Influence of the Membrane Lipid Structure on Signal Processing via G Protein-Coupled Receptors

Qing Yang, Regina Alemany, Jesús Casas, Klára Kitajka, Stephen M. Lanier, and Pablo V. Escribá

Division of Nephrology, Department of Medicine, Medical University of South Carolina, Charleston, South Carolina (Q.Y.); Laboratory of Molecular and Cellular Biomedicine, Associate Unit of the Instituto de la Grasa (Consejo Superior de Investigaciones Científicas), Department of Biology, IUNICS, University of the Balearic Islands, Palma de Mallorca, Spain (R.A., J.C., K.K., P.V.E.); and Department of Pharmacology, Louisiana State University Health Sciences Center, New Orleans, Louisiana (S.M.L.)

Received February 7, 2005; accepted April 15, 2005

ABSTRACT

We have recently reported that lipid structure regulates the interaction with membranes, recruitment to membranes, and distribution to membrane domains of heterotrimeric Gαβγ proteins, Gα subunits, and Gβγ dimers (J Biol Chem 279:36540–36545, 2004). Here, we demonstrate that modulation of the membrane structure not only determines G protein localization but also regulates the function of G proteins and related signaling proteins. In this context, the antitumor drug daunorubicin (daunomycin) and oleic acid changed the membrane structure and inhibited G protein activity in biological membranes. They also induced marked changes in the activity of the α2A/D-adrenergic receptor and adenylyl cyclase. In contrast, etidocic acid did not change the activity of the above-mentioned proteins. These fatty acids are chemical but not structural analogs of oleic acid, supporting the structural basis of the modulation of membrane lipid organization and subsequent regulation of G protein-coupled receptor signaling. In addition, oleic acid (and also daunorubicin) did not alter G protein activity in a membrane-free system, further demonstrating the involvement of membrane structure in this signal modulation. The present work also unravels in part the molecular bases involved in the antihypertensive (Hypertension 43:249–254, 2004) and anticancer (Mol Pharmacol 67:531–540, 2005) activities of synthetic oleic acid derivatives (e.g., 2-hydroxyoleic acid) as well as the molecular bases of the effects of diet fats on human health.

Plasma membrane lipids not only serve as an inert support for membrane proteins but also play an active role in cell activity that has yet to be fully understood. Membrane exo-endocytic processes, diffusion of macromolecules, and protein activities, among other cellular events, depend on the physical properties of membrane lipids. We have recently demonstrated that the hexagonal (HII) phase propensity of membrane lipids differentially influences the binding of Gαi, Gβγ, and Gαβγ proteins, indicating that membrane composition and structure can regulate cell signaling (Vögler et al., 2004). Heterotrimeric G proteins (Gαβγ, preactive) are recruited to membrane domains rich in receptor proteins, which exhibit a high nonlamellar phase propensity. Upon activation by agonists, the receptor activates the G protein. Then, the Gai subunit (active) dissociates from the Gβγ complex and because of its lesser affinity for nonlamellar structures and higher affinity for lamellar phases can be recruited to domains with ordered bilayer structure (e.g., lipid rafts) (Vögler et al., 2004), where it may activate signaling effectors. Therefore, modulation of the lipid structure (lamellar and nonlamellar phases or propensity to form them) regulates the G protein-membrane interactions and mobilization to different membrane domains upon activation. Here, we showed that

ABBREVIATIONS: HII, hexagonal; OA, oleic acid; EA, elaidic acid; SA, stearic acid; GPCR, G protein-coupled receptor; AR, adrenoceptor; AC, adenylyl cyclase; DNM, daunorubicin, daunomycin; GTPγS, guanosine 5′-O-(3-thio)triphosphate; RX821002, 2-[2-methoxy-1,4-benzodioxan-2-yl]-2-imidazoline; UK14304, 5-bromo-N-(4,5-dihydro-1H-imidazol-2-yl)-6-quinoxalineamine; CHS, cholesterol hemisuccinate; PE, phosphatidylethanolamine; POPE, 1-palmitoyl-2-oleoyl phosphatidylethanolamine; DPPC, dipalmitoyl phosphatidylcholine; L, lamellar; Gpp(NH)p, guanosine 5′-[(β,γ-imido)triphosphate.© 2005 The American Society for Pharmacology and Experimental Therapeutics. Mol Pharmacol 68:210–217, 2005

MOL Pharmacol 68:210–217, 2005

Printed in U.S.A.
not only is G protein localization regulated by the membrane structure/composition but also the function of these transducers and related proteins.

