G protein-membrane interactions II: Effect of G Protein-linked Lipids on Membrane Structure and G Protein-membrane Interactions

Jesús Casas[a], Maitane Ibarguren[a,b], Rafael Álvarez[a], Silvia Terés[a,b], Victoria Lladó[a], Stefano P. Piotto[c], Simona Concilio[d], Xavier Busquets[a,b], David J. López[a,b,*] and Pablo V. Escribá[a,b]

[a]Laboratory of Molecular Cell Biomedicine, Department of Biology, IUNICS, University of the Balearic Islands, E-07122 Palma de Mallorca, Spain.
[b]Lipopharma Therapeutics, S.L., ParcBit, 07121 Palma de Mallorca, Spain.
[c]Department of Pharmacy, University of Salerno, Via Ponte don Melillo, 84084 Fisciano (SA), Italy.
[d]Department of Industrial Engineering, University of Salerno, Via Ponte don Melillo, 84084 Fisciano (SA), Italy.

[*]To whom correspondence should be addressed:

David J. López, PhD, Laboratory of Molecular Cell Biomedicine, Department of Biology, University of the Balearic Islands, Crta. Valldemossa km. 7.5, 07122, Palma (Spain). Tel.: +34-97117 33 31; Fax +34-971 17 31 84; E-mail: davidlopez_95@hotmail.com

Abbreviations used: DiI, 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate; DSC, differential scanning calorimetry; GG, geranylgeraniol; GPCR, G protein-coupled receptor; GUV, giant unilamellar vesicle; H_{II}; inverted hexagonal; LUV, large unilamellar vesicle; MA, myristic acid; MOH, myristic alcohol; PA, palmitic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; POH, palmitic alcohol; POPC, 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine; POPE, 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine; T_H, lamellar-to-inverted hexagonal phase transition temperature.

ABSTRACT

G proteins often bear myristoyl, palmitoyl and isoprenyl moieties, which favor their association with the membrane and their accumulation in G Protein Coupled Receptor-rich microdomains. These lipids influence the biophysical properties of membranes and thereby modulate G protein binding to bilayers. In this context, we showed here that geranylgeraniol, but neither myristate nor palmitate, increased the inverted hexagonal (H_{II}) phase propensity of phosphatidylethanolamine-containing membranes. While myristate and palmitate preferentially associated with phosphatidylcholine membranes, geranylgeraniol favored nonlamellar-prone membranes. In addition, $G\alpha i_1$ monomers had a higher affinity for lamellar phases, while $G\beta\gamma$ and $G\alpha\beta\gamma$ showed a marked preference for nonlamellar prone membranes. Moreover, geranylgeraniol enhanced the binding of G protein dimers and trimers to phosphatidylethanolamine-containing membranes, yet it decreased that of monomers. By contrast, both myristate and palmitate increased the $G\alpha i_1$ preference for lamellar membranes. Palmitoylation reinforced the binding of the monomer to PC membranes and myristoylation decreased its binding to PE-enriched bilayer. Finally, binding of dimers and trimers to lamellar-prone membranes was decreased by palmitate and myristate, but it was increased in nonlamellar-prone bilayers. These results demonstrate that co/post-translational G protein lipid modifications regulate the membrane lipid structure and that they influence the physico-chemical properties of membranes, which in part explains why G protein subunits sort to different plasma membrane domains.

Keywords: G-proteins, Membranes, Palmitoylation, Myristoylation, Cell Signaling, Isoprenoids.

1. INTRODUCTION

Upon agonist-mediated activation, G protein-coupled receptor (GPCR)-mediated cell signaling is amplified through the larger number of G protein molecules present at the plasma membrane compared to the number of receptors [1]. Indeed, one agonist-activated GPCR can activate dozens and even hundreds of Gαβγ molecules [2]. Therefore, many thousands of G proteins can be found in membrane regions where there is a high density of GPCRs. In the plasma membrane, protein-lipid and lipid-lipid interactions define the membrane lipid structure, which in turn influences the type of proteins found in a given membrane region, as well as the activity of GPCRs and related signaling proteins [3, 4].

When a G protein is activated by a GPCR, the Gα subunit dissociates from the Gβγ dimer. The released dimer remains in the vicinity of the receptor and it recruits GPCR kinases, which inactivate GPCRs and regulate other signaling proteins [5]. By contrast, the Gα monomer regulates the activity of effector proteins (e.g., adenylyl cyclase, phospholipase C and ion channels) often located in different membrane domains, such as lipid rafts [6-8]. The mobilization of each subunit to the correct membrane environment largely depends on their preference for certain lipids or lipid structures. However, the molecular mechanisms underlying G protein interactions with membranes, their mobilization to different domains and their influence on membrane lipid structure remain largely unknown. In the present study we used different approaches to investigate the effect of the co- and post-translational lipid modifications of G proteins on membrane lipid structure and protein-lipid interactions.

The transmembrane domains of the GPCR, such as those of α_2-adrenergic receptor, increase the H_{II} phase propensity of the membrane [9]. Gαβγ and Gβγ proteins are also located preferentially in this nonlamellar-prone environment [4], which may partly explain why G proteins accumulate near GPCRs. On the other hand, certain Gα monomers prefer lamellar-prone regions, such as lipid rafts, explaining how they may be mobilized from the receptor to effector rich membrane domains [3, 4, 6]. Therefore, membrane lipid structure plays an important role in propagating GPCR-mediated signals.

GPCRs frequently cluster in defined membrane regions, where Gαβγ proteins co-localize in molar excess [10]. In these regions, G proteins interact with the cytosolic leaflet of the plasma membrane, aided by the myristoyl and palmitoyl moieties that are associated with the Gα subunit, and the isoprenyl moieties associated with the Gγ subunit [11]. In addition to facilitating

G protein binding to membranes, these lipid anchors may also modify the lipid bilayer environment and the G protein-membrane interactions. The effect of lipid moieties of G proteins on membrane structure and protein-lipid interactions has received little attention to date. Thus, here we have investigated the role of certain lipids on the structural properties of membranes in further detail. For this purpose, we have used model membranes that contain the lamellar-prone phospholipid phosphatidylcholine (PC) and the nonlamellar-prone phospholipid phosphatidylethanolamine (PE), testing their interactions with purified $G\alpha i_1$, $G\beta\gamma$ or $G\alpha\beta\gamma$ proteins in the presence or absence of palmitic acid (PA), myristic acid (MA) or geranylgeraniol (GG). In contrast to other studies in which point mutants were analyzed with or without G protein-anchored lipids [12], this approach enabled us to determine the effect of these lipids on wild type G protein-membrane interactions.

Accordingly, we found that these lipid moieties had different effects on membrane lipid structure, and on the interactions of the G proteins with lamellar- and nonlamellar-prone membranes. In summary, these results show the role of these lipid modifications in the complex interactions between G proteins and membranes and the possible implications in human health are discussed.

2. EXPERIMENTAL

2.1. Materials

Egg yolk PC and bovine liver PE were purchased from Avanti Polar Lipids (Alabaster, AL). MA, PA and GG were obtained from Sigma-Aldrich (Saint Louis, MO). The purified G proteins (myristoylated $G\alpha i_1$, $G\beta\gamma$ and $G\alpha i\beta\gamma$) were from Calbiochem (Darmstadt, Germany), the monoclonal anti-$G\alpha i_1$ antiserum was purchased to Santa Cruz Biotechnology (Santa Cruz, CA) and the monoclonal antibody anti-$G\beta$ was from BD Biosciences (Franklin Lakes, NJ). 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (DiI) and Alexa Fluor 488 C_5 maleimide were procured from Invitrogen (Eugene, OR). ECL Western blot detection system and Hyperfilm were from GE Healthcare (Pittsburgh, PA).

2.2. Differential Scanning Calorimetry

DSC measurements were made with a Microcal MC-2 microcalorimeter (MicroCal Inc., Northampton, MA, USA), as described elsewhere [13]. Briefly, phospholipids were dissolved in chloroform: methanol (2:1, by vol) and dried under an argon flux. Solvent traces were removed

under vacuum for at least 3 h at room temperature before hydration. Multilamellar vesicles were formed by resuspending the lipid film in 10 mM HEPES, 100 mM KCl, 1 mM EDTA, pH 7.4, following vortexing at 42ºC. The mixture was degassed for 5 min and the DSC measurements were then carried out from 10 to 50ºC at a scan rate of 1ºC/min. All samples were subjected to three consecutive scans and calorimetric transitions were found to be reversible. The transition enthalpy and temperature values shown here corresponded to the means of three independent experiments and they were obtained using the software provided by the manufacturer (Microcal Origin).

