

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

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ABSTRACT

We have recently reported that lipid structure regulates the interaction with membranes, recruitment to membranes, and distribution to membrane domains of heterotrimeric $G\alpha\beta\gamma$ proteins, $G\alpha$ subunits, and $G\beta\gamma$ 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 $\alpha_{2A/D}$ -adrenergic receptor and adenylyl cyclase. In contrast, elaidic and stearic 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 (H_{II}) phase propensity of mem-

branes differentially influences the binding of $G\alpha_i$, $G\beta\gamma$, and $G\alpha\beta\gamma$ proteins, indicating that membrane composition and structure can regulate cell signaling (Vögler et al., 2004). Heterotrimeric G proteins ($G\alpha\beta\gamma$, 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 $G\alpha_i$ subunit (active) dissociates from the $G\beta\gamma$ 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

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ABBREVIATIONS: H_{II} , hexagonal; OA, oleic acid; EA, elaidic acid; SA, stearic acid; GPCR, G protein-coupled receptor; AR, adrenoceptor; AC, adenylyl cyclase; DNM, daunorubicin, daunomycin; $GTP\gamma S$, 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-quinoxalinamine; CHS, cholesterol hemisuccinate; PE, phosphatidylethanolamine; POPE, 1-palmitoyl-2-oleoyl phosphatidylethanolamine; DPPC, dipalmitoyl phosphatidylcholine; L, lamellar; Gpp(NH)p, guanosine 5'-(β , γ -imidotriphosphate).

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 $\alpha_{2A/D}$ -adrenoceptors ($\alpha_{2A/D}$ -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 prone 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; Goñi and Alonso, 1999).

The membrane hexagonal phase propensity regulates the cellular localization of G proteins (Escribá et al., 1997; Vögler 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 $\alpha_{2A/D}$ -AR, G proteins, and adenylyl cyclase (AC). In this context, OA but not its chemical analogs EA and SA altered the membrane lipid structure and functional properties of $\alpha_{2A/D}$ -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 Bio-Rad (Madrid, Spain). [35 S]GTP γ S (1250 Ci/mmol), [3 H]rauwolscine (82.3 Ci/mmol), [3 H]RX821002 (56.0 Ci/mmol), and [3 H]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 cholesteryl 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 (10–1000 μ M), CHS (200–2000 μ M), or DNM (200–500 μ M). 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 H_{II} phases) in a stirred reaction cell (400 rpm, 1.343 ml). A series of injections (2 μ l per injection, 50–60 injections) were carried out using a 250- μ l 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 ($h_{d,i}$) of OA was subtracted from experimental data (h_i): $\delta h_i = h_i - h_{d,i}$. 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 $\alpha_{2A/D}$ -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 α_2 -selective antagonists [3 H]rauwolscine or [3 H]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 $\mu\text{g/ml}$ streptomycin, and 0.25 $\mu\text{g/ml}$ Fungizone, at 37°C and 5% CO_2 . 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 Na_2HPO_4 , and 1.8 mM KH_2PO_4 , 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 ml/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 MgCl_2 , 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.

$[\text{S}] \text{GTP}\gamma\text{S}$ binding was used to evaluate G protein activity and coupling to α_2 -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 MgCl_2 , 1 mM EDTA, 1 mM dithiothreitol, 100 mM NaCl, 1 μM guanosine diphosphate, and 1 μM propranolol, at pH 7.4) in a final volume of 100 μl , and in the presence of 0.5 nM $[\text{S}] \text{GTP}\gamma\text{S}$ and the α_2 -AR agonist UK14304 (10 nM). Reactions were incubated for 30 min at 37°C and terminated by rapid vacuum filtration through nitrocellulose filters. Radioactivity was determined by liquid scintillation counting, and nonspecific binding was defined in the presence of 100 μM GTP γS . The agonist-stimulated $[\text{S}] \text{GTP}\gamma\text{S}$ binding was determined after subtracting the basal $[\text{S}] \text{GTP}\gamma\text{S}$ binding. In another series of experiments, $[\text{S}] \text{GTP}\gamma\text{S}$ binding was performed as described above, except that the concentrations of OA, DNM, PE, and CHS were constant (100 μM), and the concentrations of UK14304 varied between 10^{-8} and 10^{-3} M. The effects of OA, EA, and SA on $[\text{S}] \text{GTP}\gamma\text{S}$ binding to 3T3-AR membranes were also studied. Finally, $[\text{S}] \text{GTP}\gamma\text{S}$ binding to purified G proteins was performed using purified bovine brain G protein heterotrimers as described in Dingus et al. (1994). Experiments were carried out as described above, using 5 nM G proteins, 0.01% Thesit, and 2.5 nM $[\text{S}] \text{GTP}\gamma\text{S}$ and in the presence or absence of 100 μM OA, DNM, PE, or CHS.