The type and abundance of membrane lipid species are regulated by dietary fat intake, which thus influences the properties of the membrane (Escudero et al., 1998). For example, oleic acid (OA), either free or bound to other molecules, confers an increased hexagonal phase propensity to the membrane (Funari et al., 2003), whereas the closely related fatty acids elaidic acid (EA) and stearic acid (SA) do not have the same effect (Funari et al., 2003). Although numerous work has studied the effect of diet fats on lipid composition, lipoprotein levels, and nutritional and biochemical status of tissues and organs, there is a lack of information about the effect of lipids on cell signaling.

We have investigated G protein-coupled receptor (GPCR)-mediated signaling to determine the effect of fatty acids on the membrane lipid structure and the propagation of signals through these receptors. Previous studies have shown that α2-AR-adrenoceptors (α2-ARs) are involved in the control of blood pressure and cell proliferation, both of which are biological processes related to the development of cardiovascular pathologies and cancer (Betuing et al., 1997; Hein et al., 1999). It is interesting that Mediterranean diets that include high amounts of OA (mainly from olive oil) (Trichopoulou et al., 1995) are associated with a reduced incidence of cardiovascular pathologies and cancer (Mata et al., 1992; Martin-Moreno et al., 1994; Ruiz-Gutiérrez et al., 1996; Tzonou et al., 1996), although the molecular bases of these effects remain largely unknown. In addition, therapies based on the interaction of synthetic fatty acid drugs with membranes have been recently developed (lipid therapy). This is an innovative pharmacological approach, because most of the marketed drugs target proteins and only a few of them target nucleic acids. The present study explains in part the molecular bases of the pharmaceutical and nutraceutical effects of OA derivatives and olive oil.

The importance of membrane lipid structure and protein-lipid interactions is evident. First, the initial steps of signaling cascades are associated with membranes and with the propagation of signals across these barriers. Second, signaling pathways are more dependent on the initial membrane-associated signaling elements (receptors, G proteins, and effectors) than on downstream signaling proteins that simply amplify the signals received (Levitzki, 1988). Third, receptors, G proteins, and effectors can be differentially recruited to certain specific membrane structures such as membrane rafts (Moffett et al., 2000), which partially define their activities. Finally, the presence of nonlamellar probe lipids modulates the localization and activity of peripheral proteins that are capable of translocating from the membrane to the cytosol, and as such, of propagating intracellular signals (Kinnunen, 1996; Goni and Alonso, 1999).

The membrane hexagonal phase propensity regulates the cellular localization of G proteins (Escribá et al., 1997; Vogler et al., 2004). For this reason, we have studied the effect of OA on membrane structure and on the subsequent changes in the activity of α2-AR, G proteins, and adenyl cyclase (AC). In this context, OA but not its chemical analogs EA and SA altered the membrane lipid structure and functional properties of α2-AR, G protein, and AC. Likewise, the antitumor drug daunorubicin (daunomycin, DNM) also alters membrane structure, cellular localization of G proteins, and GPCR-associated signaling (Escribá et al., 1995). The present results may explain in part the anticancer and hypotensive activities of fatty acids, such as the novel synthetic OA analog 2-hydroxyoleic acid (desBordes and Lea, 1995; Corl et al., 2003; Llor et al., 2003; Alemany et al., 2004; Martínez et al., 2005). In summary, this work presents relevant information and will help to understand 1) how the membrane lipid composition and structure modulate cell signaling, 2) how diet fats can influence the cell’s physiology and human health, and 3) why pharmacological approaches targeting membrane lipids can be effective against cardiovascular and tumor pathologies (Alemany et al., 2004; Martínez et al., 2005).

