and dimethyl phosphate, and the common artifact of ion aggregation was eliminated. The systems were simulated to 500 ns using the OpenMM (5) program and then extended to 5 (S1 and S2) or 3.5 (S3) μs using ANTON-2 supercomputer (6). The production runs for 5 replicas of S4 were performed using OpenMM.

Network-theoretic methods:
A network is a pair of sets $G = (N, L)$, where a set of nodes $N$ is connected by a set of links $L$. A network $G$ with $n$ nodes can be represented by its adjacency matrix $A(G)$ with $n \times n$ elements $A_{ij}$. In the PIP$_2$ networks, $A_{ij} = 1$ if $i$ and $j$ are joined by a link where each link connects two distinct nodes (PIP$_2$ molecules), otherwise $A_{ij} = 0$. The directionality of the links was not considered in the network (i.e., undirected network).

The list of the oxygen atoms considered in the link formation is as follows:
1) 4 oxygens of the non-protonated P1, ‘O1’
2) oxygen of the hydroxyl group at C2
3) oxygen of the hydroxyl group at C3
4) 4 oxygens of the singly-protonated P4, ‘O4$^p$’
5) 4 oxygens of the non-protonated P4, ‘O4’
6) 4 oxygens of the singly-protonated P5, ‘O5$^p$’
7) 4 oxygens of the non-protonated P5, ‘O5$^{np}$’
8) oxygen of the hydroxyl group at C6
9) oxygen of the carbonyl group of the sn-1 acyl chain
10) oxygen of the carbonyl group of the sn-2 acyl chain.

The 10 different oxygen types yield 55 (i.e., $10^5 = 55$) link types. The full list of link types is displayed in Table S1.

The adjacency matrix for each of the systems (S1 – S4) was constructed using the MD trajectories. The adjacency matrix was used to obtain the node degree $k$ (i.e., the number of links incident to the node) and average degree $\langle k \rangle$ (average number of links per node) which is a general descriptor of the overall connectivity and stationarity of a system (7). The PIP$_2$ cluster size was also obtained using the matrix: A PIP$_2$ cluster is a subnetwork of $G$ such that every node (i.e., PIP$_2$) is connected by one or more links to one or more nodes in the cluster. The cluster size denotes the number of nodes in the cluster. In the present analysis, two nodes were treated as connected (i.e., each node gains 1 degree) if they are connected by one or more links.
The lifetime of a cluster is defined as follows. A sequence of networks \( G(t_0), G(t_1), \ldots, G(t_{n-1}) \) exists with the same set of nodes but varying sets of links for \( N \) discrete time steps \( t_0, t_1, \ldots, t_{n-1} \). Suppose \( C \) is a cluster of network \( G(t) \) at some time step \( t \). If \( m \) and \( n \) are non-negative integers such that \((t_{i-m}, t_{i-m+1}, \ldots, t_{i+n}, t_{i+n+1}) \) is the largest contiguous sequence of time steps for which \( C \) is a subnetwork (but not necessarily a cluster) of networks \( G(t_{i-m}), G(t_{i-m+1}), \ldots, G(t_{i+n}) \), then the cluster \( C \) has lifetime \( n+m+1 \). Thus, a cluster is thought to be “alive” in the time interval during which the nodes in the cluster stay connected amongst themselves, even if at some time during the interval the nodes do not form a proper cluster (due to them being part of a larger connected subnetwork). However, by definition, it must be a proper cluster at some point during the interval.

This definition ensures that sets of connected links that are always a part of a larger set of connected links are not counted, which is desirable since the stability of such connected sets may depend on the larger clusters. However, sets of connected links that are not contained in a larger connected set at some time step are counted, even during the time steps when they are. This ensures that sets of contacts that have demonstrated self-sufficiency are counted even during times when they participate in the formation of larger clusters.

Fig. S2 illustrates the definition of cluster lifetime. The dimer \((1, 2)\) existing in time step 1 has a lifetime of 4 since its link survives to time step 4. The trimer \((1, 2, 3)\) of time step 2 has a lifetime of 1 because its 2-3 link survives for only the time step 2, while the dimer \((4, 5)\) has a lifetime of 3, since its 4-5 link survives to time step 4. The tetramer \((3, 4, 5, 6)\) of time step 3 has lifetimes of 1, since the 5-6 link survives for only the time step. The trimer of time step 4 \((3, 4, 5)\) has a lifetime of 2, since its all links were formed in the previous time step and retained until the current time step.