Binding of $[\text{H}] \text{RX821002}$ (20 nM) and $[\text{H}] \text{UK14304}$ (1 nM) to 3T3-AR cell membranes was performed in the presence or absence of OA, EA, SA, DNM, PE, or CHS, for 30 min on ice. The membrane suspensions were incubated (25 μg of protein per tube) for 30 min at 24°C in 100 μl of phosphate-buffered saline with the corresponding radioligand, and nonspecific binding was determined in the presence of 10 μM rauwolfscine. Binding was terminated by vacuum filtration through glass fiber filters, and the radioactivity was determined by liquid scintillation. In all experimental series, lipids were dissolved either in water or in ethanol. The ethanol concentration in the assays was always less than 0.5%, and control samples containing this solvent were also evaluated in all experimental series. The amount of the lipids incorporated into the cell membranes was always $\geq 90\%$ of the lipid added as determined by radioligand binding and ITC experiments (fatty acids), colorimetric assays (CHS), fluorescence spectroscopy (DNM), and thin-layer chromatography followed by gas chromatography (phospholipids) (Escribá et al., 1990, 1995).

Adenylyl Cyclase Activity Assay. AC activity was measured as described previously (Lanier et al., 1991; Duzic et al., 1992). In brief, 3T3-AR cell membranes were incubated with assay buffer (50 mM HEPES, 1 mM EDTA, 4 mM MgCl_2 , 100 mM NaCl, 0.24 mM ATP, 0.1 mM GTP, 0.2 mM cAMP, 0.2% bovine serum albumin, and 0.5 mM isobutyl-L-methylxanthine, at pH 7.4) containing $[\text{P}] \text{ATP}$ (0.5 $\mu\text{Ci/tube}$) and an ATP-regenerating system (60 mM creatinine phosphate and 10 units/ml creatinine kinase). The reactions were per-

formed with or without (basal) forskolin (2 μM); with or without OA, EA, DNM, PE, or CHS (100 μM); and in the presence or absence of UK14304 (10^{-5} M), in a final volume of 100 μl at 37°C for 30 min. Thereafter, 2% SDS was added to the incubation solution to stop the reaction, and cAMP was isolated by sequential chromatography on Dowex AG-50W-X4 and alumina columns. The 4-ml eluate from each column was equilibrated with 16 ml of Ecoscint A, and the radioactivity was determined by liquid scintillation counting. The effect of the solvent ($<0.5\%$ ethanol) was evaluated as described above.

Statistics. The results are expressed as mean \pm S.E.M. One-way analysis of variance followed by Scheffé 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 ($\text{S} \leftrightarrow \text{L}$) crystalline phase transition temperature (T_m), but rather it did induced marked changes in the lamellar-to-hexagonal ($\text{L} \leftrightarrow \text{H}_{\text{II}}$) phase transition of model membranes (Fig. 1A; Tables 1 and 2; Escribá 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 T_m and CHS-poor microdomains (Table 1). The presence of up to 100 μM OA did not significantly affect the $\text{S} \leftrightarrow \text{L}$ phase transition of POPE and DPPC membranes (Tables 1 and 2). In contrast, marked changes in POPE $\text{L} \leftrightarrow \text{H}_{\text{II}}$ transition temperature (T_h) and enthalpy (ΔH_h) 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

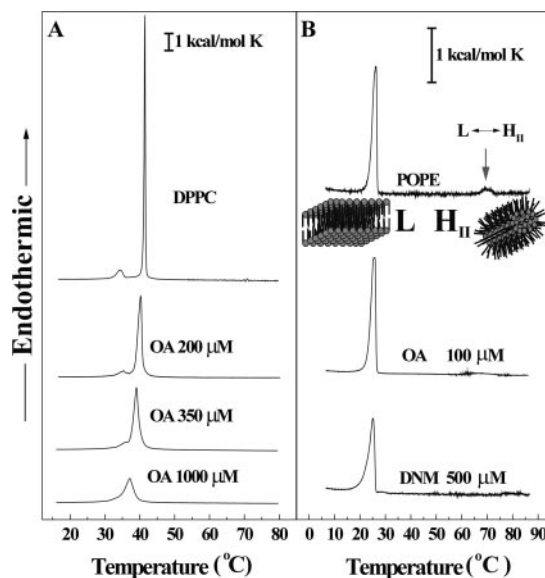


Fig. 1. Effects of OA and DNM on the thermotropic behavior of DPPC and POPE. A, effect of OA on DPPC (2 mM) $\text{S} \leftrightarrow \text{L}$ phase transition. Only OA concentrations above 200 μM induced significant changes on membrane fluidity (Table 1). B, effect of OA and DNM on POPE (2 mM) $\text{L} \leftrightarrow \text{H}_{\text{II}}$ phase transition. L and hexagonal H_{II} phases are shown below the top thermogram. For further details, see Table 2.

changes in the structure of model membranes (Funari et al., 2003).

