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Lipid Bilayer Molecular Dynamics Study of Lipid-Derived Agonists of the Putative Cannabinoid Receptor, GPR55

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Abstract

Both L- α -lysophosphatidylinositol (LPI) and 2-arachidonoyl-sn-glycero-3-phosphoinositol (2-AGPI) have been reported to activate the putative cannabinoid receptor, GPR55. Recent microsecond time-scale molecular dynamics (MD) simulations and isothiocyanate covalent labeling studies have suggested that a transmembrane helix 6/7 (TMH6/7) lipid pathway for ligand entry may be necessary for interaction with cannabinoid receptors. Because LPI and 2-AGPI are lipid-derived ligands, conformations that each assumes in the lipid bilayer are therefore likely important for their interaction with GPR55. We report here the results of 70 ns NAMD molecular dynamics (MD) simulations of LPI and of 2-AGPI in a fully hydrated bilayer of 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC). These simulations are compared with a 70ns simulation of the cannabinoid CB1 receptor endogenous ligand, N-arachidonylethanolamine (anandamide, AEA) in a POPC bilayer. These simulations revealed that (1) LPI and 2-AGPI sit much higher in the bilayer than AEA, with inositol headgroups that can at times be solvated completely by water; (2) the behavior of the acyl chains of AEA and 2-AGPI are similar in their flexibilities in the bilayer, while the acyl chain of LPI has reduced flexibility; and (3) both 2-AGPI and LPI can adopt a tilted headgroup orientation by hydrogen bonding to the phospholipid phosphate/glycerol groups or via intramolecular hydrogen bonding. This tilted head group conformation (which represents over 40% of the conformer population of LPI (42.2 $\pm$ 3.3%) and 2-AGPI(43.7 $\pm$ 1.4%) may provide a low enough profile in the lipid bilayer for LPI and 2-AGPI to enter GPR55 via the putative TMH6/7 entry port.

Keywords

cannabinoid; GPR55; lipid bilayer; molecular dynamics; GPCR

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Supporting Information **Available**: CHARMM parameter development details for P-O-C-C torsion angle between the phosphate and the inositol ring are provided.

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1. Introduction

GPR55 is a Class A G protein-coupled receptor that has been shown to be activated by cannabinoids (Johns et al., 2007; Lauckner et al., 2008; Ryberg et al., 2007). The receptor is expressed in several mammalian tissues such as adrenal gland, testis, spleen, tonsils and ileum, as well as, several regions of the brain such as the frontal cortex, striatum, hypothalamus, caudate and putamen (Johns et al., 2007; Lauckner et al., 2008; Ryberg et al., 2007; Sawzdargo et al., 1999). GPR55^{-/-} (knock-out) mice have been shown to be protected in models of inflammatory and neuropathic pain, suggesting that GPR55 antagonists may have therapeutic potential as analgesics for both these pain types (Staton et al., 2008). GPR55 has also been shown to have physiological relevance in bone resorption, suggesting a possible therapeutic use for GPR55 antagonists in the treatment of osteoporosis (Whyte et al., 2009). Initial studies conducted in three different labs suggested that GPR55 is a cannabinoid receptor (Johns et al., 2007; Lauckner et al., 2008; Ryberg et al., 2007) activated by various cannabinoid and atypical cannabinoid compounds. These studies showed that synthetic cannabinoid agonists such as HU210, CP55,940, AM251 and JWH015, endogenous cannabinoids such as N-arachidonylethanolamine (anandamide; AEA), 2-arachidonoylglycerol and virodhamine, phytocannabinoids such as Δ^9 -THC and atypical cannabinoids such as abnormal cannabidiol and O-1602, mediated either GTP γ S binding or intracellular Ca²⁺ increase, both indirect measures of GPR55 activation.

Phosphatidylinositol (PI) derived from mammalian tissues mainly consists of stearic acid esterified at the sn-1 position and arachidonic acid esterified at the sn-2 position. Oka and coworkers first reported that GPR55 is activated by L- α -lysophosphatidylinositol (LPI; **1** Chart 1) found in the brain (Oka et al., 2007). LPI was reported to mediate GTP γ S binding to GPR55, induce rapid phosphorylation of extracellular signal-regulated kinase (ERK) and increase intracellular Ca²⁺ (Oka et al., 2007). Subsequently these investigators reported that several species of LPI are found in rat brain (Oka et al., 2009). The predominant species is the stearic acid-containing species (LPI, 50.5%). However, rat brain contains a substantial amount of 2-arachidonoyl-sn-glycero-3-phosphoinositol (2-AGPI, 22.1%). In assays of GPR55 activation, Oka and co-workers reported that the activity of 2-AGPI is higher at GPR55 than the activity of LPI. These investigators concluded that 2-AGPI may be the true natural ligand for GPR55 (Oka et al., 2009).

Henstridge and co-workers reported that LPI increases GPR55 internalization and produces Ca²⁺ increase mediated by G α_{13} which leads to RhoA activation and then activation of nuclear factor of activated T cells (NFAT) family transcription factors (Henstridge et al., 2009). Kapur et al (Kapur et al., 2009) reported that LPI is a potent agonist of GPR55 in a β -arrestin assay, a direct measure of agonist-mediated receptor activation. These investigators found that AM251 and SR141716A (potent cannabinoid CB1 antagonists) acted as agonists at GPR55 in the β -arrestin assay. In the same assay, CP55,940, a potent cannabinoid agonist, antagonized the effect of LPI, AM251 and SR141716A. These investigators suggested that GPR55 is an atypical cannabinoid receptor.

Recent microsecond timescale molecular dynamics simulations have suggested that a lipid pathway for endogenous ligand entry may be necessary for interaction with the cannabinoid CB2 receptor (Hurst et al., 2010). A lipid bilayer entry pathway for ligands at both the CB1 (Picone et al., 2005) and CB2 (Pei et al., 2008) receptors has been suggested by isothiocyanate covalent labeling studies as well. Because LPI and 2-AGPI are lipid-derived ligands, conformations that each assumes in the lipid bilayer may be important for their interaction with GPR55. We report here the results of molecular dynamics simulations of LPI and 2-AGPI in a fully hydrated bilayer of 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC). These simulations are compared with those of the cannabinoid

CB1 receptor endogenous ligand, N-arachidonylethanolamine (anandamide, AEA, **3**) in a POPC bilayer. Our simulations revealed that (1) LPI and 2-AGPI sit much higher in the bilayer than AEA, with inositol headgroups that can at times be solvated completely by water; (2) the behavior of the acyl chains of 2-AGPI and AEA are similar in their flexibilities in the bilayer, while the acyl chain of LPI has reduced flexibility; and (3) LPI and 2-AGPI can also adopt a tilted headgroup orientation. This tilted head group conformation may provide a low enough profile in the lipid bilayer for LPI and 2-AGPI to enter GPR55 via the putative TMH6/7 entry port.