### Details on PIP2 clusters:

In all systems, the numbers of links among P4 and P5 are greater than P1–P1, P1–P4, and P1–P5. In general, the links between the non–protonated P4 and P5 (4p4np, 4p5np, and 5p5np) are greater than those of protonated/non–protonated (4p5np, 4p5p, 4p4p, 5p5p) or protonated pairs (4p4p, 4p5p, and 5p5p). However, the link formations are largely influenced by the nature of the cation. In cases of monovalent cation only systems, while Na+ preferentially forms the links between non–protonated P4 and P5 (i.e., 4p4np, 4p5np, and 5p5np), K+ mediates the links in the order ‘4p4np’ < ‘1np1np’ < ‘4p5np’ (for top 3)—this demonstrates that K+ has a significant affinity to the P1 phosphate group. For Ca2+-containing systems (S1 and S4), the ordering is ‘5np5np’ < ‘4np4np’ < ‘4np5np’ (top 3).

The lifetime distributions are fitted with the power-law function \( f(x) = ax^{-b} \) between 0 and 100 ns using least squares regression, and \( R^2 \) value indicating the goodness-of-fit. The value of the exponent \( b \) indicates the rate at which the probability of occurrence decays with increasing lifetimes. Monomers for all systems exhibited exponents between 1.40 and 1.48, showing similar rates of decay for lifetimes up to ~20 ns (Fig. S9, top). However, for lifetimes > 20 ns, the probabilities of finding monomers in K+ and Na+ only conditions fall rapidly below the power-law fit. For the systems with Ca2+ (S1 and S4), long-lived monomers are more prevalent than suggested by the power-law fit. This is again consistent with the higher association/dissociation rates between monomers to clusters (e.g., between monomers and dimers) in the monovalent systems (S2 and S3) compared to the Ca2+-containing systems (S1 and S4) (Fig. 4 in the main text). A similar trend is observed for dimers: while the exponents of the power laws are similar among all systems, the Ca2+ systems (S1 and S4) exhibit many more dimers with long lifetimes than the other two systems. The K+ only system (S3) has the fewest long-lived dimers, and the trend in the Na+ system (S4) lies in between (Fig. S9, middle). For large clusters (i.e., size ≥ 5), S4 has the slowest decay of probability with lifetime (\( b = 1.537 \)), S2 and S1 are next with similar exponents (\( b = 1.703 \) and 1.811, respectively), and S3 has by far the most rapid decay (\( b = 2.844 \)). This reflects the relative stability.
large clusters for each cation condition and in particular the synergistic effects of K\(^{+}\) and Ca\(^{2+}\) on promoting large clusters.

**Hydrogen bonding analysis:**

The final 2.16 μs of each simulation trajectory was split into six subset files of 360 ns to facilitate time block error analysis. The COOR HBOND facility of the CHARMM (8) program was used to evaluate the presence of both inter-and intra-molecular H-bonds, esp. for the PIP\(_2\) molecules, using the default cutoff of 2.4 Å for the proton to acceptor atom distance cutoff. In verbose mode, the program reports the mean fractional occupancy and mean duration of each specific H-bond pair, i.e., between the same two unique atoms. A csh shell script was used to post-process the data and average over instances of given pair types and molecule types, where the two protonation states of PIP\(_2\) are considered as different molecule types. The analyses were performed with respect to each specific donor type: OH of cholesterol, NH\(^{+}\) of POPE, OH groups at ring positions 2, 3, and 6 of PIP\(_2\), and the H atom on whichever phosphate group (P4 or P5) is protonated. Any oxygen atom was considered a viable H-Bond acceptor, including the water O atoms. Each of six 360 ns subset files was evaluated separately, and the results used to compute the averages and standard error. The duration values are a qualitative underestimate, due to the single count fluctuations to the ‘off’ state inherent in a cutoff method.