Materials and Methods

Materials. Tissue culture supplies were from JRH Bioscience (Lenexa, KS); acrylamide, bisacrylamide, and SDS were from BioRad (Madrid, Spain). [35S]GTPγS (1250 Ci/mmol), [3H]rauwolscine (82.3 Ci/mmol), [3H]RX821002 (56.0 Ci/mmol), and [3H]UK14304 (67.4 Ci/mmol) were all obtained from PerkinElmer Life and Analytical Sciences (Bad Homburg, Germany). Phospholipids were from Avanti Polar Lipids (Alabaster, AL), whereas OA, EA, and SA were obtained from Sigma (Madrid, Spain) and the DNM and cholesterol hemisuccinate (CHS) were from Sigma/RBI (Madrid, Spain).

Differential Scanning and Isothermal Titration Calorimetry. Differential scanning calorimetry was performed on an MCS-DSC microcalorimeter (OriginLab Corp., Northampton, MA) at a scan rate of 0.5 K/min, as described previously (Escribá et al., 1995). In brief, 1-palmitoyl-2-oleoyl phosphatidylethanolamine (POPE, 2 mM) or dipalmitoyl phosphatidylcholine (DPPC, 2 mM) was dissolved in chloroform in the presence or absence of different concentrations of OA (10–100 µM), CHS (200–2000 µM), or DNM (200–500 µM). The solvent was removed under an argon flow and submitted to a vacuum for at least 3 h. The lipid film was then resuspended in “sample buffer” (10 mM HEPES, 100 mM NaCl, and 1 mM EDTA, at pH 7.4) with agitation for 2 min at 50°C, degassed by stirring under vacuum for 10 min, and then used immediately in calorimetry experiments. Isothermal titration calorimetry was carried out on an MCS-ITC microcalorimeter (Lin et al., 1994; Heerklotz et al., 1999). During a titration experiment, 2 mM POPE vesicles were thermostated in sample buffer at either 40°C (to study the binding to lamellar phases) or 75°C (to study the binding to HII phases) in a stirred reaction cell (400 rpm, 1.543 ml). A series of injections (2 µl per injection, 50–60 injections) were carried out using a 250-µl syringe filled with 50 mM OA sodium salt in sample buffer. In control experiments, the OA sodium salt was injected into the buffer without POPE to calculate the heat of dilution, and the dilution heat (h_d) of OA was subtracted from experimental data (h_i): h_i = h_d+h_{i}. The injection time was set at 5 s and 240 s between consecutive injections to permit the equilibration of lipid binding. The first data point was disregarded (as recommended by the manufacturer), and the rest were fitted to one-, two-, and three-site binding models. Data acquisition and analysis was carried out using the software provided by the manufacturer (MCS Observer and Origin programs; OriginLab Corp.).

Radioligand Binding Studies. NIH-3T3 cells were transfected with the α2AR-AR (3T3-AR) cells as described in Lanier et al. (1991). G418 (Geneticin)-resistant clones were screened for receptor subtype expression by RNA blot analysis and by their ability to bind the α2-selective antagonists [3H]rauwolscine or [3H]RX821002. The cells were further characterized by their subtype ligand recognition properties and the molecular mass of the protein produced. 3T3-AR cells were maintained in monolayer culture in Dulbecco’s modified Eagle’s medium containing 10% bovine calf serum, 100 units/ml penicillin,
100 µg/ml streptomycin, and 0.25 µg/ml Fungizone, at 37°C and 5% CO₂. Cell membrane preparation and radioligand binding assays were performed as described in Duzic et al. (1992). In brief, confluent cells were washed twice with sterile phosphate-buffered saline (137 mM NaCl, 2.6 mM KCl, 10 mM Na₂HPO₄, and 1.8 mM KH₂PO₄, at pH 7.3), harvested with a rubber policeman, and centrifuged for 5 min at 200g and 4°C. The pellet was resuspended in lysis buffer (1 mM/dish of 5 mM Tris-HCl, 5 mM EDTA, and 5 mM EGTA, at pH 7.5) at 4°C and homogenized using a 1-mL syringe with a 26-gauge needle. The cell homogenate was centrifuged for 10 min at 14,000g and 4°C. The pellet was washed and resuspended in membrane buffer (50 mM Tris-HCl, 0.6 mM EDTA, and 5 mM MgCl₂, at pH 7.5), and the protein content of the membrane suspension was determined as described previously (Lowry et al., 1951). This protocol yields a fraction rich in plasma membranes. Other types of membranes present in this preparation did not interfere with the assays performed here because they lack of the signaling system studied. These cell membrane preparations were treated as indicated below.