2. Methods

Molecular Dynamics

The MD simulations reported here employed the NAMD 2.6 (Kalé et al., 1999) molecular dynamics software package with the CHARMM27 parameter set (Feller and MacKerell, 2000; MacKerell Jr. et al., 1998), including data for polyunsaturated lipid (Feller et al., 2002) and the TIP3P model for water. Force field parameters for LPI and 2-AGPI were constructed by the use of existing parameters for similar atom types. Only the POCC torsion angle was missing and had to be parameterized (see Supporting Information). Periodic boundary conditions were employed with the z axis normal to the lipid bilayer. Simulation conditions were similar to those employed in earlier POPC bilayer studies (Gullingsrud and Schulten, 2004), e.g. the long-range Coulombic electrostatic potential was treated with the particle mesh Ewald summation method (Darden et al., 1993), and the Lennard-Jones potential was smoothly cut off between 8.5 and 10 Å. The NAMD simulations used the r-Respa multiple time step algorithm, with a time step of 4 fs for long-range electrostatic forces, 2 fs for short-range nonbonded forces, and 1 fs otherwise. In production runs, configurations were stored every 1 ps for later analysis. All simulations were run at 310K, and this temperature was maintained using Langevin dynamics.

The system was constructed from a low hydration sample of an equilibrated POPC lipid bilayer (13.5 waters/lipid: 72 lipid molecules with 36 per leaflet) (Armen et al., 1998) by adding 3 Å of water on either side of the bilayer to achieve full hydration (~44 waters/lipid, 72 lipid molecules with 36 per leaflet) and ensure the inositol groups of LPI and 2-AGPI were fully solvated. One lipid was removed from each leaflet and one molecule of LPI or 2-AGPI was added. Each pair of molecules was placed in opposite ends of the membrane to ensure that the distance between them exceeded the 10 Å cutoff for nonbonded, short-range interactions used in the simulations. Any overlapping waters were removed and counterions were added to neutralize the system (i.e., one Na⁺ ion per LPI or 2-AGPI). For the minimization, restraints were placed on all atoms except the inserted molecule (LPI or 2-AGPI). The restraints were gradually reduced until all atoms were free to move. The system was slowly heated (NVT ensemble) to 310K with no restraints in place. The system was then allowed to equilibrate using the NPAT ensemble (P=1atm, T=310K) with the area fixed to the experimental area per lipid for pure POPC (68.3 Å²/lipid) (Kucerka et al., 2005) for 10 ns. This was followed by a production run of 35ns using the NPAT ensemble. Duplicate simulations (35ns production) were run for LPI and 2-AGPI. The same protocol was followed for AEA except that no counterions were included in the simulation since AEA is neutral.

Trajectory Analysis

The two trajectories (35 ns each) were combined for analysis. All analysis was performed utilizing VMD (Humphrey et al., 1996). The MD trajectories of LPI, 2-AGPI and AEA in POPC were analyzed for the location of the headgroup and the acyl chain terminal methyl group of each ligand. Considering the z axis perpendicular to the lipid bilayer plane, the

simulation cell was divided in 200 slices along the z axis and the number of heteroatoms of each group was counted for each slice. These measurements were repeated for each time step in the simulation producing a density per unit volume as a function of z. These group distributions were averaged over the course of the simulation.

Hydrogen Bonding

The hydrogen bonding distance cutoff between heteroatoms was 3.5 Å and the minimum hydrogen bond angle was 145°.

X-Cluster Analysis of Acyl Tail Conformations

In order to characterize the various conformations adopted by the acyl tails of LPI, 2-AGPI and AEA in the bilayer, the program XCluster was used (Maestro v9.0). Clustering was performed on atoms 1-18 for LPI and 1-20 for 2-AGPI and AEA (see Chart 1 for numbering systems). X-Cluster reorders a set of structures according to their rms deviation and groups the structures into families of similar conformers. 2000 conformers were randomly selected from the MD simulations of each ligand and used for the cluster analysis.

Head Group Tilts

Taking the normal to the lipid bilayer as the z-direction, the inositol ring tilt was measured as the projection of the vector formed between the P atom and the C4'' hydroxyl oxygen of the inositol ring onto this normal direction, i.e., its z- component. Molecules for which this component was ≤ 2.99 Å were considered to have tilted head group conformations. Molecules for which this component was 3 to 6.99 Å were considered to have upright headgroup conformations.

Interaction Energy Calculations

The energy of interaction (electrostatic and Van der Waals) for LPI or 2-AGPI with their lipid bilayer environments was calculated using the plugin NAMDEnergy from VMD v1.8.7. All lipid bilayer components (lipid or water) within a 5 Å radius of the ligand were included in the calculation. Standard errors were calculated for each category (lipid and water) and a total energy of interaction for each ligand was determined.

Statistics

Two 35 ns simulations in POPC were run for LPI, 2-AGPI and AEA here. In each 35 ns simulation, one molecule of interest was placed in the upper leaflet and one was placed in the lower leaflet of the bilayer. By considering the behavior of each molecule in each leaflet, we were able to separate the simulations of each molecule into a set of four [upper (7,000 frames) and lower leaflet (7,000 frames) in first 35ns run and upper (7,000 frames) and lower leaflet (7,000 frames) in the second 35 ns run for a total of 28,000 frames] from which statistics for each property measured, could be calculated (hydrogen bonding, headgroup tilts and lipid bilayer interaction energy). The average and standard error for each property was then calculated.

3. Results

3.1. Group Distributions

Figures 1-4 illustrate the resulting group distributions for POPC, LPI, 2-AGPI and AEA and their components.

POPC Group Distributions—The POPC group distribution graph presented (Figure 1) is from the LPI simulation since all MD simulations gave very similar results for all three

ligands studied. The group distribution of the whole simulation cell (Figure 1, magenta line) was found to be in agreement with X-ray scattering studies of pure POPC reported by Kucerka and co-workers (Figure 1, purple line) (Kucerka et al., 2005). Figure 1 shows that water (dark brown) penetrates the bilayer as deeply as the glycerol region (green). Water penetration will be more thoroughly addressed in the discussion section.

LPI Group Distributions—Figure 2 illustrates the group distribution of LPI in POPC. The inositol group of LPI (blue, maxima at ± 21.5 Å) penetrates the POPC choline, phosphate and glycerol regions of the bilayer, but also has density that slightly exceeds the POPC choline region (cyan). This suggests that the inositol group may extend at times above the bilayer phospholipids into the aqueous phase. The group distributions of the phosphate (red, maxima at ± 20 Å) and glycerol (light green, maxima at ± 18 Å) groups of LPI overlap the corresponding distributions of the phosphate (orange, maxima at ± 18 Å) and glycerol (dark green, maxima at ± 15 Å) regions of POPC, however, the maxima for the phosphate and glycerol regions of LPI are shifted at least 2 Å further from the membrane center compared to the POPC phosphate and glycerol densities. The results for the inositol and phosphate groups of LPI in Figure 2 suggest that the headgroup (inositol and phosphate) of LPI is located, on average, slightly higher in the bilayer than the POPC headgroup (choline and phosphate) (see Figure 1). The acyl chain terminal methyl of LPI (black, maximum at $+2$ Å) lies near the center of the bilayer, the hydrophobic core, and has very slight overlap with the headgroup and glycerol group distributions of LPI.