The PIP\(_2\) headgroup has both hydrogen bond donors (the protons on O2, O3, O6, and either P4 or P5) and acceptors (the oxygens on the ring phosphates). Hence, numerous intramolecular hydrogen bonds are possible, including between adjacent phosphate and hydroxyl groups, and between P5 and the axial hydroxyl on position 2 (Fig. S10). That cross-ring H-bond is the dominant internal one, and is more persistent when the P5 phosphate is unprotonated. Intermolecular H-bonds can be formed with water and other lipids, including PIP\(_2\), and in the latter case could be a factor in cluster stabilization. Hence, it is of interest to determine the correlation of clustering and hydrogen bonding for PIP\(_2\).

Table S5 lists the hydrogen bond fractions for the principal acceptors from donors in the inositol group of PIP\(_2\). On average nearly 60% are internal and 25% are with water, accounting for 85% of hydrogen bonds. Inter-molecular PIP\(_2\) hydrogen bonds only account for approximately 1% of the total. There is a variation with ion type: with the exception of OH2, intra-PIP\(_2\) fractions decrease in the order K\(^{+}\), Na\(^{+}\), Ca\(^{2+}\), with the largest drop associated with protonated P4 and P5. However, the fractions of intermolecular PIP\(_2\) hydrogen bonds are nearly independent of ion type, and there is an increase in hydrogen bonds with water proceeding from K\(^{+}\) to Ca\(^{2+}\). This finding implies that hydrogen bonds do not play a significant role in PIP\(_2\) clustering. In fact, they are anticorrelated because internal hydrogen bonds with protonated P4 and P5 are lost when these moieties participate in the larger clusters for three of the four proton donors. PIP\(_2\) hydrogen bonds to POPC, PSM, and POPA are also at the 1% level, and those to cholesterol are near 0%. In contrast, POPE accounts for ~7% of intermolecular PIP\(_2\) hydrogen bonds (Table S5), which is larger than the mole fractions in Table 1 would predict if these interactions were random. Since POPE is a common lipid in the inner leaflet of cells its interactions with PIP\(_2\) are worth further study.

**Lipid diffusion:**

The final 2.16 μs of each simulation trajectory was split into six subset files of 360 ns to facilitate time block error analysis for the diffusion coefficient calculations. Six new files for each simulation were created from the subset files described above, via image unfolding of all six in a single pass to remove any periodic boundary artifacts from the lipid motions. The unfolded trajectories were then used to compute the center of mass time series for each lipid and for each leaflet. After subtracting the respective leaflet center of mass from each lipid, the z component of each vector time series was set to zero, and the 2D mean squared displacement (MSD) time series was
computed for each lipid, then stored on disk; this was done for each subset file and for the concatenation of all six. This analysis was performed separately for each lipid type and for each leaflet. A Fortran program was then used to read the stored leaflet corrected lipid MSD data, compute an average MSD over the lipids, and use the variance at each MSD time point to perform a weighted linear fit over the range from 10 ns to half of the input time points. For the 2D case, the diffusion constant D_{MSD} is the slope divided by 4; standard errors were computed using D_{MSD} from each of the six subsets. The correction for periodic boundary condition artifacts (9, 10) was not applied here because of uncertainties in the leaflet viscosities and interleaflet friction in these asymmetric systems. The observed qualitative trends are expected to hold.

Table S6 lists the diffusion constants for each lipid type in the upper (uncharged) and lower (charged, containing both PIP_2 and POPA) leaflets. Diffusion constants in the uncharged leaflet are essentially independent of ion type. Cholesterol diffuses most rapidly, consistent with its absence of a headgroup, POPC and POPE are similar, and PSM is the slowest, as expected from its ability to form hydrogen bond networks (11, 12). Diffusion constants are lower and much more sensitive to ion type in the charged leaflet, with an average reduction is 32, 56, and 63% in K^+, Na^+, and Ca^{2+}, respectively, for the lipids also present in the uncharged leaflet. The diffusion constants of PIP_2 are not dramatically different from the other lipids indicating that there is an overall increase in leaflet viscosity that reduces the diffusion of all species, and it is reasonable to infer that this viscosity increase is associated with PIP_2 clustering. Some other trends are evident. D for PIP_2 is similar to the other lipids in S1, where it is mostly unclustered, but lower than all except PSM in S3 where its clustering is highest. Hence, some evidence of clustering may be obtained from diffusion constants, but the clustering affects the other lipids as well.