[^35S]GTP binding was used to evaluate G protein activity and coupling to α₂-adrenoceptors. Membranes prepared from transfected NIH-3T3 cells were preincubated in the presence or absence of OA, EA, SA, DNM, PE, or CHS for 30 min on ice. The reaction was then initiated by adding the membrane suspensions to assay buffer (50 mM Tris-HCl, 5 mM MgCl₂, 1 mM EDTA, 1 mM dithiothreitol, 100 mM NaCl, 1 mM guanosine diphosphate, and 1 mM propanolol, at pH 7.4) in a final volume of 100 µL, and in the presence of 0.5 nM[^35S]GTP and the α₂-AR agonist UK14304 (10 nM). Reactions were incubated for 30 min at 37°C and terminated by rapid vacuum filtration through glass fiber filters, and the radioactivity was determined by liquid scintillation. In all experimental series, lipids were dissolved through glass fiber filters, and the radioactivity was determined by liquid scintillation. The effect of the solvent (<0.5% ethanol) was evaluated as described above.

Statistics. The results are expressed as mean ± S.E.M. One-way analysis of variance followed by Scheffe or Fisher tests was used for statistical evaluations. The level of significance was established at p = 0.05.

**Results**

**Effect of OA on Phospholipid Thermal Transitions.** The effect of OA on the thermotropic behavior of 2 mM DPPC was different from that of DNM and CHS. DNM did not induce significant changes in the solid-to-liquid (S→L) crystalline phase transition temperature (Tm), but rather it induced marked changes in the lamellar-to-hexagonal (L→HII) phase transition of model membranes (Fig. 1A; Tables 1 and 2; Escribí et al., 1995). These results indicated that at low concentrations (up to 100 µM), OA had little effect on membrane fluidity but induced important changes in membrane lipid organization. As described previously for cholesterol (Mabrey et al., 1978), CHS (a cholesterol analog) produced a segregation of membrane lipids, generating CHS-rich domains with a higher Tm and CHS-poor microdomains (Table 1). The presence of up to 100 µM OA did not significantly affect the S→L phase transition of POPE and DPPC membranes (Tables 1 and 2). In contrast, marked changes in POPE L→HII transition temperature (Tm) and enthalpy (ΔHₗ) were observed in the presence of OA (Fig. 1B; Table 2), further indicating that the fatty acid had a greater effect on the membrane lipid structure than on membrane fluidity (or viscosity). By contrast, neither EA nor SA induces important...
The presence of OA affected differently the S→L (fluidity) and L→H₁₁ (nonlamellar phase propensity) transitions, suggesting a differential interaction with different membrane lipid organizations. For this reason, we studied the binding of OA to both lipid structures, the lamellar and H₁₁ phases. The OA-POPE interactions were assessed by isothermal titration calorimetry as previously used to determine the binding of other compounds to liposomes (Heerklotz et al., 1999). The binding of OA to POPE organized into lamellar and hexagonal phases fitted best a two-site binding model (Table 3), suggesting the existence two OA binding sites (high and low affinity) or interaction architectures. The higher affinity exhibited by OA occurred when interacting with nonlamellar structures (Kₐ¹ = 3100 M⁻¹; Kₐ² = 322 μM) rather than with lamellar structures (Kₐ¹ = 786 M⁻¹; Kₐ² = 1.27 mM). In addition, the binding capacity (nₛ) of OA to hexagonal structures was greater than that corresponding to lamellar structures (Table 3). These results explain the higher affinity-site (Kₐ₂ values for L and H₂₃ structures were similar. Overall, calorimetry experiments demonstrate that OA binds to membrane phospholipids, and OA binding affinity and effects on the latter depend on the lipid type and organization.