2-AGPI Group Distributions—Group distributions of the constituents of 2-AGPI in POPC are illustrated in Figure 3. The inositol group of 2-AGPI (blue, maxima at ± 20 Å) penetrates the POPC choline, phosphate and glycerol regions of the bilayer. The inositol density of 2-AGPI exceeds the POPC choline region less than LPI, suggesting that the inositol group of 2-AGPI is somewhat less solvated by the aqueous phase. The phosphate group of 2-AGPI (red, maxima at ± 19 Å) overlaps with the POPC choline (cyan) and phosphate (orange) group distributions, while the glycerol group of 2-AGPI (green, maxima at ± 17 Å) is in the POPC glycerol region (dark green). The terminal methyl group distribution of 2-AGPI (black) is centered on the bilayer hydrophobic core with maximum density at $+1$ Å and is very broad (± 16.5 Å) showing slight overlap with the glycerol, phosphate and inositol regions of 2-AGPI. This may indicate that 2-AGPI can adopt more curved conformations that cause the terminal methyl of the acyl chain to sit higher in the bilayer than the LPI terminal methyl.

AEA Group Distributions—Figure 4 illustrates the group distribution for constituents of AEA. The $-\text{CH}_2\text{OH}$ group distribution of AEA (blue, maxima at ± 15.5 Å) is centered on the POPC glycerol density (dark green) and, in addition, overlaps with the POPC choline (cyan) and phosphate (orange) group distributions. However, this overlap occurs closer to the bilayer center than seen for LPI or 2-AGPI. This indicates that the headgroup of AEA sits lower in the bilayer compared to the position of the inositol group in LPI or 2-AGPI. The terminal methyl distribution of AEA (black) has its maximum on -1 Å, close to the membrane center and is almost as broad (± 15.5 Å) as that of 2-AGPI (± 16.5 Å), suggesting that AEA can adopt curved conformations that place the acyl chain terminal methyl high in the bilayer. This distribution overlaps with the $-\text{CH}_2\text{OH}$ group of AEA more than the terminal methyl of 2-AGPI overlaps with its headgroup (inositol and phosphate).

3.2. Hydrogen Bonding

Both LPI and 2-AGPI have great hydrogen bonding potential because each contains six hydroxyl groups (see Chart 1) that can potentially both donate and accept hydrogen bonds (five on the inositol ring at C2'', C3'', C4'', C5'' and C6'' and one glycerol hydroxyl at C2' for

LPI and C1' for 2-AGPI), as well as, six oxygens that can potentially act as hydrogen bond acceptors (four phosphate oxygens at OP1, OP2, OP3, OP4 and two ester oxygens at O1 and O2). In contrast, AEA has only one hydroxyl at C2' that can be both a hydrogen bond donor and acceptor, an amide NH that can act as a hydrogen bond donor and a carboxamide oxygen (O1) that can act as a hydrogen bond acceptor.

The three 70ns MD trajectories were analyzed for the occurrence and frequency of both inter- and intramolecular hydrogen bonds. Tables 1-3, present the complete hydrogen bonding analysis for the constituents of LPI, 2-AGPI and AEA with the waters and lipids surrounding them, as well as, an analysis of intramolecular hydrogen bonding in each. Values reported in Tables 1-3 represent the percentage of the MD simulation in which each different hydrogen bond is present along with the standard error associated with each.

Intermolecular Bonds—Table 1 contains the breakdown of the hydrogen bonding between water and regions of LPI, 2-AGPI and AEA. No attempt has been made here to separate the water into two categories (external water vs. water that penetrates the bilayer). The inositol group of LPI and 2-AGPI forms multiple hydrogen bonds with water (6 or more hydrogen bonds with water) to the same extent. Similarly, the number of phosphate and glycerol hydrogen bonds with water for both ligands are very comparable. Although AEA sits lower in the bilayer which reduces exposure to external water, it still forms a significant percentage of hydrogen bonds (C2' OH $86.0 \pm 1.9\%$; NH $41.0 \pm 4.0\%$; O1 $60.0 \pm 6.5\%$) with water that has penetrated into the POPC headgroup region during most of the simulation time.

Table 2 contains a breakdown of the hydrogen bonding between POPC moieties and regions of LPI, 2-AGPI and AEA. The inositol rings of LPI and 2-AGPI form hydrogen bonds with equal frequency to the phosphate and glycerol moieties of POPC. Table 2 also shows that LPI and 2-AGPI significantly differ in the ability of their glycerol groups to hydrogen bond to POPC phosphate and glycerol moieties. The glycerol group of LPI forms more hydrogen bonds with the POPC phosphate groups ($19.5 \pm 2.6\%$) than does the glycerol of 2-AGPI ($3.2 \pm 0.7\%$). The difference in the position of the glycerol hydroxyl (C2' in LPI; C1' in 2-AGPI) determines its availability to form intramolecular hydrogen bonds (see below). Due to intramolecular hydrogen bonding, the glycerol C1' hydroxyl of 2-AGPI is less available to hydrogen bond to the POPC phosphate and glycerol moieties (see Chart 1). In contrast, the glycerol hydroxyl group of LPI at C2' is not able to form an intramolecular hydrogen bond because the geometry for such formation is poor. The glycerol group of LPI is therefore free to hydrogen bond with the POPC phosphate and glycerol.

Both the C2' OH and amide NH moieties of AEA can hydrogen bond with POPC. The C2' hydroxyl ($26.5 \pm 1.0\%$) and amide NH ($3.5 \pm 1.1\%$) of AEA hydrogen bond to POPC phosphate, while the C2' hydroxyl ($6.2 \pm 1.4\%$) forms fewer hydrogen bonds with the POPC glycerol than does the NH moiety of AEA ($16.6 \pm 4.0\%$). This hydrogen bonding pattern indicates that the C2' hydroxyl of AEA favors POPC phosphate hydrogen bonding, while the amide NH favors POPC glycerol hydrogen bonding.

Intramolecular Bonds—Table 3 presents a break down of the intramolecular hydrogen bonding for LPI, 2-AGPI and AEA. All three ligands were built initially without any internal hydrogen bonds. During the MD simulations, however, despite the presence of water and lipid moieties, intramolecular hydrogen bonding was found to occur both in LPI and 2-AGPI, but to different extents. The difference in the position of the glycerol hydroxyl (C2' in LPI; C1' in 2-AGPI) determines its availability to form intramolecular hydrogen bonds (see below). The highest occurrence of intramolecular hydrogen bonding for LPI ($15.0 \pm 0.2\%$) is between an inositol hydroxyl (C2'' or C6'') and a phosphate oxygen OP3 or

OP4. Intramolecular hydrogen bonding for LPI was also observed between a phosphate oxygen OP3 or OP4 and the glycerol C2' hydroxyl ($10.5\pm0.5\%$).

The glycerol hydroxyl group of 2-AGPI at C1' is very frequently at the same level in the membrane as the phosphate OP2 oxygen of 2-AGPI and has very good geometry for hydrogen bond formation. This hydrogen bond contributes (49.4%) to the total intramolecular bonding between the glycerol and phosphate groups of 2-AGPI listed in Table 3. The remaining percentage is produced by intramolecular hydrogen bonding of the glycerol with OP3 or OP4. The glycerol to OP2 intramolecular hydrogen bond does not occur in the LPI trajectory because the glycerol hydroxyl group C2' location in LPI has a less favored geometry for hydrogen bond formation with the OP2 oxygen of the phosphate group, but can hydrogen bond with OP3 or OP4 ($10.5\pm0.5\%$). In contrast, intramolecular hydrogen bonds involving the inositol hydroxyl groups of LPI ($15.0\pm0.2\%$ to phosphate group; $2.6\pm0.5\%$ to glycerol group) and 2-AGPI ($15.0\pm0.7\%$ to phosphate group; $5.7\pm1.7\%$ to glycerol group) form to a similar extent and are low in incidence due to extensive hydrogen bonding to water and POPC.