2.3. 31P-Nuclear Magnetic Resonance

Multilamellar vesicles were prepared by mixing 56 mg of bovine liver PE with deionized deuterated water (D_2O, 15% w/w) in the presence or absence of 5 mol% MA, PA or GG. Lipid suspensions were hydrated and homogenized with a pestle-type minihomogenizer (Sigma), and vortexed to homogeneity. The suspensions were then subjected to 10 cycles of heating (60°C) and freezing (-80°C), and then equilibrated before data acquisition, as reported previously [14]. ^{31}P-NMR measurements were made in 5 mm tubes on an Advance-300 multinuclear NMR spectrometer (Bruker Instruments). Data were acquired every 5ºC between 5 and 55ºC, equilibrating the temperature for 15 min before each measurement. The accumulated ^{31}P-NMR free induction decay was obtained for 128 transients using a 4.4 μs 90° radio-frequency pulse, a 24.3 kHz sweep width and 65,000 data points. The delay between the transients was 2 s and spectra were obtained by scanning from lower to higher temperatures.

2.4. Molecular dynamics

Two all-atom lipid bilayers were used for symmetric membrane models containing 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphatidylcholine: 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphatidylethanolamine (POPC:POPE) (6:4; mol ratio) and POPC. The POPC membrane was made with 98 POPC molecules, 33 Na^+ ions and 33 Cl^- counter ions, and 11,301 water molecules. The POPC:POPE membrane was made with 58 POPC and 40 POPE molecules, 28 Na^+ ions and 28 Cl^- counter ions, and 9,825 water molecules. In both cases, the water density was of 0.997 g/mL. Simulations were performed using the YASARA program [15] at 310 K and 1 atm under a NPT ensemble, coupling the system to a Berendsen thermostat and barostat [16] combined with a control of solvent density as implemented in the software Yasara. The AMBER03 force field was used and the geometry of the molecules was optimized by the semi-

empirical AM1 method, using the COSMO solvation model [17]. Partial atomic charges were calculated using the same level of theory as the Mulliken point charge approach [18]. Electrostatic interactions were calculated with a 10.48 Å cut-off, and the long-range electrostatic interactions were handled by the particle mesh Ewald (PME) algorithm [19] using a sixth-order B-spline interpolation and a grid spacing of 1 Å. The leapfrog algorithm was used in all simulations with a 1.25 fs step time for intramolecular forces and a 2.5 fs step time for intermolecular forces.

Five types of molecules (MOL_SET) were included to both membrane systems: GG, MA, PA, myristic alcohol (MOH) and palmitic alcohol (POH). The lipid bilayers were assembled and relaxed, reducing the box dimension until the Van der Waals energy of the system started to increase, and the structural parameters of the membranes were then compared using the experimental data [20]. To avoid atom-atom bumps and abnormal non-covalent interactions, the size of the MOL_SET molecules was initially reduced to 20% of the original size and the non-covalent interactions to 10% of their normal value [21]. The MOL_SET molecules were then placed at 160 different positions across the membrane using a software specially designed for this purpose [22]. The size and energy constants were then gradually increased until they reached normal values through cycles of steepest descent minimization, and a cycle of annealing was undertaken until the speed of the fastest atom dropped below 500 m/s. For each membrane/lipid system, the minimum in potential energy was then heated to 310 K and equilibration dynamics of 50 ns were completed. The lateral pressure profile was calculated as described [23-25] and the result are expressed as the average of 5 snapshots of the last 5 ns of simulations.

Free energy of insertion was estimated by means of metadynamics calculations [26] using the Desmond program [27] under periodic boundary conditions. Simulations were performed in an isothermal-isobaric ensemble (1 atm, 310 K) with Langevin barostat and thermostat. The metadynamics simulations were carried out after equilibration. The free energy profile G(z) associated with lipid molecule translocation through the lipid bilayer was calculated along the z-component of the distance vector joining the membrane and the lipid molecule center of mass (z-dist). For this study, the distance between one headgroup oxygen of each molecule of MOL_SET and the center of the membrane was chosen as collective variable. A second collective variable was the distance between the terminal carbon of each molecule of MOL_SET and the center of the membrane. The time interval between the addition of two Gaussian functions, τ, as well as

the Gaussian height, w, and Gaussian width, δ, were tuned to optimize the ratio between accuracy and computational cost. We used: τ = 100 fs, w = 0.2 kJ/mol, δ = 0.5 Å. The free energy of the insertion of lipid molecules in the membrane was calculated as the difference between the minimum energy of the molecule inserted in membrane and the free energy in water.

2.5. G protein binding to large unilamellar vesicles (LUVs)

LUVs containing different molar ratios of PC:PE were prepared in the presence or absence of 5 mol% PA, MA or GG. The lipids were dissolved in chloroform/methanol (2:1) and mixed at the appropriate volumes. The solvent was evaporated under argon flux and solvent traces were removed under vacuum for at least 3 h. Lipid films were hydrated in 10 mM HEPES, 100 mM KCl, 0.1 mM EDTA, pH 7.4 at 42°C for 1 h, with vigorous vortexing every 15 min. The lipid suspension was submitted to five freeze/thaw cycles and was sonicated in a probe-type sonicator from Branson (Danbury, CT) for 10 s at 15 W. The LUVs were then incubated for 1 h at 37°C with purified Gαβγ (300 ng), Gβγ dimers (100 ng) or $G\alpha i_1$ monomers (150 ng) in a total volume of 200 μl. The binding of Gαβγ to membranes was carried out in the presence of GDPβS (50 μM) and that of the $G\alpha i_1$ monomers in the presence of GTPγS (50 μM). Unbound G proteins were then separated from membrane-bound G proteins by centrifugation for 1 h at 25°C at 100,000 × g. Finally, the membrane pellets were resuspended in electrophoresis loading buffer (84 mM Tris-HCl, pH 6.8, 4% SDS, 1% 2-mercaptoethanol, 5% glycerol, 0.01% bromophenol blue) and boiled for 5 min. In all experiments, myristoylated Gα subunits and geranylgeranylated Gγ subunits were used.

Immunoblot and quantification of bound G proteins- Immunoblotting was performed as described elsewhere [28]. Briefly, samples from the binding experiments were resolved on 10-20% gradient SDS-polyacrylamide gels and the proteins were then transferred to nitrocellulose membranes. The membranes were blocked with PBS containing 5% non-fat dry milk, 0.5% bovine serum albumin and 0.02% Tween-20 (blocking solution), and they were then incubated with anti-$G\alpha i_1$ (1:1000 dilution in fresh blocking solution) to detect $G\alpha i_1$ and Gαβγ, or anti-Gβ (1:1000) to detect Gβγ. Antobody binding was detected with a horseradish peroxidase-linked anti-mouse IgG (1:2000) in fresh blocking solution, which was visualized by ECL. The immunoreactive bands on the films were quantified by image analysis and the binding of G proteins to pure PC liposomes in the absence of other lipids was considered as the control value (100%). For each ratio of PE, the relative effect of PA, MA and GG on G protein binding was

compared to the same membrane without any lipid moiety and indicated in parentheses in Table 1.

2.6. Confocal microscopy

Giant unilamellar vesicles (GUVs) were prepared using the electroformation method [29, 30]. For this purpose, lipid solutions containing 0.3 mM total lipid supplemented with 0.4 mol% DiI were prepared in chloroform: methanol (2:1; v:v). Three µl of the lipid mixture were added to the surface of platinum electrodes and solvent traces were removed under vacuum for 60 minutes. Platinum electrodes were covered with 400 µl of 25 mM HEPES, pH 7.4, previously heated at 50ºC. The platinum wires were connected to an electric wave generator at 50ºC under the following AC field conditions: 500 Hz, 0.22 V for 5 min; 500 Hz, 1.9 V for 20 min and finally 500 Hz, 5.3 V for 90 min. After GUV formation, the chamber was placed on a Leica TCS SPE inverted confocal fluorescence microscope (Barcelona, Spain).