Effects of OA and DNM on α₂-Adrenoceptor Function. To determine the influence of the lipid structure on signal processing by GPCRs, we evaluated the effect of OA and DNM on α₂AR-AR function in membranes from 3T3 cells overexpressing the α₂AR-AR. This model of biological membranes is more complex than the above-described model membranes because it contains the first elements of the GPCR signaling machinery, but it is less complex than a whole cell. In this model similarly to cells, the interaction of the G protein with the receptor stabilizes the conformation of the α₂AR-AR such that it exhibits a high affinity for agonists. This interaction is disrupted by the exchange of GDP by GTP or its analogs in the Gα subunit, such as Gpp(NH)p (an event that activates the G protein). At low concentrations, ~90% of [³H]UK14304 agonist binding was sensitive to Gpp(NH)p. Adrenergocceptor agonist binding was significantly diminished in the presence of OA (a reduction of 81.2 ± 13%, p < 0.001) and DNM (a reduction of 61.9 ± 7%, p < 0.01; Fig. 2A). The α₂-AR antagonist [³H]RX821002 is less sensitive to Gpp(NH)p, and its binding to 3T3-AR cell membranes in the presence of OA was reduced by 41 ± 7% (p < 0.01), whereas DNM provoked a slightly smaller decreases of 36.3 ± 12% (p < 0.01; Fig. 2B). In contrast, neither PE nor CHS induced significant changes in the binding of [³H]UK14304 or [³H]RX821002 to 3T3-AR membranes (Fig. 2). Furthermore, the OA analogs EA and SA (300 μM) did not alter the radioligand recognition properties of α₂-ARs, despite the fact that EA, CA (cis- and trans-stereoisomers of 9-octadecenoic acid, respectively), and SA (octadecanoic acid) are 18-C fatty acids (Fig. 3). trans/unsaturated fatty acids (e.g., EA and SA) have a linear “molecular shape”, whereas the prominent kink induced by the cis-double bonds in OA induces important differences in its molecular shape with respect to these analogs.

Effect of OA on G Protein Activity. The relationship between the modification in membrane structure and G protein function was addressed by determining the influence of OA on the agonist-induced increases in [³⁵S]GTPΓS binding to G proteins in membranes. The presence of OA markedly reduced agonist-induced [³⁵S]GTPΓS binding to 3T3-AR membranes in a concentration-dependent manner (Fig. 4A). Whereas DNM induced a similar effect, CHS and PE only induced modest changes in agonist-induced activation of G proteins. We also analyzed the inhibitory effect of OA (100 μM) and DNM (100 μM) on [³⁵S]GTPΓS binding at increasing concentrations of UK14304 (10⁻⁸–10⁻¹⁰ M). Both, OA and DNM induced a significant decrease in [³⁵S]GTPΓS binding to 3T3-AR membranes at all concentrations of UK14304 examined (Fig. 4B). In contrast, PE and CHS did not markedly alter the binding of this GTP analog (Fig. 4B), and EA and SA did not alter receptor-mediated activation of G protein (Fig.

### Table 1

<table>
<thead>
<tr>
<th>OA (μM)</th>
<th>0</th>
<th>100</th>
<th>200</th>
<th>350</th>
<th>500</th>
<th>1000</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tm (°C)</td>
<td>41.5</td>
<td>41.2</td>
<td>40.3</td>
<td>39.0</td>
<td>38.3</td>
<td>37.1</td>
</tr>
<tr>
<td>ΔH₂ (kcal/mol)</td>
<td>10.9</td>
<td>10.7</td>
<td>10.3</td>
<td>9.9</td>
<td>9.1</td>
<td>9.5</td>
</tr>
<tr>
<td>CHS (μM)</td>
<td>0</td>
<td>200</td>
<td>500</td>
<td>1000</td>
<td>2000</td>
<td></td>
</tr>
<tr>
<td>CHS-to-DPPC ratio (mol/mol)</td>
<td>0</td>
<td>1:10</td>
<td>1:4</td>
<td>1:2</td>
<td>1:1</td>
<td></td>
</tr>
<tr>
<td>Tm (°C)</td>
<td>41.5</td>
<td>39.2</td>
<td>40.2</td>
<td>47.3</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>ΔH₂ (kcal/mol)</td>
<td>10.9</td>
<td>8.1</td>
<td>6.3</td>
<td>3.6</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>ΔH₂ (kcal/mol)</td>
<td>53</td>
<td>61.8</td>
<td>63.4</td>
<td>2.2</td>
<td>0</td>
<td></td>
</tr>
</tbody>
</table>