In a previous short MD simulation of AEA in DOPC conducted in our lab (Lynch and Reggio, 2005) a very low occurrence of intramolecular hydrogen bonding of AEA (between the amide NH and the C2' hydroxyl oxygen) was reported. In the present simulation of AEA in POPC, no intramolecular hydrogen bonding was detected.

3.3. Conformational Analysis from MD

X-Cluster Analysis: Acyl Chain Conformations—X-cluster analysis was used to assess the conformations that the acyl tails of LPI, 2-AGPI and AEA adopt in the POPC bilayer during the simulations reported here. In previous work on the acyl chain conformations of AEA, we have used the torsion angle conventions of Applegate and Glomset (Applegate and Glomset, 1986) to characterize the pattern of dihedral angles along the polyunsaturated arachidonic acid acyl chain that involve vinyl methylene carbons for 2-AGPI and AEA (Barnett-Norris et al., 1998; Lynch and Reggio, 2005). These patterns lead to 5 basic overall acyl chain conformations: extended, U/extended U, J/L (curvature/bend in acyl tail region) and J' (curvature near headgroup). Figure 5 illustrates each of these categories. The saturated acyl chain of LPI was also categorized following this system. Table 4 reports the percentage population for LPI, 2-AGPI and AEA for which the acyl chain is in the extended, U-shaped, J-shaped and J'-shaped conformations.

LPI has a saturated fatty acid chain with 18 carbons (stearic) attached on the C1' carbon of glycerol. Our MD simulations in POPC showed that the acyl chain of LPI overwhelmingly prefers to be in an extended conformation (75.4%; see Table 4) in the bilayer, as would be expected for a saturated acyl chain. However, its saturated acyl tail can also assume different, more compact conformations but to a much lesser extent as can be seen by the small clusters of U/extended U (2.5%), J/L (3.0%) and J' (1.4%) conformations.

The arachidonic acid acyl chain of 2-AGPI attached on the C2' carbon of the glycerol, has 20 carbons with four double bonds each separated by a methylene carbon. The low Csp³ to Csp² rotational barrier (≤ 1 kcal/mol) (Rich, 1993) in the acyl chain of 2-AGPI results in a very flexible molecule with slightly more than one-third (35.2%) of the population in the extended conformation cluster. The second largest cluster with 15.3% of the conformers is the J/L cluster, followed by the J' (7.7%) and U/extended U (5.9%) clusters.

AEA has the same flexible arachidonic acid chain as 2-AGPI. The acyl chain cluster pattern for AEA follows a trend similar to 2-AGPI. The largest cluster is the extended conformation (45.4%; see Table 4), followed by the J/L (8.9%) cluster, U/extended U (6.0%), J' (5.8%).

Bond Order Parameters—The average structure of the acyl chain segments of phospholipid bilayers can be characterized with the orientational bond order parameter. This same measure can be used to characterize the acyl chain segments of the lipid-like ligands, LPI, 2-AGPI and AEA. C-H bond order parameters are obtained experimentally by either deuterium substitution NMR, ^2H NMR, or ^{13}C NMR spectroscopies (Eldho et al., 2003; Feller et al., 2002). These order parameters may be expressed and computed from a MD trajectory using the following expression (Feller et al., 2002; Saiz and Klein, 2001):

$$S_{\text{CH}} = 1/2 \langle 3\cos^2\theta - 1 \rangle$$

in which θ is the angle between the C-H bond vector and the normal to the bilayer surface, and the brackets indicate averaging over time (in a trajectory) and the number of molecules in the system.

In Figure 6, we report the C-H bond order parameters for LPI (Figure 6A), 2-AGPI (Figure 6B) and AEA (Figure 6C) from our MD trajectories in the POPC bilayer. The carbon numbers on the x axis represent the carbon numbers in the stearyl acyl chain of LPI (C1-C18, see Chart 1) and the arachidonoyl acyl chain of 2-AGPI and AEA (C1-C20, see Chart 1). The error bars indicate the standard error in each. The LPI order parameters in Figure 6A suggest that the acyl carbons in LPI have a relatively high amount of order (lower flexibility), but that order decreases as one moves toward C-18. These order parameters are similar to those reported by Holte and co-workers for a series of phosphatidylcholines at 37 °C with stearate in the sn-1 position (Holte et al., 1995). The value for the first carbon of LPI is lower than reported by Holte and co-workers, but this is likely a function of the headgroup difference.

The magnitude of the order parameters for 2-AGPI (Figure 6B) are similar to the order parameters reported by Feller and co-workers for the polyunsaturated docosahexaenoic acid (DHA) chain in SDPC (Feller et al., 2002). These order parameters are smaller than those typically seen for saturated lipid fatty acid chains (see Figure 6 in (Feller et al., 2002) and LPI in Figure 6A), indicating increased flexibility of the chain in 2-AGPI. The order parameters reported for 2-AGPI in Figure 6B are also smaller for carbons closer to the bilayer center (i.e., carbons near the end of the acyl chain of 2-AGPI) than for those closer to the phospholipid headgroup/glycerol region (i.e., acyl chain carbons near the headgroup). This suggests that there is a decrease in the order going from the water/lipid interface toward the center of the bilayer (i.e., C1 to C20).

The order parameters of AEA (Figure 6C) are also similar to those reported by Feller and co-workers (Feller et al., 2002) for DHA in SDPC. Compared to the 2-AGPI order parameters in Figure 6B, the AEA order parameters closer to the phospholipid headgroup region are smaller, suggesting less order even at C1. This likely is due to the difference in headgroup size between 2-AGPI and AEA, with the small size of the AEA headgroup affording it greater mobility in the bilayer.

Head Group Conformations—Two strikingly different conformations of the inositol ring with respect to the rest of the ligand and the membrane emerged in the simulations of LPI and 2-AGPI: an upright and a tilted conformation. The upright conformation occurs when the ligand is high enough in the bilayer for the inositol ring to be solvated by water. Figure 7 illustrates this conformation for LPI (Figure 7A) and 2-AGPI (Figure 7B).

The tilted conformation can be produced by the inositol ring resting one face or side on top of the POPC headgroups and hydrogen bonding to a lipid headgroup phosphate or glycerol oxygen or to waters embedded in the POPC headgroup region. Both LPI and 2-AGPI could create a tilted head group conformation in these ways. This is illustrated in Figure 8 for LPI (Figure 8A) and 2-AGPI (Figure 8B).

Table 5 summarizes the percent times that the headgroups of LPI and 2-AGPI spend in these different conformations. LPI and 2-AGPI have, on average, a slightly higher propensity to be upright with respect to the lipid bilayer ($57.8 \pm 3.3\%$, LPI; $56.3 \pm 1.4\%$ 2-AGPI, see Figures 7A and 7B) versus tilted ($42.2 \pm 3.3\%$ LPI; $43.7 \pm 1.4\%$ 2-AGPI) (see Figures 8A and 8B).