The GVMD software from the Beckman Institute (University of Illinois) was used to localize free cysteine residues on the surface of $G\alpha i_1$, $G\beta\gamma$ and $G\alpha i\beta\gamma$ in order to be used for protein labeling (see Fig. S1) [31]. In brief, free cysteine residues were labeled with Alexa 488 C_5-maleimide by mixing 10 µl of 0.8 µg/ml protein with 0.5 µl Alexa Fluor 488 (10 µg/ml stock solution) for 10 min at RT. Fluorescently-labeled G proteins were added to GUVs at a final concentration of 15 ng/ml. The binding of $G\alpha\beta\gamma$ to membranes was carried out in the presence of GDPβS (50 µM) and that of the $G\alpha i_1$ monomers in the presence of GTPγS (50 µM). The excitation wavelength for DiI was 532 nm and the emission was collected at 555-750 nm; the excitation for Alexa Fluor 488 was 488 nm and the emission 500-533 nm. The binding of fluorescently labeled G protein subunits to the GUVs was measured using the software provided with the microscope. The fluorescence signal sorrounding the lipid membrane was used as background.

2.7. Data analysis

The data shown correspond to mean ± SEM values from the number of experiments indicated. One-way ANOVA followed by a Bonferroni test or two-tailed *t*-test was used for statistical evaluation. Differences were considered statistically significant at $p < 0.05$.

3. RESULTS

3.1. Effects of PA, MA and GG on membrane lipid structure

In the temperature range studied (15 - 45ºC), DSC showed a lamellar-to-inverted hexagonal (H_{II}) phase transition peak for bovine liver PE at 22.4ºC (Fig. 1A). The presence of MA and PA increased the lamellar-to-inverted hexagonal phase transition temperature (T_H) value to 31.2ºC and 28.5ºC, respectively. By contrast, when GG is added no transition is observed in the studied range of temperatures, although ^{31}P-NMR experiments indicated that this lipid decreased the T_H value, suggesting that it favored the occurrence of nonlamellar phases (Fig. 1B) [32]. When assessed by ^{31}P-NMR, PE organized into lamellar phases at temperatures below 20°C; between 20ºC and 25ºC, both lamellar and H_{II} phases co-existed, and at higher temperatures ($\geq$30°C), PE molecules adopted H_{II} phases. ^{31}P-NMR scans also showed that MA and PA increased the T_H, whereas GG decreased it about 10ºC (Fig. 1B).

The binding free energies of GG, PA, POH, MA and MOH to POPC:POPE and POPC membranes were calculated by computational analysis (Fig. 2 and S2). MOH and POH were studied to isolate the effect of the acyl chain and to compare their effect on lipid membranes with GG, which shares the same alcohol headgroup. MA and PA, which are negatively charged at pH 7.4, exhibited greater binding energies than GG, POH and MOH, which lack of net charge. On the other hand, the binding of GG to POPC:POPE membranes was ~6 kcal/mol higher than that to POPC bilayers, indicating a preference of GG moieties for H_{II}-prone domains. This difference is due to the balance between the hydrophobic match of the isoprenyl chain in the membrane and the hydrogen bonds between the hydroxyl group and water molecules. The differences in free energies for MA binding to POPC and POPC:POPE membranes were larger than those of GG, although the former preferred lamellar (POPC) membranes. In addition, MA showed an even higher propensity to associate with lamellar membranes than PA. These results suggest that GG rapidly segregates to membrane domains rich in the nonlamellar prone phospholipid PE, while PA and MA prefer lamellar-prone membrane domains. In addition, this binding behavior also contributes to explain the membrane microdomain preference of the Gγ-containing G proteins (Gαβγ and Gβγ complexes with an isoprenyl moiety) and Gα monomers containing MA and/or PA.

The presence of each type of lipid has an important effect on the lipid membrane organization. Indeed, POPC membranes displayed an altered stress profile after the addition of any of G protein lipids, MA, PA and GG (Fig. 3A), indicating that regions rich in G proteins may undergo structural lipid regulations that could contribute to control the localization and activity of certain

proteins [23]. In the surface region, at circa 23 Å away from the center of the membrane, MA and PA reduced the positive pressure among charged choline headgroups, whereas GG showed no effect. Immediately under the membrane surface, between 15 and 18 Å away from the membrane barycenter, PA increased the negative stress of more than 100 bars. This reduction, large in absolute terms, might have an impact in the membrane permeability [33]. MA showed only a minor shift of the minimum position from 13 to 18 Å away from the center. GG, that is shorter than PA, had a reduced effect on the lateral stress at 17 Å. Notably, because of the length of PA, MA and GG, the inner core of the membrane maintained the same level of positive stress.

By contrast, H_{II}-prone membranes composed of POPC:POPE seemed to resist the perturbation caused by G protein lipids better than lamellar-prone membranes (Fig. 3B). Only GG, contrarily to what observed in pure POPC membrane, reduced the lateral stress among choline groups. The binding energies and lateral pressure profiles further indicated that GG preferentially interacts with H_{II}-prone regions, inducing only minor perturbations in these membrane domains. However, MA and PA accumulated in lamellar-prone domains, consequently altering the physical states of these domains. As shown in Fig. S3, the effects of MOH and POH on POPC and POPC:POPE membranes were similar to those of MA and PA, respectively.

3.2. The effect of lipids on the binding of Gαi1 monomers to model membranes

Under the experimental conditions used (25°C), PC membranes organized into lipid bilayers and PE mainly into H_{II} structures (Fig. 1). Both structures were formed in the PC:PE mixtures at a ratio that reflected their relative abundance [4]. The presence of the nonlamellar-prone phospholipid, PE, inhibited the binding of $G\alpha i_1$ monomers to membranes in a concentration-dependent manner. Thus, considering that the amount of $G\alpha i_1$ monomers bound to pure PC membranes was 100%, PC:PE vesicles containing 20% PE (8:2; mol ratio) and 40% PE (6:4; mol ratio) bound only 89.5 ± 3 .9% and 68.7 ± 2.7% of the monomers, respectively (Fig. 4, Table 1). Higher proportions of PE reduced $G\alpha i_1$ binding to 36.6 ± 5.5% and 22.9 ± 3.2% for PC:PE molar ratios of 4:6 and 2:8, respectively (Table 1). Confocal microscopy analysis of Gαi1 binding to GUVs showed higher affinity of the protein to pure PC (11.18 ± 1.23 a.u., arbitrary units) than to PC:PE (6:4, mol:mol) membranes (0.14 ± 0.3 a.u.). Moreover, $G\alpha i_1$ binding to lipid membranes could not be detected when the PE content increased (Fig. 5 and Table 2).

In the presence of PA, the binding of $G\alpha i_1$ monomers to lamellar-prone PC membranes increased to 123.6 ± 7.4% when compared to control membranes (Fig. 4 and Table 1). By contrast, the presence of this fatty acid did not significantly affect the binding of G protein monomers to H_{II}-prone (PE-rich) membranes. Conversely, MA significantly decreased the binding of $G\alpha i_1$ to membranes with low PE content (-27.6% and -21.4% at 8:2 and 6:4 mol ratios, respectively), while it increased monomer binding to membranes with higher PE content (31.1% and 41.5% at 4:6 and 2:8 mol ratios, respectively, Fig. 4 and Table 1) with respect to the same molar ratio of control vesicles (without PA). Finally, GG reduced the binding of $G\alpha i_1$ proteins to nonlamellar-prone membranes, an effect that was particularly significant for membranes with a PC: PE molar ratio of 6: 4, similar to that found at the inner leaflet of the plasma membrane [34] (Fig. 4, Table 1).

3.3. The effect of lipids on the binding of Gβγ dimers to model membranes

The binding affinity of Gβγ proteins to LUVs and GUVs increased directly with PE content (Fig. 4 and 5, Tables 1 and 2), reaching the maximum binding at PC:PE molar ratios of 2:8 in LUVs (334.7 ± 9.8%) (Table1) and GUVs (14.82 ± 1.08 a.u.) (Table 2). The presence of PA increased the binding of Gβγ dimers to membranes with a PE content similar to that found in biological membranes. Relative increases of 21.9% and 20.4% were observed for PC:PE molar ratios of 8:2 and 6:4, respectively (Fig. 4, Table 1). However, MA only caused a significant increase in the binding of Gβγ to model membranes at 8:2 (PC:PE) mol ratio (Fig. 4, Table 1). Finally, the isoprenoid GG enhanced Gβγ binding to model membranes containing little or no PE (Fig. 4, Table 1).