**Simulations at high and low Ca^{2+} concentration:**

As noted in the main text, systems S1 and S4 contained 150 mM and 25 mM bulk Ca^{2+} concentrations, respectively. Given that cellular bulk calcium concentrations are less the 1 mM, it is important to demonstrate that clustering and other properties observed in these simulations were not significantly perturbed by these high calcium concentrations.

To this end, two symmetric lipid model systems with 0.15 PIP_2:0.85 POPC and 0.15 PIP_2:0.375 POPE:0.475 POPC 180 mM excess CaCl_2 were constructed via CHARMM-GUI (13), and two more with the same lipid composition were later created by replacing most of the bulk CaCl_2 ions with water molecules from the 500 ns time point of the two initial systems to yield ~10 mM bulk concentration (4 Ca^{2+} ions in the water region). All systems contained 320 lipids, and approximately 19,000 waters. The simulations at high Ca^{2+} concentration contained 160 Ca^{2+} and 128 Cl^−; those at low Ca^{2+} concentration contained 101 Ca^{2+} and 8 Cl^−. There was sufficient Ca^{2+} on the membrane surface to neutralize the PIP_2; this is the so-called Stern layer of tightly bound ions. To improve the statistics for comparisons, four replicates of each lipid system were created by sampling coordinate sets from the initial simulations at time points 125 ns apart for a total of 20 simulations (5 replicates of 4 simulation conditions). Relevant properties were calculated the final 500 ns of each simulation. The diffusion constants were not corrected for periodic boundary conditions artifacts given that the relative quantities are of interest here.