### Table 2

<table>
<thead>
<tr>
<th>OA (μM)</th>
<th>0</th>
<th>100</th>
<th>200</th>
<th>350</th>
<th>500</th>
<th>1000</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tm (°C)</td>
<td>25.9</td>
<td>26.1</td>
<td>26.0</td>
<td>25.8</td>
<td>25.3</td>
<td>24.1</td>
</tr>
<tr>
<td>ΔH (kcal/mol)</td>
<td>5.9</td>
<td>6.4</td>
<td>6.4</td>
<td>5.9</td>
<td>5.1</td>
<td>5.9</td>
</tr>
<tr>
<td>ΔH₂ (kcal/mol)</td>
<td>71.0</td>
<td>72.2</td>
<td>66.1</td>
<td>64.3</td>
<td>64.9</td>
<td>64.9</td>
</tr>
<tr>
<td>ΔH₂ (kcal/mol)</td>
<td>0.61</td>
<td>0.49</td>
<td>0.41</td>
<td>0.31</td>
<td>0.17</td>
<td></td>
</tr>
</tbody>
</table>
4C). The effect of OA (and DNM) on G protein function was not caused by a direct interaction of the fatty acid with the protein, because the $[^{35}S]GTPyS$ binding to purified G proteins in the absence of membranes was not altered (Fig. 4D).

**Effects of OA and DNM on Adenylyl Cyclase Activity.** Finally, we extended this study to evaluate the influence of manipulating the lipid phase on the activity of AC. A decrease was observed in the basal activity of AC in the presence of OA (a decrease of 92.8 ± 3.5%; p < 0.001) and DNM (a decrease of 30.9 ± 13.7%; p < 0.05) and of the forskolin-stimulated activity of AC (decreases of 98 ± 1%, p < 0.001 and 57.2 ± 10%, p < 0.01 for OA and DNM, respectively) (Fig. 5A). As expected, the $\alpha_2$-AR agonist UK14304 also inhibited the basal and forskolin-stimulated activity of AC (37.8 ± 9.7%, p < 0.01 and 38.3 ± 12%, p < 0.05, respectively) (Fig. 5B). In contrast, PE and CHS did not significantly alter either the basal or the forskolin-stimulated activity of AC (Fig. 5). Furthermore, no synergism was observed between UK14304 and OA, EA, DNM, PE, or CHS because in combination AC activity was not inhibited more than in the presence of any of these compounds alone (data not shown). Again, the effect of OA was structure-specific because the congener EA had no effects on basal or forskolin-stimulated AC activity.

### Discussion

The effects of the membrane lipid composition/structure on the physiology of the cell remain largely unknown. This knowledge is important in understanding 1) how lipid alterations influence cell signaling in pathologies where lipid changes have been described, 2) the mechanisms involved in the effects of diet fats on health, and 3) the molecular bases underlying “lipid therapy” (Martínez et al., 2005). Fatty acids are the most important molecules in the formation of the core of cell membranes, either in their free state or as a moiety of other molecules (e.g., phospholipids). However, not all fatty acids have the same structural properties or confer the same properties to membranes. Among the characteristics of membranes, the propensity to form nonlamellar (HII) structures is facilitated by the cis-monounsaturated fatty acid OA (Funari et al., 2003). In contrast, trans-monounsaturated (EA) or saturated (SA) fatty acids do not affect this property in model membranes, indicating that HII propensity modulation depends on the molecular shape of the fatty acid and relies on highly structural bases (Funari et al., 2003). It is interesting that, certain phosphatidylethanolamine derivatives with OA, such as dioleoyl phosphatidylethanolamine, have a very high HII propensity ($T_{h}$ of $-16°C$). In POPE, used here, the high $T_{h}$ value ($-71°C$; Table 2) suggests that the palmitoyl moiety in position 1 inhibits the motion of the oleoyl residue, also indicating that the free fatty acid (OA) has greater effect on HII propensity. We recently demonstrated that activated G proteins prefer lamellar-prone membranes, whereas heterotrimeric Gi ($\alpha$) proteins and G$\beta$ dimers prefer HII phases (Vögler et al., 2004). Here, we show that OA modulated the HII phase propensity (Fig. 1) and regulated the activities of GPCRs and G proteins (Figs. 2 and 4). In contrast, the OA analogs EA and SA did not significantly alter either membrane structure or GPCR-mediated signaling. Although OA and DNM induced important changes on G protein activity in