3.4. The effect of lipids on the binding of heterotrimeric Gαβγ protein to model membranes

In line with the data for Gβγ, the presence of PE also significantly augmented the binding of Gαβγ heterotrimers to membranes. Indeed, its maximal binding was observed at 2:8 (PC:PE) mol ratios in both LUVs (Fig. 4, Table 1), and GUVs (Fig. 5, Table 2). The effect of PA and MA on Gαβγ binding to membranes was similar to that observed for Gβγ binding. PA increased the binding of Gαβγ to membranes with 6:4 (PC:PE) molar ratio, while MA did not affect heterotrimer binding (Fig. 4, Table 1). Finally, GG induced a significant increase in the binding of Gαβγ to membranes containing the nonlamellar-prone phospholipid PE, with a maximum at a

PC:PE mol ratio of 6:4 (Fig. 4, Table 1). Moreover, the isoprenoid did not significantly alter the binding of Gαβγ to lamellar-prone vesicles of pure PC.

4. DISCUSSION

G proteins can bear various lipid moieties in their different monomeric or oligomeric forms. In membrane regions enriched in GPCRs, where G protein dimers and trimers can be found, or in effector regions where monomeric Gα proteins accumulate, thousands of G protein-linked lipids are inserted into the bilayer structure. PA is reversibly linked to cysteine residues in the amino-terminal region of Gαi and other Gα protein subunits [35]. By contrast, MA is irreversibly linked to glycine residues in the amino-terminal region of various Gα proteins and it appears along with PA in the Gαi, Gαo and Gαz proteins [11]. The isoprenoid GG is covalently and irreversibly linked to cysteine residues in C-terminal region of most types of Gγ subunits [36]. Hence, we studied the effects of these lipids on the structure of model membranes and their interactions with G proteins. The different lipid moieties of G proteins mentioned above, MA, PA and GG, are present in Gαiβγ in equimolar amounts and therefore this complex is an ideal model for the study of the role that lipid modifications play in the signal transduction through G proteins. Thus, G proteins of other families may respond in a similar way than Gαiβγ when comparing the effect of lipid moieties.

4.1. Membrane lipid structure and protein-lipid interactions

Cell membranes are formed by a wide variety of lipids that confer specific physico-chemical properties. These lipids are not homogeneously distributed in the membrane but rather, they form membrane microdomains that have a distinct protein and lipid composition to the surrounding membrane regions, as well as different functions. Indeed, it is not uncommon for GPCRs to cluster in receptor-rich domains [37] that can also concentrate large number of G proteins and their associated lipids. The accumulation of these associated proteins and lipids further contributes to the biophysical properties of the lipid bilayer, thereby constituting another mechanism that regulates the sorting of these and other membrane proteins to different microdomains. The present study was designed to study the effect of the lipids present in G proteins on the structural properties of membranes and the binding behavior of G protein monomers, dimers and trimers to model membranes with lamellar- and nonlamellar-prone structures.

The transmembrane regions of GPCRs can increase the H_{II} phase propensity of membranes [9]. The inner leaflet of most mammalian cell membranes, the region where GPCRs and G proteins interact, contains a 30-50 mol% of the H_{II}-prone phospholipid PE [34]. Hence, the membrane areas surrounding some transmembrane receptors are likely to have a high nonlamellar phase propensity. We investigated here the effect of G protein lipid modifications on membrane structure and we found that such modifications influenced the lamellar–to-H_{II} phase transition. Thus, the isoprenyl moiety associated with most Gγ subunits, GG, favored the formation of nonlamellar-prone structures in PE-containing membranes, unlike MA and PA. Accordingly, the presence of large amounts of G proteins in a given membrane region may induce important changes in the lipid structure, influencing the interaction and function of these transducers. It has become apparent in recent years that receptors, transducers, effectors and other proteins involved in cell signaling are confined to, or are enriched in, defined membrane regions [38, 39]. In this context, protein-lipid interactions play a relevant role in the localization of G proteins to specific membrane microdomains, thereby influencing their activity [40].

4.2. G protein binding to model membranes

We used membranes containing 100% PC as a model for lamellar membrane structures. Furthermore, we examined other situations resembling the bulk of the inner leaflet of the membrane (PC: PE, 6:4; mol:mol) or discrete membrane regions or microdomains with different nonlamellar phase propensities (different PE contents). An increase in the proportion of PE induced gradual decreases in $G\alpha i_1$ monomer binding to model membranes. The higher binding energies of PA and MA to POPC over POPC: POPE membranes were consistent with their distribution to lamellar-prone regions confirmed in our binding studies and by the fact that Gα proteins localize to lipid rafts (5 and references therein). By contrast, heterotrimeric Gαβγ subunits exhibit a greater affinity for nonlamellar (H_{II}) phases, most likely due to the Gβγ dimer exhibiting greater affinity for membranes containing PE. Thus, the Gβγ dimer is responsible for transporting the Gα monomer to the receptor molecule in H_{II}-prone membranes.

4.3. The effect of fatty acids on the binding of G proteins to model membranes

N-terminal thioacylation aids the membrane docking of G proteins and it plays a crucial role in triggering GPCR signaling [11, 41]. Moreover, fatty acids regulate the structure of the lipid bilayer [42], which in turn controls G protein-membrane interactions. The binding of Gα to lamellar-prone membranes (100 mol% PC) is increased when membranes are enriched in PA

(Table I). Indeed, there is considerable Gα protein associated to highly ordered lamellar phases (membrane rafts) that interacts with signaling effectors to propagate incoming messages [43]. However, PA only favored the binding of Gβγ dimers and Gαβγ heterotrimers to nonlamellar-prone membranes (PC:PE, 8:2 and 6:4, mol ratio), the preferred lipid structure of both G protein oligomers that are consequently found in the vicinity of GPCRs. These results suggest that palmitoylation could be an additional selective mechanism to segregate G protein monomers, dimers and trimers into different membrane environments. This is consistent with previous studies showing that G protein palmitoylation is necessary for the propagation of signals through adrenergic receptors [41]. In fact, palmitoylation of membrane proteins is known to be involved in regulating both membrane lipid structure and membrane lipid-protein interactions [44].

MA reduced $G\alpha i_1$ binding to PC:PE membranes containing 8:2 and 6:4 molar ratios, although it did not significantly alter the binding of heterodimeric proteins to lamellar- and nonlamellar-prone membranes. Thus, MA may further contribute to the mobilization of G protein monomers from receptor environments to effector microdomains together with PA. In addition, modifications to lateral pressure provoked by the presence of G protein lipids in model bilayers suggest that they could favor the accumulation of G protein oligomers in the membrane regions where they are present (Fig. 3, Fig. S3).

In summary, MA and PA induced changes in the binding of G proteins that depend on the type of G protein (monomeric, dimeric or trimeric) and the membrane composition (PC: PE molar ratio). These results imply that the fatty acids associated with Gα subunits not only participate in the attachment of G proteins to membranes but also, in the interactions and sorting of G proteins to different membrane domains.

4.4. The effect of GG on G protein binding to model membranes

In general, GG induced the most relevant changes in the binding of the different forms of G proteins to lipid bilayers, further demonstrating the relevance of the Gγ subunit in membrane binding. In nonlamellar-prone membranes, GG significantly reduced the binding of Gαi protein to membranes with a PC:PE content similar to that found at the inner leaflet of the plasma membrane (6:4 and 4:6, mol ratio). By contrast, this lipid significantly increased the binding of Gβγ and Gαβγ to nonlamellar prone membranes. Gβγ dimers and Gαβγ trimers bear a GG moiety and associate preferentially with nonlamellar-prone regions, whereas the anchorage of Gα monomers is favored in lamellar-prone structures. Moreover, GG moieties could be

responsible for the preference of G protein dimers and trimers for nonlamellar phases (such as those containing PE) since the bulky branched structure of this lipid is excluded from highly ordered bilayer structures (e.g., membrane rafts) [45]. Furthermore, the C-terminal region of the Gγ subunit favors the formation of nonlamellar-prone membrane domains enriched in isoprenyl moieties [46, 47]. Indeed, this and other studies support the important role of the C-terminal region of the Gγ subunit, including the GG moiety, in the localization and activity of G proteins [4, 46, 47]. The fact that Gβ does not participate in G protein-membrane interactions [48] illustrates the pivotal role of the Gγ subunit in the binding of G protein oligomers to membranes.