The percentage that LPI and 2-AGPI form the upright versus the tilted headgroup conformation does not appear to be influenced by the location of the ligand and hence that of its headgroup in the bilayer. Figure 9 provides a comparison of the headgroup positions of LPI (blue), 2-AGPI (magenta) and AEA (purple) relative to the POPC bilayer. This figure shows that the inositol of LPI is shifted away from the hydrophobic center of the bilayer to a greater extent than that of 2-AGPI by approximately $1.5 \text{ \AA}$. Interestingly, for 2-AGPI, which relative to LPI is more centered on the POPC phosphate region, proximity to the phospholipid headgroups does not lead to higher hydrogen bond formation with the POPC phosphates and glycerol (the main driving force for the inositol tilted conformation). Minor contributions to the tilted headgroup conformation are created by intramolecular hydrogen bonding between the inositol C2" or C6" hydroxyls and O1 of 2-AGPI ($<5\%$; see Figure 10). One important consequence of the tilted conformation is that the phosphate groups on LPI and 2-AGPI are more exposed. For example, solvent accessible surface area calculations on a representative pair of conformers for each compound showed that the phosphate group was 50.8% more exposed in the tilted conformation of LPI compared to its upright conformation, while for 2-AGPI, the phosphate group was 44.6% more exposed in the tilted conformation.

Interaction Energy with Lipid Bilayer as a Function of Headgroup Orientation

—Table 6 summarizes the energy of interaction that the two different headgroup conformations of LPI and 2-AGPI have with their surroundings. Only the lipids and water molecules that were within $5 \text{ \AA}$ of the ligands were included in these calculations. These energy calculations were performed for the whole 70ns production run for each ligand and for both ligands present in the simulation.

The average difference in interaction energy between the tilted and upright conformations of both LPI and 2-AGPI with surrounding lipid molecules was found to be approximately 3 kcal/mol lower (i.e., more negative) for the tilted conformation. However this difference was not found to be statistically significant. In contrast, the energy of interaction of the tilted and upright headgroups with the surrounding water did show a significant difference. LPI's upright headgroups had 7.3 kcal/mol better (i.e., more negative) energy of interaction with water compared to its tilted headgroup conformations and the same trend occurred for 2-AGPI (6.4 kcal/mol more negative for upright headgroups). The average total interaction energies for both LPI and 2-AGPI in the bilayer suggest that the upright conformation is favored in the bilayer over the tilted conformation for each (LPI, $\Delta E = -3.9 \text{ kcal/mol}$) and 2-AGPI ($\Delta E = -3.6 \text{ kcal/mol}$), however this difference was not found to be statistically significant.

4. Discussion

4.1. MD Simulation Analysis

4.1.1. POPC Bilayer—The group distribution of the POPC simulation cell shown in Figure 1 (magenta line) is in excellent agreement with X-ray scattering studies of pure

POPC conducted by Kucerka and coworkers (Kucerka et al., 2005) as illustrated in Figure 1 (purple line).

Water Penetration: Figure 1 illustrates that in our POPC simulations, water penetrates into the polar head group glycerol region of the POPC bilayer. The extent of water penetration into a fluid DOPC bilayer has been demonstrated in the joint X-ray and neutron diffraction studies of Wiener and White (Wiener and White, 1992). Their analysis highlights the dynamic nature of a lipid bilayer in the fluid phase, wherein the atomic locations of the various chemical components are described by distributions which overlap to varying degrees. For example, the distribution of water is seen to overlap significantly with the choline, phosphate, glycerol, and ester regions (see Figure 4 in reference (Wiener and White, 1992)). It is important to note that these diffraction experiments were run at very low hydration. The effect of hydration on water penetration has been studied by Simon and McIntosh (Simon and McIntosh, 1986) using X-ray diffraction and specific capacitance measurements on phosphatidylethanolamine. In these studies, water was observed to penetrate into the ester carbonyl regions of the phospholipid. Moreover, water contact with these ester carbonyls has also been observed via FT-IR spectroscopy of ^{13}C labeled C=O groups in various phospholipids (Blume et al., 1988). In general, water penetration is limited to the extent of the carbonyl region of the ester group, e.g. *most* water molecules do not penetrate further into the hydrophobic core of the bilayer than these carbonyl groups. This is in good agreement with our present results, as well as, earlier high hydration simulations of DOPC and POPC (Chiu et al., 1999) and of SDPC (Saiz and Klein, 2001).

4.1.2. Location and Conformations of Ligands in Lipid

Location of LPI, 2-AGPI and AEA in POPC Bilayer: The MD studies suggest that the headgroups of LPI and 2-AGPI are located in the lipid/water interface with inositol at least partly solvated. The inositol group distribution for LPI and 2-AGPI is on average 2.5Å and 1Å respectively beyond the POPC choline density. The ligands' phosphate is in the POPC choline/phosphate region and the acyl chain terminal methyl group is in the hydrophobic core as would be expected. The group distributions for LPI and 2-AGPI (See Figures 2 and 3) indicate that the terminal methyl distribution is fairly broad, especially for 2-AGPI, with some overlap with the POPC headgroup region. AEA has a much smaller headgroup with significantly less hydrogen bonding capability than LPI or 2-AGPI. This allows AEA to be situated lower in the lipid bilayer. The -CH₂OH group of AEA never exists high enough in the POPC bilayer to be solvated by water like LPI and 2-AGPI (see Figure 7). However, AEA can hydrogen bond to waters that have penetrated the POPC headgroup region. The group distribution of AEA (Figure 4) shows that the -CH₂OH group is centered on the POPC glycerol region, but can extend into the phosphate and choline regions of the bilayer. The terminal methyl group in the hydrophobic region has some overlap with the POPC headgroup region. This location for AEA is in agreement with earlier MD simulations in DOPC (Lynch and Reggio, 2005).

Acyl Chain Conformational Preferences of LPI in POPC: XCluster analysis of acyl chain conformations of LPI, 2-AGPI and AEA revealed the striking difference between the behavior of a saturated fatty acid acyl chain in the bilayer vs. a polyunsaturated fatty acid acyl chain in the bilayer. The predominant acyl chain conformer for LPI, which has a saturated stearic acid acyl chain, in POPC was found to be an extended conformation (75.4%, see Table 4). The preference for more extended acyl chain conformations is likely due to the combination of two factors: (1) the high energy barrier for rotation about Csp³ to Csp³ bonds and (2) the higher energy of the *gauche* conformations relative to the *trans* conformations. For example, the global minimum energy conformation about the central Csp³ to Csp³ bond in n-butane is the *anti* or *trans* conformation. *Gauche* conformations are

0.628 kcal/mol higher in energy than the *trans* conformation. Moreover, the *trans-gauche* barrier is 3.303 kcal/mol, while the *cis* barrier which results in eclipsed methyl groups is 5.460 kcal/mol (Hafezi and Sharif, 2007). Such a potential energy surface favors the global minimum energy *anti* or *trans*-conformation (torsion angle = 180°), thus biasing the population towards more extended conformations. The order parameters of LPI (Figure 6A) indicate the rigidity and high order of the saturated stearic acid acyl chain. These results are in good agreement with experimentally obtained order parameters (Holte et al., 1995) and other MD studies (Saiz and Klein, 2001).