<table>
<thead>
<tr>
<th>Table 3</th>
<th>Binding parameters for OA-POPE interaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Binding Parameter</td>
<td>L Phase</td>
</tr>
<tr>
<td>$n_1$ (mol of OA/mol of POPE)</td>
<td>1.03 ± 0.08</td>
</tr>
<tr>
<td>$K_{n_1}$ (M$^{-1}$)</td>
<td>786 ± 49</td>
</tr>
<tr>
<td>$H_1$ (kcal/mol)</td>
<td>413 ± 16</td>
</tr>
<tr>
<td>$n_2$ (mol of OA/mol of POPE)</td>
<td>0.75 ± 0.05</td>
</tr>
<tr>
<td>$K_{n_2}$ (M$^{-1}$)</td>
<td>122 ± 8</td>
</tr>
<tr>
<td>$H_2$ (kcal/mol)</td>
<td>$-1180 ± 58$</td>
</tr>
<tr>
<td>$n_3$ (mol of OA/mol of POPE)</td>
<td>1.78 ± 0.08</td>
</tr>
</tbody>
</table>

* p < 0.05, ** p < 0.01.
3T3 membranes, they did not change the activity of purified G proteins in a membrane-free system, further supporting the involvement of membranes in their effects. Therefore, this work shows that the membrane structure regulates not only G protein localization but also the activity of G proteins. On the other hand, OA regulated not only $[^{3}H]$UK14304 but also $[^{3}H]$RX821002 binding (insensitive to Gpp(NH)p) to $\alpha_{2\varepsilon}$-ARs (Fig. 2). These results suggest that the effect on the receptor could be caused by the contribution of the membrane environment on G proteins and by a direct effect on the receptor molecule. This issue is currently under investigation. Again, neither EA nor SA influenced $\alpha_{2\varepsilon}$-AR activity, further indicating the pivotal role of the membrane structure in the modulation of GPCR-associated signaling.

Why are biological membranes so abundant in type and diversity of composition if they only define barriers? Why is the membrane composition so finely regulated and why can membrane lipids organize into more secondary structures than proteins or nucleic acids if they mainly serve as support for proteins? Membrane lipids have more functions than those usually attributed to them. Hexagonally prone lipids modulate the activity and interaction of peripheral proteins with the plasma membrane (Escribá et al., 1995, 1997; Giorgione et al., 1995; Soulages et al., 1995). Our data indicate that the effects of OA and DNM on cell signaling pathways are related to the propensity of the membrane to enter the $H_{II}$ phase, because OA binds more readily to nonlamellar phases (Table 3). Therefore, DSC data indicated stronger effect of OA on the hexagonal phase propensity of the membrane (defined by the $L_{c} \leftrightarrow H_{II}$ transition) than on membrane fluidity (defined by the $S \leftrightarrow L$ transition) (Tables 1 and 2).

In the present study, we show that the membrane structure also modulates AC activity (Fig. 5). To our knowledge, this is the first time that AC activity has been shown to be regulated by $H_{II}$ phase propensity, and this finding is in agreement with its regulation by changes in the membrane lipid composition (Calorini et al., 1993). The signaling cas-

### Fig. 4

Influence of agents that modify the lipid phase on agonist-induced binding of $[^{35}S]$GTP$\gamma$S to 3T3-AR cell membranes and to purified brain G proteins. A, specific agonist-stimulated binding of $[^{35}S]$GTP$\gamma$S (mean ± S.E.M.) was measured in the presence or absence (control) of increasing concentrations of OA, DNM, PE, or CHS. B, specific agonist-stimulated binding of $[^{35}S]$GTP$\gamma$S to 3T3-AR membranes in the absence (C, control) or presence of 100 $\mu$M OA, DNM, PE, or CHS at increasing concentrations of the $\alpha_{2\varepsilon}$-AR agonist UK14304. C, effect of $100 \mu$M OA, EA, and SA on the specific agonist-stimulated binding of $[^{35}S]$GTP$\gamma$S to 3T3-AR membranes. D, specific binding of $[^{35}S]$GTP$\gamma$S to G proteins purified from bovine brain, in the absence of membranes and in the presence or absence (control) of $100 \mu$M OA, DNM, PE, or CHS. Basal $[^{35}S]$GTP$\gamma$S (control) binding to 3T3-AR membranes was 182.9 ± 28.9 and in UK14304-stimulated (5 × 10$^{-6}$ M) membranes binding reached 280.35 ± 35.4 fmol/mg of protein. Binding to control purified G proteins was 101 ± 4 fmol/mg of protein. *, $p < 0.05$; **, $p < 0.001$. 