4.5. Lipids in signal transduction and their implication in human pathologies and therapies

Co- or post-translational lipid modifications of G proteins have been associated with their anchorage to membranes. This study highlights other roles of these lipid moieties as regulators of membrane structure and modulators of G protein-membrane interactions, suggesting their participation in G protein sorting to different membrane microdomains *in vivo*. Thus, G protein lipids would determine G protein location in membranes and G protein location in specific microdomains would be necessary for their interaction with proteins. In particular, Gαβγ-GDP complexes (inactive state) may prefer PE-rich membrane microdomains with a high non-lamellar propensity [4]. Upon agonist-mediated activation, each receptor molecule can activate several G proteins and then activated Gαi1 dissociates from Gβγ. Gβγ dimer may remain in PE-rich membrane domains while the Gα monomer would prefer lamellar regions with a higher PC content. Marked preference of Gαi1 for raft-like lamellar microdomains would facilitate its interaction with effector proteins located in those membrane regions, such as adenylyl cyclase [4, 8].

Like other membrane proteins, G proteins can interact with various downstream proteins. The correct and alternative propagation of messages through different effectors depends to some degree on the co-localization of G proteins with other proteins for productive protein-protein interactions. In this context, the most prominent effect on membrane lipid structure was observed with GG, which markedly increased the nonlamellar phase propensity and might explain why this lipid significantly increased the binding of Gβγ and Gαβγ to nonlamellar-prone membranes. However, MA favors the mobilization of $G\alpha i_1$ away from the receptor rich (nonlamellar-prone, PE-rich) environment in membranes. By contrast, PA appears to help $G\alpha i_1$ subunits localize to

lamellar-prone regions, and Gβγ and Gαβγ to nonlamellar-prone microdomains, where these proteins can participate in productive interactions with specific signaling effectors. Finally, both MA and PA regulate membrane fluidity, which could modulate the activity of GPCRs and other membrane proteins [49].

It has been shown recently that membrane lipid composition and its structural regulation influences physiological processes such as blood pressure, platelet aggregation, cell proliferation and apoptosis, as well as underlying the mechanism of action of certain drugs [50-52]. The regulatory effect of membrane lipid composition on the localization and activity of peripheral and integral proteins can be partly explained by changes in the lipid bilayer lateral pressure [53] or fluidity [54]. Treatment with lipids or lipid-interacting molecules can regulate the composition and structure of membranes, reversing important pathological alterations such as cancer, hypertension or obesity [32, 50]. This novel therapeutic strategy, called "membrane-lipid therapy", is based on the regulation of the activity of important signaling proteins by modulating the reorganization of membrane microdomains [55] and the subsequent protein-lipid interactions [50, 56]. By contrast to the general opinion that interventions on membranes could affect a large number of processes, this approach has been shown to be highly specific [52, 57], further demonstrating that the structure-function relationships of membrane lipids can be finely regulated. Thus, the present study sheds further light on the molecular mechanisms governing pharmaceutical and nutraceutical therapies targeting membrane lipids.

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Table 1 Binding of G proteins to LUVs in the presence or absence of PA, MA and GG

Binding of $G\alpha i_1$, $G\beta\gamma$ and $G\alpha\beta\gamma$ to LUVs of PC and PE at various molar ratios, in the presence or absence of 5 mol% PA, MA or GG. The binding of these G proteins to PC:PE (10:0, mol:mol) membranes in the absence of PA, MA or GG was considered 100%. Results are expressed as average values ± SD of 3 experiments. *t*-tests were used to determine statistical significance * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. The data in parentheses are the percent change (positive or negative) with respect to the corresponding control model membrane (same PC:PE ratio), considering the binding of each G protein to each membrane composition without PA, MA or GG as 0% change.

$G\alpha i_1$				
PC:PE	**Control**	**PA**	**MA**	**GG**
10:0	100.0± 2.6 (0)	123.6 ± 7.4* (23.6)	109.3 ± 7.1 (9.3)	86.8 ± 7 (-13.2)
8:2	89.5 ± 3.9 (0)	98.4 ± 11.4 (9.9)	64.8 ± 8.1* (-27.6)	74.3 ± 12.8 (-17)
6:4	68.7 ± 2.7 (0)	66.1 ± 9.7 (-3.8)	54.0 ± 7.4* (-21.4)	52.8 ± 7.1* (-23.2)
4:6	36.6 ± 5.5 (0)	41.3 ± 7.5 (12.8)	48.0 ± 6* (31.1)	28.5 ± 6.6 (-22.2)
2:8	22.9 ± 3.2 (0)	23.8 ± 3.6 (3.9)	32.1 ± 3.9* (41.5)	20.1 ± 6.7 (-12.3)

$G\beta\gamma$				
PC:PE	**Control**	**PA**	**MA**	**GG**
10:0	100.0± 1.9 (0)	86.0 ± 12.7 (-14.0)	104.5 ± 16.6 (4.5)	125.0 ± 12.2* (25.0)
8:2	134.9 ± 15.8 (0)	164.5 ± 5.5* (21.9)	170.1 ± 12.3* (26.1)	169.6 ± 5.4* (25.7)
6:4	207.9 ± 16.7 (0)	250.3 ± 12.2* (20.4)	210.3 ± 24.5 (1.1)	266.6 ± 5.9** (28.2)
4:6	289.3 ± 24.8 (0)	287.5 ± 47.2 (-0.6)	309.7 ± 51.0 (7.5)	298.4 ± 29.6 (9.1)
2:8	334.7 ± 9.8 (0)	277.3 ± 33.9 (-17.2)	374.5 ± 13.5 (11.9)	339.5 ± 40.3 (1.4)

$G\alpha i_1\beta\gamma$				
PC:PE	**Control**	**PA**	**MA**	**GG**
10:0	100.0± 3.4 (0)	118.1 ± 18.5 (18.1)	95.2 ± 18.2 (-4.8)	108.8 ± 8.8 (8.8)
8:2	140.1 ± 5.4 (0)	175.3 ± 20.9* (25.1)	141.3 ± 26.8 (0.8)	179.0 ± 6.7* (27.8)
6:4	166.3 ± 14.5 (0)	228.8 ± 28.7** (37.6)	172.2 ± 23.9 (3.5)	288.8 ± 23.0** (73.7)
4:6	225.7 ± 17.9 (0)	233.2 ± 35.7 (3.3)	224.3 ± 30.9 (-0.6)	331.2 ± 32.1* (46.7)
2:8	272.5 ± 13.0 (0)	285.7 ± 30.1 (4.8)	267.7 ± 36.8 (-1.8)	371.1 ± 51.3* (36.2)

Table 2 Binding of G proteins to GUVs assessed by confocal microscopy.

$G\alpha i_1$, $G\beta\gamma$ and $G\alpha i_1\beta\gamma$ were fluorescently labeled with Alexa Fluor 488 and the binding to GUVs composed of PC:PE was determined from the amount of fluorescence in the membrane vesicle (a.u., arbitrary units), using the fluorescence of surrounding areas as background. Data are expressed as average values ± S.D of at least 5 vesicles per condition. *t*-tests were used to determine statistical significance * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

PC:PE	$G\alpha i_1$	$G\beta\gamma$	$G\alpha i_1\beta\gamma$
10:0	11.18 ± 1.23	0.53 ± 0.23	1.07 ± 0.76
8:2	3.84 ± 0.88***	1.18 ± 0.18	2.94 ± 0.98
6:4	0.41 ± 0.3***	3.6 ± 0.56*	3.35 ± 1.11*
4:6	No binding	6.04 ± 0.66**	7.47 ± 0.37***
2:8	No binding	14.82 ± 1.08***	10.51 ± 0.59***

FIGURE LEGENDS

Figure 1 The effect of G protein lipids on membrane lipid structure.

(**A**) DSC thermograms of bovine liver PE membranes in the presence or absence of 5 mol% PA, MA or GG. The peaks correspond to the lamellar-to-hexagonal phase transition. (**B**) ^{31}P-NMR of bovine liver PE membranes in the presence or absence of 5 mol% PA, MA or GG. NMR scans were recorded at the temperatures indicated on the left of the panel.

Figure 2 Binding energies of GG, MA and PA to model membranes.

Binding energies of the G protein lipids to POPC (filled bars) and POPC:POPE (6:4, mol:mol) (grey bars) membranes. The bars correspond to the mean binding energy values ± S.D. of three simulation experiments.