Acyl Chain Conformational Preferences of 2-AGPI and AEA in POPC: The XCluster analysis of the 2-AGPI and AEA conformers shows that even though the largest cluster for each molecule is the extended conformation (35.2% and 45.4% respectively see Table 4), the population of the extended cluster is significantly smaller than that for LPI. Instead, the population has shifted to more compact conformations, J/L, U/extended U and J' (2-AGPI (15.3%, 5.9% and 7.7% respectively, see Table 4) and AEA (8.9%, 6.0% and 5.8% respectively, see Table 4)). Order parameter results for 2-AGPI (Figure 6B) and AEA (Figure 6C) are consistent with these X-cluster results, as the C-H order parameters for the arachidonyl chains are lower than seen for the LPI stearic acid acyl chain, suggesting greater flexibility in the bilayer. Rabinovich and Ripatti found that polyunsaturated acyl chains such as arachidonic acid (in which adjacent double bonds are separated by a methylene carbon linker) are characterized by the highest equilibrium flexibility compared with other unsaturated acyl chains (Rabinovich and Ripatti, 1991). Rich reported that the barrier to rotation about Csp² to Csp³ bonds in arachidonic acid is ≤ 1.0 kcal/mol (Rich, 1993). This low torsional energy barrier has also been quantified using *ab-initio* quantum chemistry by Feller and co-workers (Feller et al., 2002). Molecular simulations for other polyunsaturated fatty acids (PUFAs) including, docosahexaenoic acid (DHA) (Applegate and Glomset, 1986; Rabinovich and Ripatti, 1991) and for PUFAs incorporated into lipid, including 1-stearoyl-2-docosahexaenoyl-sn-glycero-3-phosphocholine (SDPC) and 1-palmitoyl-2-docosahexaenoyl-sn-glycero-3-phosphocholine (PDPC) (Feller et al., 2002; Gawrisch et al., 2003; Huber et al., 2002; Saiz and Klein, 2001) have characterized the effects of the low torsional energy barrier for rotation about the Csp² to Csp³ bonds. Huber and co-workers (Huber et al., 2002) noted that the DHA chain in PDPC samples all possible conformational states about the 5 methylene linkers in the PUFA chain in a 6.0 ns MD simulation. Analogous conclusions have been reached by Saiz and Klein for SDPC (Saiz and Klein, 2001). Our results are in agreement with results reported in the earlier studies of PDPC and SDPC, as these studies show that the acyl chain of PUFAs adopts a large number of energetically accessible conformations ranging from extended to more compact conformations, such as the U shape. Our results are also in agreement with our previous MD study of AEA in DOPC that also demonstrated the extreme conformational flexibility of the polyunsaturated (20:4, n-6) arachidonic acid acyl chain of AEA (Lynch and Reggio, 2005).

In a rotational echo double resonance (REDOR) NMR study of AEA in a DPPC multilamellar model membrane bilayer system, Tian and co-workers (Tian et al., 2005) found that AEA adopts an extended conformation with its terminal methyl located near the bilayer center. These NMR results represent an average conformation for AEA over the time of measurement, rather than the instantaneous positions for the acyl chain that can be calculated in an MD simulation. Our simulations show that the fatty acid acyl chain of AEA can adopt many different conformations over the course of the 70ns simulation, including quite compact conformations. The group distribution results for AEA illustrated in Figure 4 however, do show that the average position for the terminal methyl of AEA in POPC is near the bilayer center, in agreement with the results of Tian and co-workers (Tian et al., 2005).

Head Group Conformations: The headgroups of LPI and 2-AGPI are much larger than that of AEA. In addition, the LPI and 2-AGPI headgroups are charged and have tremendous hydrogen bonding potential. Our MD simulations show here that the headgroup inositol ring of LPI and 2-AGPI can assume different conformations with respect to the rest of the molecule and also the membrane. Taking the normal to the lipid bilayer as the z-direction, the inositol ring can be either fully solvated in the water in an upright conformation which is parallel to the z axis (see Figure 7) or the inositol ring can be tilted (up to 100° angle with respect to the z axis) and laying across the headgroups of the bilayer with only one side exposed to water (see Figure 8). In the MD studies reported here, LPI and 2-AGPI were found to have a slightly higher propensities for formation of the upright headgroup conformation in POPC (see Table 5). Unlike LPI and 2-AGPI, the headgroup of AEA is small and does not have any distinct or preferred conformations it can assume in the lipid bilayer.

The interaction energy analysis for tilted vs. upright headgroup conformations of LPI and 2-AGPI obtained from the MD simulations suggests that the upright conformation has higher interactions (on average) with the bilayer (LPI, $\Delta E = -3.9$ kcal/mol) and 2-AGPI ($\Delta E = -3.6$ kcal/mol). While it is tempting to speculate that this higher energy of interaction for the upright conformation with the bilayer may favor its retention in the bilayer and thus prime the tilted head group conformation for entry into GPR55, the fact that the energy difference between upright and tilted conformations did not reach statistical significance makes any conclusion about predisposition for receptor entry impossible.

4.1.3. Hydrogen Bonding—The headgroups of both LPI and 2-AGPI hydrogen bond extensively to waters and lipids that surround them and these interactions produce two very distinct overall conformations for LPI and 2-AGPI in the bilayer. In contrast, the overall conformation of AEA is largely influenced by its fatty acid chain conformation due to the small head group size of AEA.

Hydrogen Bonding of LPI, 2-AGPI and AEA with Water: Both the inositol and phosphate moieties of LPI and 2-AGPI hydrogen bond to external water throughout the simulation, while glycerol, since it is deeper in the bilayer, hydrogen bonds to water that has penetrated the bilayer as far as the POPC glycerol and ester region (see discussion of water penetration above). AEA does hydrogen bond to water, but to a smaller extent. The headgroup of AEA has fewer hydrogen bonding capable moieties than LPI and 2-AGPI and AEA sits lower in the lipid bilayer compared to the LPI species, only exposed to the water molecules that penetrate the POPC headgroup region.

Hydrogen Bonding of LPI, 2-AGPI and AEA to POPC Lipid Moieties: The extent of hydrogen bonding to the bilayer is similar for both LPI and 2-AGPI, but the glycerol group of LPI hydrogen bonds to lipid moieties more frequently compared to the glycerol group of 2-AGPI. Since the headgroup of AEA is situated lower in the bilayer, the POPC phosphate/glycerol region, it hydrogen bonds to those groups through its hydroxyl and amide NH though to a lesser degree compared to LPI and 2-AGPI that contain multiple hydrogen bonding groups.