Using the CHARMM (14) program, the two initial (high Ca^{2+}) systems were minimized with lipid headgroup and C=C bond restraints, and then briefly equilibrated for 1 ns of molecular dynamics. The remaining simulations were all performed using the Rickflow (15) package for OpenMM(5) 7.5, with a Nosé-Hoover thermostat and the anisotropic membrane barostat. Each simulation used a unique random seed for the assignment of the initial atom velocities; coordinate sets were stored at 0.1 ns intervals. A 1 fs timestep was employed for all simulations, at a temperature of 310° K, with 8-12 Å force switched VDW truncation, and PME for the long-range electrostatics beyond 12 Å.
The results of this test are shown in Table S7 and Fig. S11. It is evident that average areas/lipid, compressibility moduli, lipid diffusion constants, and cluster distributions for the 180 mM and 10 mM bulk CaCl$_2$ solutions are either within statistical error or otherwise very close. This is because these properties are dominated by the tightly bound ions at the membrane surface, and therefore relatively independent of the bulk concentrations. Of note, the average surface areas of the POPE containing bilayers are significatively smaller than those with only POPC and PIP$_2$, as are the individual lipid diffusion constants.
Figure S1. Snapshots of MD system S4. (left) Initial condition of S4, when Ca$^{2+}$ (red) was added to the K$^+$ (green sphere) only system (S3) at the end of the 3.5 μs trajectory of S3, and (right) 1 μs frame of S4. PIP$_2$ are spheres otherwise line representations: the phosphorus atom of P1 phosphate and that of P4/P5 phosphates of PIP$_2$ are highlighted in yellow and orange, respectively. The sizes of the phosphorus atoms and cations are increased for better visibility.
**Figure S2.** Schematic illustration of the cluster lifetime. A simple system with six nodes is shown over four time steps. The filled and unfilled (numbered) circles indicate clustered and non-clustered nodes, respectively, where the links (distinct colored lines) vary over time steps. Dotted lines indicate links that have disappeared. The table under each network lists the clusters (first column), cumulative cluster lifetime (second), links constituting the clusters (third), and the cumulative lifetime of each link (fourth). Coloring of each of the clusters and links in the table corresponds to that of graphs.
Figure S3. Occurrences of specific link types. The fifty-five different link types on the PIP$_2$ networks of each system (S1–S4) are displayed, e.g., ‘1np1np’ denotes the edge between O1$^{ns}$–O1$^{np}$ interaction. The full description of link types is displayed in Table S1. The link occurrences were obtained by taking the averages over the last 2.5 μs trajectories. The last five 500 ns blocks of the trajectory were used for S1-S3. For S4, the last 500 ns segments of 5 replicas were used. The error bars denote standard errors.
**Figure S4.** Time evolution of PIP₂ clusters for (A) S1-S3 and (B) five replicas (R1-R5) of S4. The number of monomers and clusters of each size (from dimers up to decamers) is shown for each trajectory. Dots and bars indicate the mean and (standard deviation) s.d., respectively, of counts within a 48 ns window.
Figure S5. Clustering histories of all PIP$_2$ molecules in the Ca$^{2+}$ only system (S1). Each panel tracks the history of one of 60 PIP$_2$ molecules during the last 500 ns of the simulation. Each line traces the size of the cluster that the PIP$_2$ molecule constitutes (or 1 if monomer). Gray dashed lines are visual guides at the height of monomers (lower lines) and pentamers (higher lines).
Figure S6. Clustering histories of all PIP₂ molecules in the Na⁺ only system (S2). Each panel tracks the history of one of 60 PIP₂ molecules during the last 500 ns of the simulation. Each line traces the size of the cluster that the PIP₂ molecule constitutes (or 1 if monomer). Gray dashed lines are visual guides at the height of monomers (lower lines) and pentamers (higher lines).
Figure S7. Clustering histories of all PIP$_2$ molecules in the K$^+$ only system (S3). Each panel tracks the history of one of 60 PIP$_2$ molecules during the last 500 ns of the simulation. Each line traces the size of the cluster that the PIP$_2$ molecule constitutes (or 1 if monomer). Gray dashed lines are visual guides at the height of monomers (lower lines) and pentamers (higher lines).
Figure S8. Clustering histories of all PIP_2 molecules in the Ca^{2+}/K^{+} system (S4). Each panel tracks the history of one of 60 PIP_2 molecules during the last 500 ns of the simulation. Each line traces the size of the cluster that the PIP_2 molecule constitutes (or 1 if monomer). Gray dashed lines are visual guides at the height of monomers (lower lines) and pentamers (higher lines). Replica 1 of the five replicas is shown.
Figure S9. Lifetime distributions of monomers, dimers, and larger clusters. Large clusters are defined to be pentamers and larger (size ≥ 5). Circles indicate the average number of occurrences of a lifetime. Coloring is as follows: S1, red; S2, blue; S3, green; S4, cyan. The solid lines of each of the systems are from power-law fitting in the range of 0 – 100 ns using least squares regression. The last 2.5 μs trajectories were used: for S1-S3, five 500 ns blocks were taken, and the last 500 ns segments of each of 5 replicas were used for S4.
Figure S10. H-bond locations on the inositol ring of PIP$_2$. The inositol ring of PIP$_2$ with the ring positions labeled in the left image, and H-bond locations indicated via dashed lines. The left structure has the P4 phosphate protonated, and P5 is protonated for the right one. Polar H atoms are shown in cyan, ring C atoms are gray, and all O atoms are purple. The P atoms are color coded by position, with P1 brown, P4 yellow, and P5 orange.
Figure S11. Cluster size distributions for the two symmetric bilayers at high and low Ca\(^{2+}\) concentrations.
Table S1. All link types used to define PIP<sub>2</sub> clusters.