Figure 3 Effects of GG, MA and PA on the bilayer lateral pressure.

The figure shows the lateral pressure in (**A**) POPC and (**B**) POPC:POPE (6:4, mol:mol) membranes in the absence (black line) or presence of GG (red line), MA (green line) and PA (blue line). The X-axis indicates the distance from the center of the membrane.

Figure 4 Effects of G protein lipids on the binding of G proteins to lipid bilayers.

Model membranes composed of PC:PE (10:0) or PC:PE (6:4; molar ratio), in the presence (gray bars) or absence (filled bars) of 5 mol% PA, MA or GG. Bars correspond to the mean ± SEM of five independent experiments for the binding of $G\alpha i_1$, $G\beta\gamma$ and $G\alpha\beta\gamma$ to lipid bilayers. Representative immunoblots of each graph are shown above each histogram. Anti-$G\alpha i_1$ was used to detect $G\alpha i_1$ and $G\alpha\beta\gamma$, or anti-$G\beta$ to detect $G\beta\gamma$. * indicates statistical significance ($p < 0.05$) compared to the same membrane composition in the absence of PA, MA or GG.

Figure 5 Confocal images of G protein binding to GUVs.

$G\alpha i_1$, $G\beta\gamma$ and $G\alpha\beta\gamma$ were fluorescently labeled with Alexa Fluor 488 and incubated with GUVs composed of different mol ratios of PC:PE (10:0, 8:2, 6:4, 4:6, 2:8). Lipid membranes were stained with DiI and are shown in yellow, while the fluorescently labeled G proteins appear in red. Binding of G protein subunits to lipid vesicles was assessed by quantification of the Alexa 488 fluorescence signal associated to membranes. The fluorescence surrounding the vesicle was used as background. Bar=10 μm.

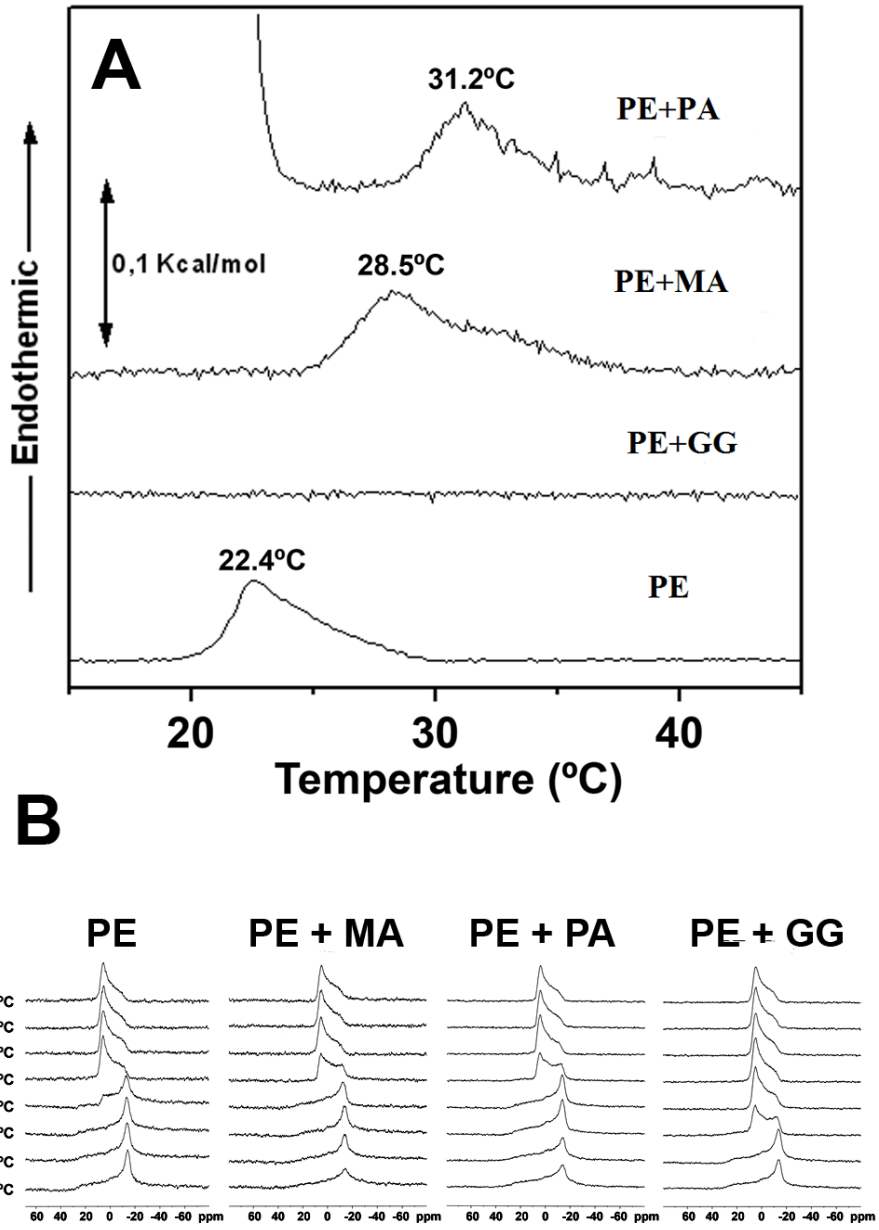

A
31.2ºC
PE+PA
0,1 Kcal/mol
28.5ºC
PE+MA
PE+GG
22.4ºC
PE
Endothermic
20
30
40
Temperature (ºC)
B
PE
PE + MA
PE + PA
PE + GG
40ºC
35ºC
30ºC
25ºC
20ºC
15ºC
10ºC
5ºC
60 40 20 0 -20 -40 -60 ppm

Figure 1

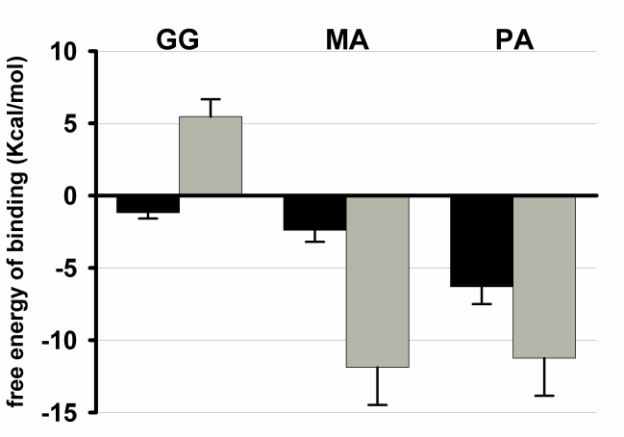
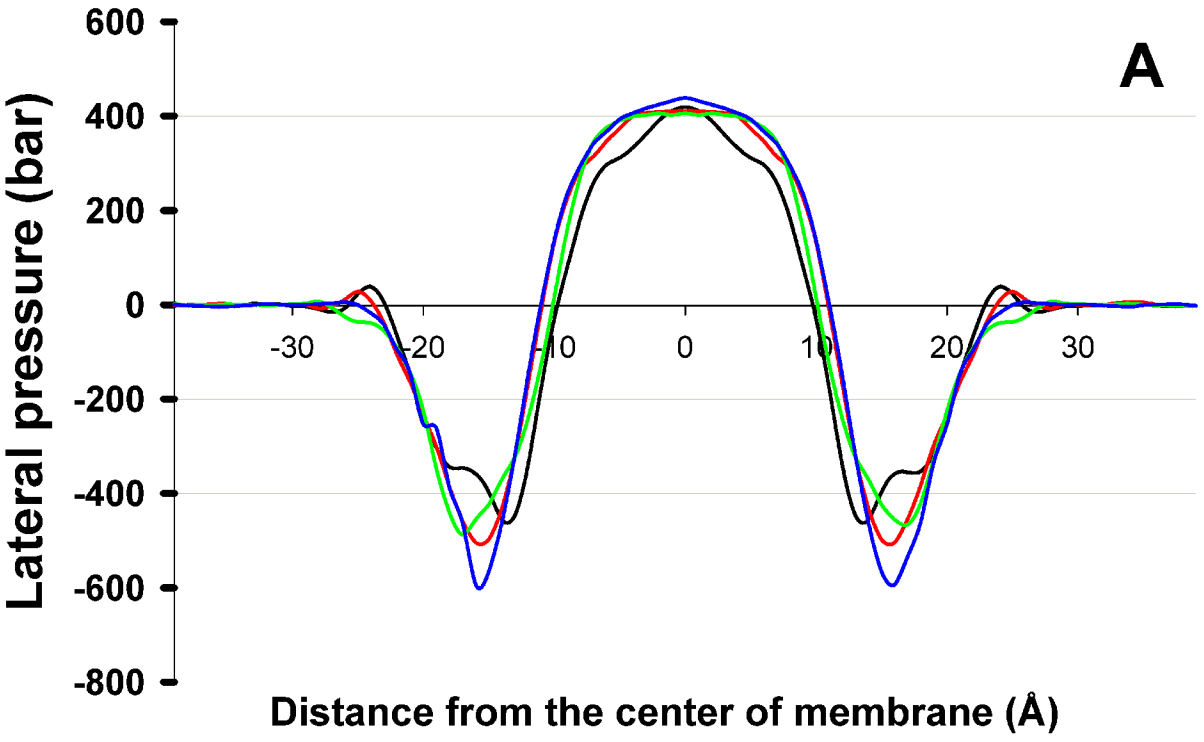