Membrane Structure on G Protein-Coupled Receptor Signaling 215
cades controlled by \( \alpha_{2A/D} \)-ARs often use Gi proteins as transducers and AC as the effector.

In addition to their apparent effect on signal processing by GPCRs, \( H_{II} \) structures are involved in many other cellular functions, including exo/endocytic processes (Vidal and Hoekstra, 1995). Nonlamellar structures also modulate membrane permeability, elasticity, and fluid shear stress, which regulate the activity of membrane proteins (Keller et al., 1993; Gudi et al., 1998). The \( H_{II} \)-prone phospholipid PE has been shown to accumulate at the cleavage furrow, during cytokonic cell division (Emoto et al., 1996), and in \textit{Escherichia coli} it exhibits a chaperone-like activity (Bogdanov et al., 1996). Indeed, PE is required for the membrane packaging of integral proteins (de Kruijff, 1997), and it has also been used for biomedical and biotechnological purposes (Landau and Rosenbusch, 1996; Perkins et al., 1996; Zelphati and Szoka, 1996). The high proportion of this phospholipid in membranes and the precise regulation of its levels indicate that the hexagonal phase propensity is of great functional importance (Wieslander et al., 1986; Goldfine et al., 1987).

This study further supports the mechanism of action proposed for daunorubicin (Escriba et al., 1995). Using this molecular mechanism, we have designed new compounds (2-hydroxyoleic acid) with a great anticancer activity (Martínez et al., 2004). This work also explains in part the molecular mechanisms underlying the protective effects of cis-unsaturated fatty acids against cardiovascular and tumor pathologies (desBordes and Lea, 1995; Perez et al., 2003). Based on these mechanisms, we have designed new anticancer and antihypertensive drugs structurally related to OA (Alemany et al., 2004; Barceló et al., 2004; Martínez et al., 2005). One of the main targets of these compounds is membrane lipids (Funari et al., 2003; Barceló et al., 2004), thus defining a new pharmacological approach termed lipid therapy (Escriba and Bean, 2002). The present study also explains the molecular bases of the effects of diet fats on human health. For example, high OA intake, mainly through consumption of olive oil (containing approximately 80% OA) typical of Mediterranean diets, has been associated with a decrease in the incidence of cardiovascular (Heyden, 1994; Ruiz-Gutiérrez et al., 1996) and tumor pathologies (Martin-Moreno et al., 1994; Tzonou et al., 1996). Finally, the differential effects of OA, EA, and SA on membrane structure and cell signaling indicated that lipid-protein and lipid-lipid interactions are driven by structural biology principles.

Acknowledgments

We thank Dr. John Hildebrandt (Department of Pharmacology, Medical University of South Carolina, Charleston, SC) for providing the purified brain G proteins.

References


Fig. 5. AC activity. Basal (A) and forskolin-stimulated (B) AC activity measured in 3T3-AR cells, in the presence or absence (C, control) of 50 \( \mu \)M OA (OA50), 100 \( \mu \)M OA (OA), 100 \( \mu \)M EA (EA), 100 \( \mu \)M DNM (DNM), 100 \( \mu \)M CHS (CHS), or 10 \( \mu \)M UK14304 (UK). The level of control basal AC activity was 15.37 ± 2.8 pmol cAMP/min mg of protein and control forskolin-stimulated AC activity was 275.0 ± 36.9 pmol cAMP/min mg of protein. The results are expressed as mean ± S.E.M. *, \( p < 0.05; **, p < 0.01; \bullet, p < 0.001. \)


**Address correspondence to:** Dr. Pablo V. Escribá, Laboratory of Molecular and Cellular Biomedicine, Department of Biology, IUNICS, University of the Balearic Islands, Ctra. de Valldemossa km 7,5, E-07122 Palma de Mallorca, Spain. E-mail: pablo.escriba@uib.es