| Oxygen-Oxygen interaction type | Link      | Interaction<sup>a</sup> |
|--------------------------------|-----------|-------------------------|
| 1                              | 1np1np    | O<sup>1np</sup>–O<sup>1np</sup> |
| 2                              | 1np2p     | O<sup>1np</sup>–O2     |
| 3                              | 1np6p     | O<sup>1np</sup>–O2     |
| 4                              | 2p2p      | O2–O2                  |
| 5                              | 2p6p      | O2–O6                  |
| 6                              | 6p6p      | O6–O6                  |
| 7                              | 1np3p     | O<sup>1np</sup>–O3     |
| 8                              | 1np4p     | O<sup>1np</sup>–O4<sup>p</sup> |
| 9                              | 1np4np    | O<sup>1np</sup>–O4<sup>np</sup> |
| 10                             | 1np5p     | O<sup>1np</sup>–O5<sup>p</sup> |
| 11                             | 1np5np    | O<sup>1np</sup>–O5<sup>np</sup> |
| 12                             | 2p3p      | O2–O3                  |
| 13                             | 2p4p      | O2–O4<sup>p</sup>     |
| 14                             | 2p4np     | O2–O4<sup>np</sup>     |
| 15                             | 2p5p      | O2–O5<sup>p</sup>     |
| 16                             | 2p5np     | O2–O5<sup>np</sup>     |
| 17                             | 3p6p      | O3–O6                  |
| 18                             | 4p6p      | O4<sup>p</sup>–O6      |
| 19                             | 4np6p     | O4<sup>np</sup>–O6     |
| 20                             | 5p6p      | O5<sup>p</sup>–O6      |
| 21                             | 5np6p     | O5<sup>np</sup>–O6     |
| 22                             | 3p3p      | O3–O3                  |
| 23                             | 3p4p      | O3–O4<sup>p</sup>     |
| 24                             | 3p4np     | O3–O4<sup>np</sup>     |
| 25                             | 3p5p      | O3–O5<sup>p</sup>     |
| 26                             | 3p5np     | O3–O5<sup>np</sup>     |
| 27                             | 4p4p      | O4<sup>p</sup>–O4<sup>p</sup> |
| 28                             | 4p4np     | O4<sup>p</sup>–O4<sup>np</sup> |
| 29                             | 4p5p      | O4<sup>p</sup>–O5<sup>p</sup> |
| 30                             | 4p5np     | O4<sup>p</sup>–O5<sup>np</sup> |
| 31                             | 4np4np    | O4<sup>np</sup>–O4<sup>np</sup> |
| 32                             | 4np5p     | O4<sup>np</sup>–O5<sup>p</sup> |
| 33                             | 4np5np    | O4<sup>np</sup>–O5<sup>np</sup> |
| 34                             | 5p5p      | O5<sup>p</sup>–O5<sup>p</sup> |
| 35                             | 5p5np     | O5<sup>p</sup>–O5<sup>np</sup> |
| 36                             | 5np5np    | O5<sup>np</sup>–O5<sup>np</sup> |
| 37 | 1npt1 | O1<sub>np</sub>–O<sub>1e</sub> |
| 38 | 1npt2 | O1<sub>np</sub>–O<sub>2e</sub> |
| 39 | 2pt1 | O2–O<sub>1e</sub> |
| 40 | 2pt2 | O2–O<sub>2e</sub> |
| 41 | 3pt1 | O3–O<sub>1e</sub> |
| 42 | 3pt2 | O3–O<sub>2e</sub> |
| 43 | 4pt1 | O4<sub>np</sub>–O<sub>1e</sub> |
| 44 | 4pt2 | O4<sub>np</sub>–O<sub>2e</sub> |
| 45 | 4npt1 | O4<sub>np</sub>–O<sub>1e</sub> |
| 46 | 4npt2 | O4<sub>np</sub>–O<sub>2e</sub> |
| 47 | 5pt1 | O5<sub>np</sub>–O<sub>1e</sub> |
| 48 | 5pt2 | O5<sub>np</sub>–O<sub>2e</sub> |
| 49 | 5npt1 | O5<sub>np</sub>–O<sub>1e</sub> |
| 50 | 5npt2 | O5<sub>np</sub>–O<sub>2e</sub> |
| 51 | 6pt1 | O6–O<sub>1e</sub> |
| 52 | 6pt2 | O6–O<sub>2e</sub> |
| 53 | t1t1 | O<sub>1e</sub>–O<sub>1e</sub> |
| 54 | t1t2 | O<sub>1e</sub>–O<sub>2e</sub> |
| 55 | t2t2 | O<sub>2e</sub>–O<sub>2e</sub> |

<sup>a</sup>"O1<sub>np</sub>" denotes oxygen atoms on the non–protonated P1; "O4<sub>np</sub>" and "O4<sub>np</sub>" those on the protonated and non–protonated P4; "O5<sub>np</sub>" and "O5<sub>np</sub>" those on the protonated and non–protonated P5, respectively. "O2", "O3", and "O6" are hydroxyl oxygen attached to C2, C3, and C6 of the inositol ring. "O<sub>1e</sub>" and "O<sub>2e</sub>" denote sn-1 carbonyl oxygen and sn-2 carbonyl oxygen, respectively.
Table S2. PIP<sub>2</sub> cluster size distributions.