Figure 2


A
Lateral pressure (bar)
600
400
200
0
-200
-400
-600
-800
-30
-20
-10
0
10
20
30
Distance from the center of membrane (Å)

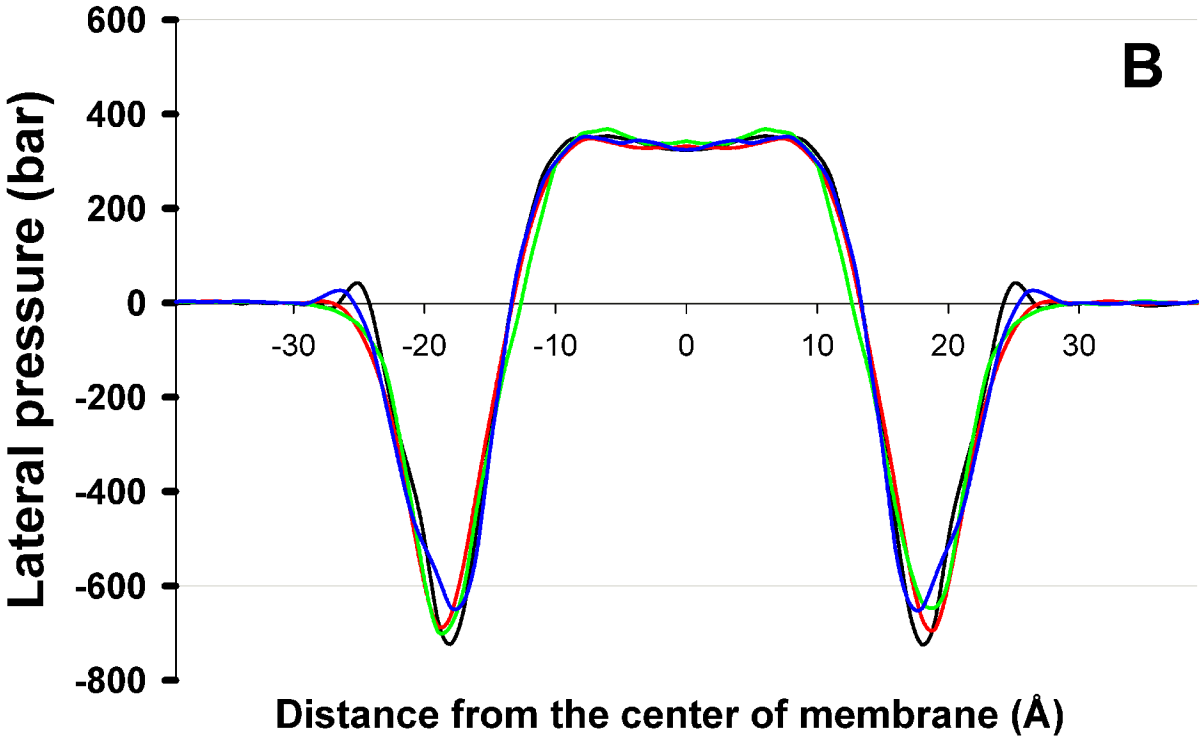

B
Lateral pressure (bar)
600
400
200
0
-200
-400
-600
-800
-30
-20
-10
0
10
20
30
Distance from the center of membrane (Å)

Figure 3

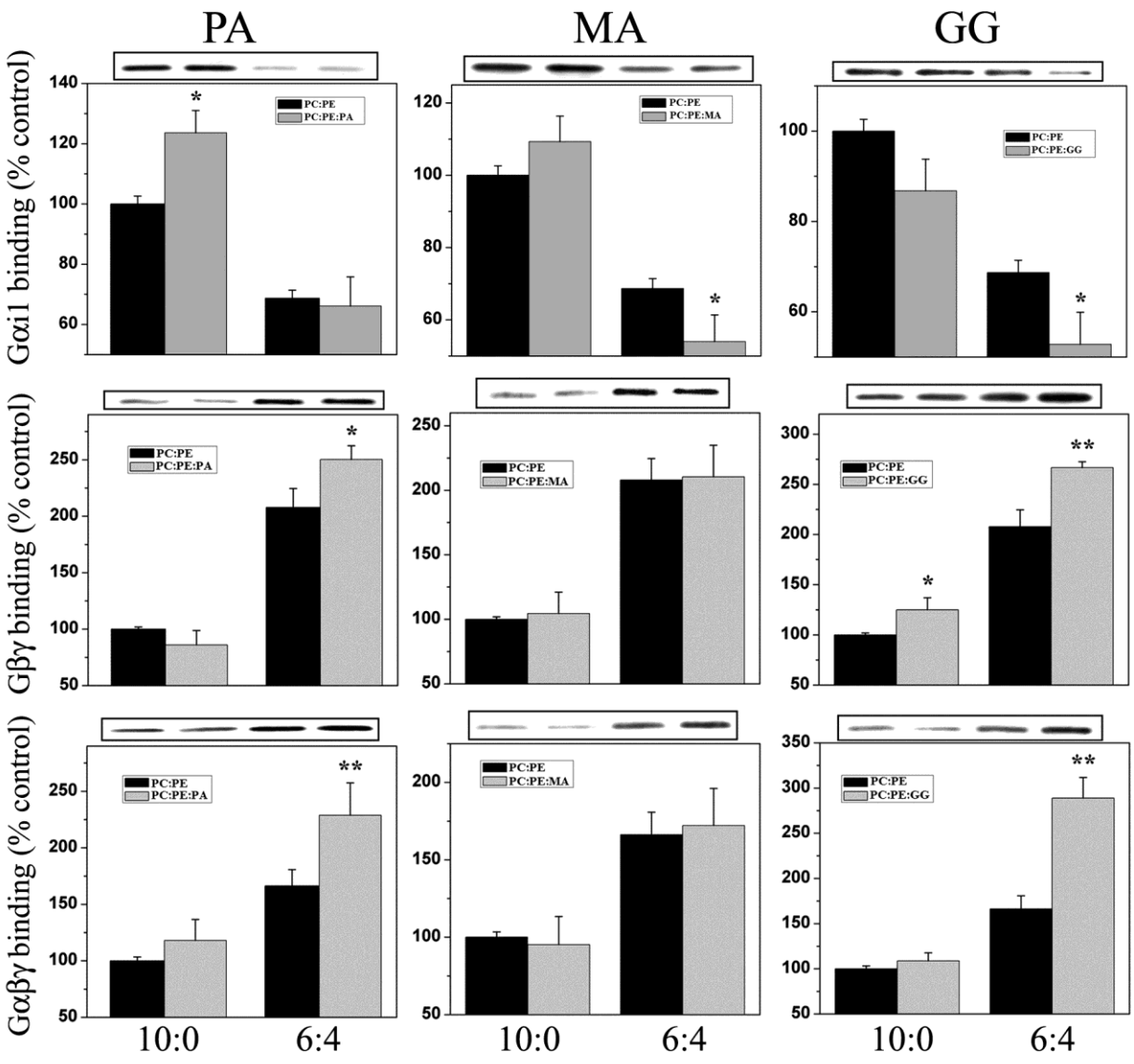

PA
MA
GG
Gαi1 binding (% control)
Gβγ binding (% control)
Gαβγ binding (% control)
PC:PE
PC:PE:PA
PC:PE:MA
PC:PE:GG
10:0
6:4