Intramolecular Hydrogen Bonding in LPI, 2-AGPI and AEA: In addition to intermolecular hydrogen bonds, both LPI and 2-AGPI also form intramolecular hydrogen bonds. 2-AGPI forms intramolecular hydrogen bonds at least twice as frequently as LPI. This difference is due to the greater phosphate to glycerol hydroxyl hydrogen bonding capability of 2-AGPI vs. LPI. Inspection of the 3D structure of these molecules (Chart 1) shows that this difference is due to the geometry of the glycerol/phosphate segment. In LPI, the hydroxyl attached on C2' does not have the proper geometry to hydrogen bond to a

phosphate oxygen (OP2). Instead, the hydroxyl attached on C2' can only hydrogen bond to the free oxygens of the phosphate (OP3 and OP4). In 2-AGPI, the phosphate is at the same place (C3' of glycerol) as in LPI, but C2' is attached to the ester oxygen of arachidonic acid (O2) and the hydroxyl is on C1' of the glycerol. The geometry between the glycerol hydroxyl at C1' and the phosphate (OP2) is conducive to formation of an internal hydrogen bond. As a result, this interaction forms quite readily. This hydroxyl oxygen can also form a hydrogen bond with the two free oxygens of the phosphate (OP3 and OP4).

No intramolecular hydrogen bonding was identified for AEA in the POPC simulations reported here. Intramolecular hydrogen bonding between the AEA amide oxygen and hydroxyl group, which has been identified via MC simulations in DMSO (Bonechi et al., 2001) and suggested in the NMR studies of Eldho and co-workers (Eldho and Gawrisch, 2003) was not observed in our simulation. In addition, a previous MD simulation of AEA in a 1,2-dioleoyl-sn-glycerophosphocholine (DOPC) lipid bilayer conducted by our laboratory (Lynch and Reggio, 2005), indicated only a relatively weak and infrequent occurrence of an intramolecular hydrogen bond between the amide NH and the hydroxyl oxygen of AEA. Apparently, the competition with water and phospholipid components is sufficiently strong that intramolecular hydrogen bonding of AEA is not significant in POPC.

4.2. Interaction with GPR55

Recent microsecond time-scale molecular dynamics (MD) simulations and isothiocyanate covalent labeling studies have suggested that a lipid pathway for ligand entry may be necessary for interaction with cannabinoid receptors (Hurst et al., 2010; Pei et al., 2008; Picone et al., 2005). In each case, the putative entry pathway is between TMH6 and TMH7. Interestingly, the EC-3 loop (connecting the extracellular ends of TMH6 and TMH7) which must be able to accommodate such a ligand passage, is significantly longer (GPR55: RNSFIVECRAKQSI) than the EC-3 loop of CB1 (CB1: KMNKLI) or CB2 (CB2: TLSdqV). Because of the number of charged amino acids this larger EC-3 loop contains, it is very possible the loop will be mostly solvated by water and not packed against the top of the receptor allowing a ligand with large headgroup (such as LPI or 2-AGPI) to enter the receptor between TMHs 6 and 7. In preliminary modeling studies of GPR55, we have found that EC-3 loop in GPR55 can assume such a conformation (Hurst et al., 2006). Given its smaller size, it is not surprising that AEA has been found to activate GPR55 (Johns et al., 2007; Lauckner et al., 2008; Ryberg et al., 2007). While detailed docking studies of LPI or 2-AGPI in GPR55 are beyond the scope of this paper, it is important to note that the tilted head group conformation (which represents over 40% of the conformer population of LPI and 2-AGPI) may provide a low enough profile in the POPC bilayer for LPI and 2-AGPI to enter under this EC-3 loop.

5. Conclusions

Recent microsecond time-scale molecular dynamics (MD) simulations and isothiocyanate covalent labeling studies have suggested that a lipid pathway for ligand entry may be necessary for interaction with cannabinoid receptors (Hurst et al., 2010; Pei et al., 2008; Picone et al., 2005). Although AEA, LPI and 2-AGPI all activate the putative cannabinoid receptor, GPR55, the profiles of LPI and 2-AGPI, are quite different in the lipid bilayer from that of AEA. The headgroups of LPI and 2-AGPI are much larger, charged and with great hydrogen bonding potential, while their acyl chains are similar in size (LPI) or identical (2-AGPI) to AEA. Due to these structural differences, the headgroups of LPI and 2-AGPI are situated higher in the bilayer and can be more easily solvated by water compared to AEA. An upright head group conformation represents over 50% of the conformer population for LPI (57.8±3.3%) and 2-AGPI (56.3±1.4%) and places the inositol group largely above the POPC phospholipid headgroups. The remaining population adopts a tilted headgroup

conformation [LPI ($42.2 \pm 3.3\%$) and 2-AGPI ($43.7 \pm 1.4\%$)] that places the head group at the level of the POPC headgroups. If LPI and 2-AGPI follow the putative TMH6/7 entry pathway for cannabinoid ligands, this tilted headgroup conformation may present a low enough profile in the bilayer for LPI and 2-AGPI to enter GPR55.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviation List

MD	molecular dynamics
LPI	L- α -lysophosphatidylinositol
2-AGPI	2-arachidonoyl-sn-glycero-3-phosphoinositol
POPC	1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine
AEA	N-arachidonylethanolamine
TMH	transmembrane helix
GPCR	G protein-coupled receptor
PUFA	polyunsaturated fatty acid

Figure 1. Information about group distributions of the POPC lipid bilayer simulation cell is provided here. The group distribution of the simulation cell (magenta line) was found to be in agreement with experimental measurements for pure POPC reported both by Kucerka and co-workers (purple line) (Kucerka et al., 2005). Group distributions for water (dark brown) and the various POPC moieties, phosphate (orange), choline (cyan) and glycerol (dark green) are also illustrated here. It is clear here that water (dark brown) penetrates the bilayer as deeply as the glycerol region (dark green) of the bilayer.

Figure 2.

The group distribution of LPI in POPC is illustrated here. The inositol (blue) of LPI penetrates the POPC choline (cyan), phosphate (orange) and glycerol (dark green) regions of the bilayer, but also has density that slightly exceeds the POPC choline region (cyan). This suggests that the inositol group may extend at times above the bilayer phospholipids into the aqueous phase. The phosphate (red) and glycerol (light green) group distributions of LPI overlap the corresponding densities of the phosphate (orange) and glycerol (dark green) regions of POPC, however, the maxima for the phosphate and glycerol regions of LPI are shifted at least 2 Å further from the membrane center compared to the POPC phosphate and glycerol densities. The acyl chain terminal methyl of LPI (black) lies near the center of the bilayer, the hydrophobic core, with very slight overlap with the headgroup and glycerol group distributions of LPI.

Figure 3.

The group distribution of 2-AGPI in POPC is illustrated here. The inositol region (blue) of 2-AGPI penetrates the POPC phosphate (orange), choline (cyan) and glycerol (dark green) regions of the bilayer. The inositol density of 2-AGPI exceeds the POPC choline region less than LPI, suggesting that the inositol group of 2-AGPI is somewhat less solvated by the aqueous phase. The phosphate region (red) of 2-AGPI overlaps with the POPC choline (cyan) and phosphate (orange) group distributions, while the glycerol region (light green) of 2-AGPI is centered on the POPC glycerol region (dark green). The terminal methyl group distribution (black) of 2-AGPI is centered on the bilayer hydrophobic core and is very broad, showing slight overlap with the glycerol (light green), phosphate (red) and inositol (blue) regions of 2-AGPI. This may indicate that 2-AGPI can adopt more curved conformations that cause the terminal methyl of the acyl chain to sit higher in the bilayer.

Figure 4.