| Cluster Size | Probability (Mean and S.E.) |
|--------------|-----------------------------|
|              | S1(Ca<sup>2+</sup>) | S2(Na<sup>+</sup>) | S3(K<sup>+</sup>) | S4(Ca<sup>2+</sup>/K<sup>+</sup>) |
| 1            | 0.26397 0.01114 | 0.60645 0.01694 | 0.69762 0.01365 | 0.21405 0.03893 |
| 2            | 0.35414 0.01775 | 0.22700 0.00817 | 0.09286 0.00478 | 0.22700 0.01365 |
| 3            | 0.21236 0.00593 | 0.08286 0.00295 | 0.06890 0.00153 | 0.21236 0.00478 |
| 4            | 0.11198 0.01154 | 0.04343 0.00153 | 0.02075 0.00153 | 0.11198 0.00478 |
| 5            | 0.01955 0.00543 | 0.01997 0.00108 | 0.00644 0.00018 | 0.01955 0.00478 |
| 6            | 0.03032 0.00518 | 0.00569 0.00156 | 0.02073 0.00016 | 0.03032 0.00478 |
| 7            | 0.00344 0.00127 | 0.00290 0.00077 | 0.00562 0.00070 | 0.00344 0.00478 |
| 8            | 0.00249 0.00037 | 0.00146 0.00016 | 0.00107 0.00004 | 0.00249 0.00478 |
| 9            | 0.00168 0.00127 | 0.00016 0.00007 | 0.00009 0.00004 | 0.00168 0.00478 |
| 10           | 0.00004 0.00004 | 0.00005 0.00003 | 0.00009 0.00006 | 0.00004 0.00478 |
| 11           | 0.00004 0.00004 | 0.00003 0.00003 | 0.00000 0.00006 | 0.00004 0.00478 |
Table S3. Average lifetimes (ns) of monomers, dimers, and large clusters (s.e. in parentheses).

| Cluster size          | S1 (Ca²⁺) | S2 (Na⁺) | S3 (K⁺) | S4 (Ca²⁺/K⁺) |
|-----------------------|-----------|----------|---------|--------------|
| Monomers              | 33.0 (4.0)| 20.5 (1.1)| 11.9 (0.3)| 21.1 (2.9)  |
| Dimers                | 311 (20)  | 35.6 (2.3)| 12.1 (0.4)| 291 (19)    |
| Large clusters        | 6.1 (0.3) | 5.7 (0.2) | 3.1 (0.1) | 7.8 (0.5)   |
| (size ≥ 5)            |           |          |         |              |
Table S4. Power-law distributions of cluster lifetimes.

| Size          | System |   |   |   |
|---------------|--------|---|---|---|
| Monomers      |        |   |   |   |
| S1            | 145.3  | 1.474 | 0.996 |
| S2            | 816.7  | 1.471 | 0.998 |
| S3            | 1794.8 | 1.405 | 0.995 |
| S4            | 197.4  | 1.476 | 0.995 |
| Dimers        |        |   |   |   |
| S1            | 10.5   | 1.361 | 0.940 |
| S2            | 210.3  | 1.339 | 0.995 |
| S3            | 629.8  | 1.269 | 0.992 |
| S4            | 15.3   | 1.287 | 0.948 |
| Large clusters|        |   |   |   |
| S1            | 423.2  | 1.811 | 0.998 |
| S2            | 317.2  | 1.703 | 0.998 |
| S3            | 670.6  | 2.844 | 1.000 |
| S4            | 353.4  | 1.537 | 0.994 |
Table S5. Fractional occupancy of principal H-bond acceptor types by donors from PIP₂ (s.e. in parentheses).