Figure 4

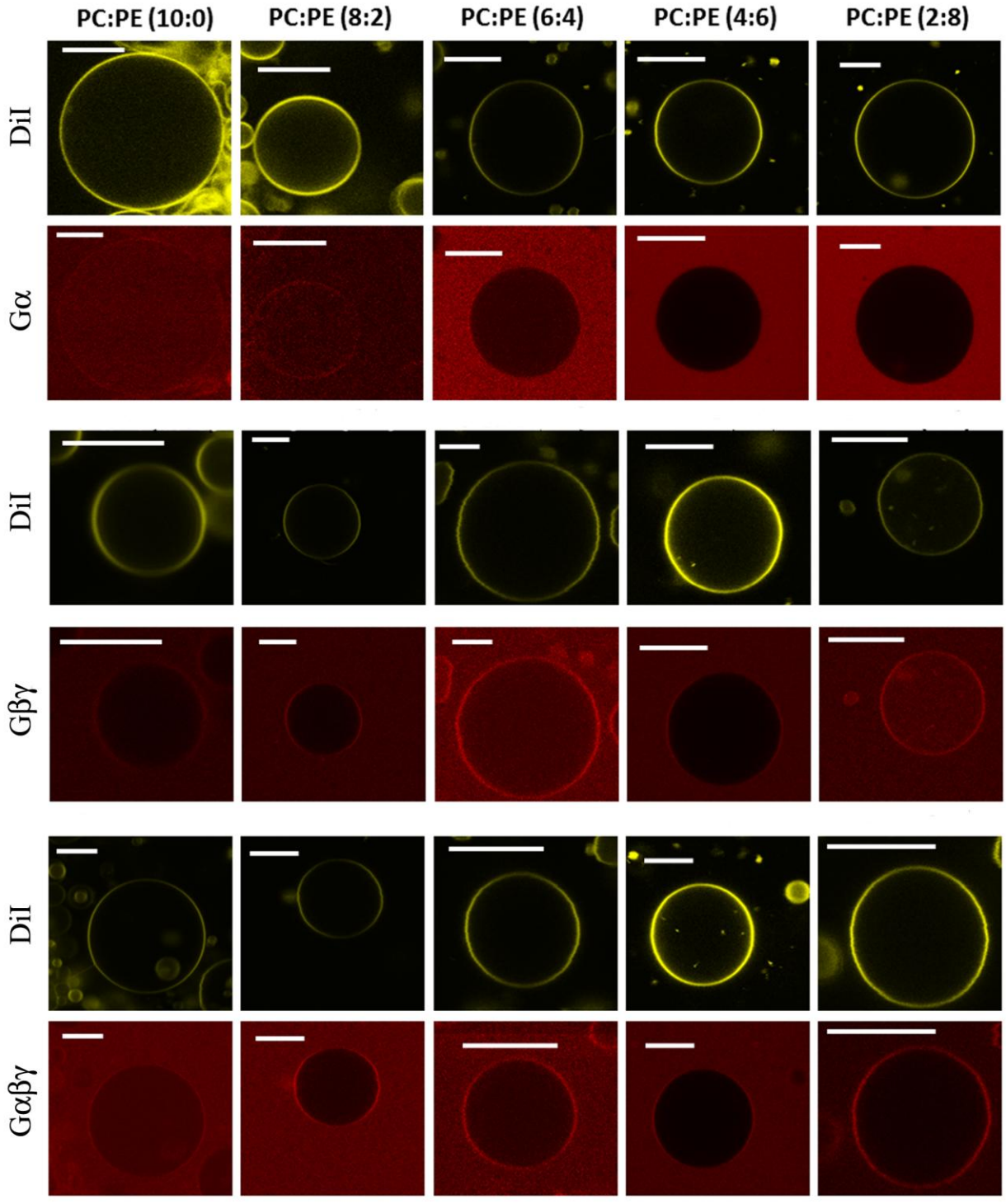


Figure 5

G protein-membrane interactions II: Effect of G Protein-linked Lipids on Membrane Structure and G Protein-membrane Interactions

Jesús Casas[a], Maitane Ibarguren[a,b], Rafael Álvarez[a], Silvia Terés[a,b], Victoria Lladó[a], Stefano P. Piotto[c], Simona Concilio[d], Xavier Busquets[a,b], David J. López[a,b,*] and Pablo V. Escribá[a,b]

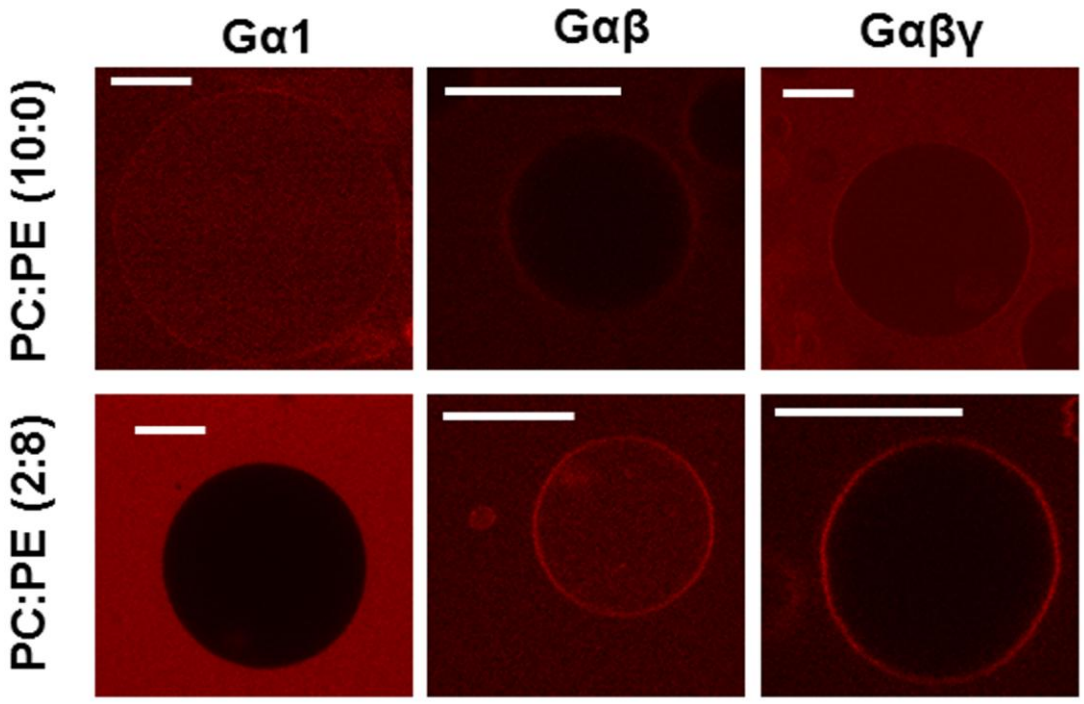
[a]Laboratory of Molecular Cell Biomedicine, Department of Biology, IUNICS, University of the Balearic Islands, E-07122 Palma de Mallorca, Spain.
[b]Lipopharma Therapeutics, S.L., ParcBit, 07121 Palma de Mallorca, Spain.
[c]Department of Pharmacy, University of Salerno, Via Ponte don Melillo, 84084 Fisciano (SA), Italy.
[d]Department of Industrial Engineering, University of Salerno, Via Ponte don Melillo, 84084 Fisciano (SA), Italy.

[*]To whom correspondence should be addressed:

David J. López, PhD, Laboratory of Molecular Cell Biomedicine, Department of Biology, University of the Balearic Islands, Crta. Valldemossa km. 7.5, 07122, Palma (Spain). Tel.: +34-97117 33 31; Fax +34-971 17 31 84; E-mail: davidlopez_95@hotmail.com

CONFLICT OF INTEREST

The authors declare no conflict of interest



Graphical abstract

G protein-membrane interactions II: Effect of G Protein-linked Lipids on Membrane Structure and G Protein-membrane Interactions

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Highlights

- Myristoyl, palmitoyl and isoprenyl groups influence biophysical properties of membranes.
- Myristoyl, palmitoyl and isoprenyl moieties of G proteins modulate their binding to membranes.
- The isoprenyl moieties favor the accumulation of heterotrimeric G proteins in phosphatidylethanolamine-enriched domains.
- Changes in the lipid composition of G proteins might modulate cell signaling.