The group distribution of AEA in POPC is illustrated here. The $-\text{CH}_2\text{OH}$ group distribution (blue) is centered on the POPC glycerol density (dark green) and, in addition, overlaps with the POPC choline (cyan) and phosphate (orange) group distributions. The overlap occurs closer to the bilayer center than seen for LPI or 2-AGPI. This indicates that the headgroup of AEA sits lower in the bilayer compared to the position of the inositol group in LPI or 2-AGPI. The terminal methyl group distribution (black) of AEA has its maximum close to the membrane center and is almost as broad as that of 2-AGPI, suggesting that AEA can adopt curved conformations that place the acyl chain terminal methyl high in the bilayer. The terminal methyl group distribution of AEA reaches as far as the maximal value of the $-\text{CH}_2\text{OH}$ group (blue) distribution, suggesting extreme flexibility in the acyl chain.

Figure 5.

Acyl chain conformations for LPI, 2-AGPI and AEA were grouped by X-cluster analysis into several categories: Extended, U-shaped, J/L and J' shaped (see Table 4). This figure illustrates a representative conformer from each of these clusters for 2-AGPI.

Figure 6.

The C-H bond order parameters for LPI (Figure 6A), 2-AGPI (Figure 6B) and AEA (Figure 6C) are reported here. The carbon numbers on the x axis represent the carbon numbers in the stearoyl tail of LPI (C1-C18, see Chart 1) and the arachidonoyl tail of 2-AGPI and AEA (C1-C20, see Chart 1). The error bars indicate the standard error in each.

Figure 7.

This figure illustrates the upright headgroup orientation for LPI (Figure 7A) and 2-AGPI (Figure 7B). The upright orientation occurs when the ligand is high enough in the bilayer for the inositol ring to be solvated by water. Here the lipid acyl chains are shown in tube display and colored cyan. The phosphorous atoms of the POPC headgroups are shown as gold spheres, while the nitrogens of the headgroups are shown as blue spheres. Water molecules are shown in tube display. The carbons of LPI are colored orange, while the carbons of 2-AGPI are colored green.

Figure 8.

This figure illustrates the tilted headgroup orientation for LPI (Figure 8A) and 2-AGPI (Figure 8B). The tilted headgroup orientation occurs primarily when the inositol ring rests one face or side on top of the POPC headgroups and hydrogen bonds to a lipid headgroup phosphate or glycerol. Here the lipid acyl chains are shown in tube display and colored cyan. The phosphorous atoms of the POPC headgroups are shown as gold spheres, while the nitrogens of the headgroups are shown as blue spheres. Water molecules are shown in tube display. The carbons of LPI are colored orange, while the carbons of 2-AGPI are colored green. In each case, the yellow arrow(s) point to hydrogen bonds (shown in yellow) between the inositol ring and the phospholipid headgroups.

Figure 9.

This figure provides a comparison of group distributions of the inositol headgroup of LPI (blue) and 2-AGPI (magenta) versus the position of the $-\text{CH}_2\text{OH}$ headgroup of AEA (purple) relative to the POPC bilayer.

Figure 10.

This figure illustrates that 2-AGPI can also create a tilted headgroup conformation by forming an intramolecular hydrogen bond between the C2'' or C6'' inositol hydroxyl group and the carboxyl oxygen (O1) (see Chart 1 for numbering system). This type of intramolecular hydrogen bonding is not seen in LPI because the C2'' and C6'' hydroxyls are further away from the carboxyl oxygen due to the additional carbon between them (at C1'). Here the lipid acyl chains are shown in tube display and colored cyan. The phosphorous atoms of the POPC headgroups are shown as gold spheres, while the nitrogens of the headgroups are shown as blue spheres. Water molecules are shown in tube display. The carbons of 2-AGPI are colored green. The intramolecular hydrogen bond is shown in yellow.

Percent time over the 70ns combined MD trajectory that hydrogen bonds exist between water and ligand headgroup moieties. Standard errors for each measurement are also reported.

Table 1

Water	LPI			2'-AGPI			AEA		
	Inos	Phos	Glyc	Inos	Phos	Glyc	C2'OH	NH	OI
1 to 5 HBs	39.4±1.6	87.5±1.1	87.8±1.3	42.0±2.2	89.0±0.9	88.8±1.0	86.0±1.9	41.0±4.0	60.0±6.5
6 or more	60.6±1.7	12.5±1.1		58.0±2.2	10.9±0.9				

Table 2

Percent time over the combined 70ns MD trajectory that hydrogen bonds exist between POPC constituents and ligand headgroup moieties. Standard errors for each measurement are also reported.

POPC moieties	LPI			2-AGPI			AEA		
	Inos	Phos	Glyc	Inos	Phos	Glyc	OH	NH	CO
Phosphate	31.2±2.9		19.5±2.6	31.9±1.8		3.2±0.7	26.5±1.0	3.5±1.1	
Glycerol	7.8±2.8		4.9±1.4	8.2±3.1		1.6±0.6	6.2±1.4	16.6±4.0	

Table 3

Percent time over the combined 70ns MD trajectory that intramolecular hydrogen bonds exist for each ligand. Standard errors for each measurement are also reported.

Ligand moieties	LPI			2-AGPI			AEA		
	Inos	Phos	Glyc	Inos	Phos	Glyc	OH	NH	CO
Phosphate	15.0±0.2			15.0±0.7					
Glycerol	2.6±0.5	10.5±0.5	1.8±0.4	5.7±1.7	49.4±2.3	1.8±0.9			
NH							0.0		0.0
CO							0.0	0.0	

Table 4

XCluster analysis of acyl tail conformations using a 2000-conformer sample from the trajectory of each ligand. The clustering was performed on C1-C18 for LPI and C1-C20 of 2-AGPI and AEA (see Chart 1 for numbering systems).

Conformations	LPI (level 80)	2-AGPI (level 60)	AEA (level 60)
	Percentage (%) ^a	Percentage (%) ^a	Percentage (%) ^a
Extended	75.4	35.2	45.4
U/extended U	2.5	5.9	6.0
J ^b /L	3.0	15.3	8.9
J ^c	1.4	7.7	5.8

^aThe remaining structures (to complete 100%) had random shapes.

^bCurvature in acyl tail region.

^cCurvature nearer headgroup region.

Table 5

Percentage of population for the two different inositol ring orientations over the combined 70ns MD trajectories for LPI and 2-AGPI. Standard errors for each measurement are also reported.

Headgroup Orientation	LPI	2-AGPI
Tilted	42.2±3.3	43.7±1.4
Upright	57.8±3.3	56.3±1.4

Table 6

Energies of interaction (kcal/mol) between LPI or 2-AGPI conformers with POPC bilayer lipids and waters within 5Å of each ligand.

NAMD Energy	LPI		2-AGPI	
kcal/mol	Tilted	Upright	Tilted	Upright
Energy	Mean±Std error	Mean±Std error	Mean±Std error	Mean±Std error
Lipid	-96.2±6.3	-92.7±4.2	-94.2±2.0	-91.4±1.5
Water	-141.3±3.8	-148.6±2.4	-142.3±1.8	-148.7±2.0
Total Energy	-237.5±3.9	-241.4±2.2	-236.5±3.2	-240.1±0.9