| Donorᵃ | Acceptor          | S1 (Ca²⁺)   | S2 (Na⁺)   | S3 (K⁺)   |
|--------|-------------------|-------------|-------------|-------------|
| OH2    | Intra-PIP₂         | 0.659 (0.007) | 0.606 (0.005) | 0.550 (0.006) |
|        | Water              | 0.223 (0.005) | 0.256 (0.005) | 0.299 (0.004) |
|        | POPE               | 0.036 (0.004) | 0.046 (0.004) | 0.047 (0.003) |
|        | Inter-PIP₂         | 0.009 (0.001) | 0.009 (0.001) | 0.009 (0.001) |
| OH3    | Intra-PIP₂         | 0.474 (0.013) | 0.557 (0.008) | 0.580 (0.002) |
|        | Water              | 0.424 (0.011) | 0.334 (0.003) | 0.336 (0.002) |
|        | POPE               | 0.034 (0.002) | 0.061 (0.006) | 0.055 (0.003) |
|        | Inter-PIP₂         | 0.010 (0.003) | 0.010 (0.002) | 0.007 (0.001) |
| OH6    | Intra-PIP₂         | 0.432 (0.010) | 0.505 (0.009) | 0.513 (0.006) |
|        | Water              | 0.321 (0.008) | 0.276 (0.006) | 0.278 (0.002) |
|        | POPE               | 0.129 (0.005) | 0.150 (0.010) | 0.145 (0.007) |
|        | Inter-PIP₂         | 0.004 (0.002) | 0.009 (0.001) | 0.005 (0.001) |
| P-OH   | Intra-PIP₂         | 0.598 (0.012) | 0.707 (0.005) | 0.778 (0.007) |
|        | Water              | 0.240 (0.006) | 0.202 (0.003) | 0.178 (0.002) |
|        | POPE               | 0.056 (0.010) | 0.045 (0.003) | 0.038 (0.003) |
|        | Inter-PIP₂         | 0.013 (0.001) | 0.016 (0.002) | 0.007 (0.001) |

ᵃThe donor: acceptor pair data were tabulated without regard to which phosphate bears the proton.
Table S6. Lipid diffusion constant ($10^{-8}$ cm$^2$/s) in upper (uncharged) and lower (charged and PIP$_2$ containing) leaflets for systems S1-S3 (s.e. in parentheses).

| Leaflet          | Lipid | S1 (Ca$^{2+}$) | S2 (Na$^+$) | S3 (K$^+$) |
|------------------|-------|----------------|-------------|------------|
| **Upper** (uncharged) | Chol  | 0.55 (0.03)    | 0.52 (0.03) | 0.52 (0.03) |
|                  | POPC  | 0.54 (0.03)    | 0.46 (0.02) | 0.50 (0.03) |
|                  | POPE  | 0.62 (0.05)    | 0.43 (0.04) | 0.48 (0.01) |
|                  | PSM   | 0.43 (0.02)    | 0.35 (0.02) | 0.41 (0.04) |
| **Lower** (charged) | PIP$_2$ | 0.15 (0.02)   | 0.18 (0.02) | 0.35 (0.03) |
|                  | POPA  | 0.19 (0.02)    | 0.22 (0.02) | 0.31 (0.01) |
|                  | Chol  | 0.25 (0.01)    | 0.25 (0.01) | 0.40 (0.03) |
|                  | POPC  | 0.20 (0.01)    | 0.20 (0.02) | 0.34 (0.02) |
|                  | POPE  | 0.19 (0.01)    | 0.19 (0.02) | 0.32 (0.03) |
|                  | PSM   | 0.15 (0.01)    | 0.14 (0.02) | 0.24 (0.02) |
Table S7. Average areas/lipid, area compressibility, and diffusion constant for the two symmetric bilayers at high and low Ca$^{2+}$ concentrations.

| System              | Ca$^{2+}$ conc (mM) | $<\text{Area}>$/lipid ($\text{Å}^2$) | $K_A$ (dyn/cm) | $D$ ($10^{-8}$ cm$^2$/s) PIP$_2$ | PIP$_2$ POPC | PIP$_2$ POPE |
|---------------------|---------------------|-------------------------------------|----------------|----------------------------------|--------------|--------------|
| 0.15 PIP$_2$:0.85 POPC | 180                 | 65.41 (0.06)                       | 246 (12)       | 3.9 (0.5)                        | 7.4 (0.3)    | -            |
|                     | 10                  | 65.95 (0.05)                       | 259 (9)        | 4.3 (0.2)                        | 7.9 (0.2)    | -            |
| 0.15 PIP$_2$:0.375 POPE:0.475 POPC | 180 | 61.74 (0.05)                       | 252 (5)        | 2.9 (0.2)                        | 6.1 (0.2)    | 5.2 (0.4)    |
|                     | 10                  | 61.94 (0.05)                       | 272 (9)        | 2.9 (0.4)                        | 6.0 (0.2)    | 5.1 (0.6)    |
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