The presence of OA affected differentially the $S \leftrightarrow L$ (fluidity) and $L \leftrightarrow H_{II}$ (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 H_{II} 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 ($K_{a1} = 3100 \text{ M}^{-1}$; $K_{d1} = 322 \text{ } \mu\text{M}$) rather than with lamellar structures ($K_{a1} = 786 \text{ M}^{-1}$; $K_{d1} = 1.27 \text{ mM}$). In addition, the binding capacity (n_b) of OA to hexagonal structures was greater than that corresponding to lamellar structures (Table 3). These results explain the more significant effect of OA on membranes in the hexagonal phase than in the lamellar phase at micromolar concentrations. They also constitute the first evidence indicating that the membrane organization regulates fatty acid-phospholipid interactions. In contrast with the high-affinity site, for the low-affinity-site, K_{a2} values for L and H_{II} 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 α_2 -Adrenoceptor Function. To determine the influence of the lipid structure on signal processing by GPCRs, we evaluated the effect of OA and DNM on $\alpha_{2A/D}$ -AR function in membranes from 3T3 cells overexpressing the $\alpha_{2A/D}$ -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 $\alpha_{2A/D}$ -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 $G\alpha$ subunit, such as Gpp(NH)p (an event that activates the G protein). At low concentrations, $\sim 90\%$ of [^3H]UK14304 agonist binding was sensitive to Gpp(NH)p. Adrenoceptor agonist binding was significantly diminished in the presence of OA (a reduction of $81.2 \pm 13\%$, $p < 0.001$) and DNM (a reduction of $61.9 \pm 7\%$, $p < 0.01$; Fig. 2A). The α_2 -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 $41 \pm 7\%$ ($p < 0.01$), whereas DNM provoked a slightly smaller decreases of $36.3 \pm 12\%$ ($p < 0.01$; 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 ($300 \text{ } \mu\text{M}$) did not alter the radioligand recognition properties of α_2 -ARs, despite the fact that OA, EA (*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 [^{35}S]GTP γ S binding to G proteins in membranes. The presence of OA markedly reduced agonist-induced [^{35}S]GTP γ S 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 ($100 \text{ } \mu\text{M}$) and DNM ($100 \text{ } \mu\text{M}$) on [^{35}S]GTP γ S binding at increasing concentrations of UK14304 (10^{-8} – 10^{-3} M). Both, OA and DNM induced a significant decrease in [^{35}S]GTP γ S 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

OA and CHS on DPPC thermodynamic parameters

T_m values for DPPC in the presence of 200 to 500 μM DNM were 41.1 – 41.4°C , and ΔH values were 10.6 to 10.9 kcal/mol. DPPC (2 mM) was always used.

OA (μM)	0	100	200	350	500	1000
OA-to-DPPC ratio (mol/mol)	0	1:20	1:10	7:40	1:4	1:2
T_m ($^\circ\text{C}$)	41.5	41.2	40.3	39.0	38.3	37.1
ΔH_2 (kcal/mol)	10.9	10.7	10.3	9.9	9.1	9.5
CHS (μM)	0	200	500	1000	2000	
CHS-to-DPPC ratio (mol/mol)	0	1:10	1:4	1:2	1:1	
T_{m1} ($^\circ\text{C}$)	41.5	39.2	40.2	47.3		
ΔH_1 (kcal/mol)	10.9	8.1	6.3	3.6	0	
T_{m2} ($^\circ\text{C}$)		53	61.8	63.4		
ΔH_2 (kcal/mol)		1.2	1.5	2.2	0	

TABLE 2

OA on POPE thermodynamic parameters

Effects of DNM on POPE and OA on bovine brain PE were also measured (see text). DNM effects on $L \leftrightarrow H_{II}$ transition were described previously (Trichopoulos et al., 1995). POPE (2 mM) was always used.

OA (μM)	0	10	20	50	100	200	500
OA-to-POPE ratio (mol/mol)	0	1:200	1:100	1:40	1:20	1:10	1:4
T_m ($^\circ\text{C}$)	25.9	26.1	26.0	25.8	25.3	24.1	22.7
ΔH (kcal/mol)	5.9	6.4	6.4	5.9	5.1	5.9	6.2
T_h ($^\circ\text{C}$)	71.0	72.2	66.1	64.3	64.9		
ΔH_h (kcal/mol)	0.61	0.49	0.41	0.31	0.17		

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]GTP γ S 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 $92.8 \pm 3.5\%$; $p < 0.001$) and DNM (a decrease of $30.9 \pm 13.7\%$; $p < 0.05$) and of the forskolin-stimulated activity of AC (decreases of $98 \pm 1\%$, $p < 0.001$ and $57.2 \pm 10\%$, $p < 0.01$ for OA and DNM, respectively) (Fig. 5A). As expected, the α_2 -AR agonist UK14304 also inhibited the basal and forskolin-stimulated activity of AC ($37.8 \pm 9.7\%$, $p < 0.01$ and $38.3 \pm 12\%$, $p < 0.05$, respectively) (Fig. 5B). In contrast, PE and CHS did not significantly alter either the basal or the forskolin-stimulated

TABLE 3

Binding parameters for OA-POPE interaction

Binding of OA (70 μ M to 4 mM) to POPE (2 mM) liposomes was measured by isothermal titration calorimetry, where n_1 , n_2 , and n_t are binding site stoichiometry values, K_{a1} and K_{a2} are association binding constants, and H_1 and H_2 are reaction enthalpies. The overall OA binding corresponds to n_t values. Results are means of three independent experiments.

Binding Parameters	L Phase	H _{II} Phase
n_1 (mol of OA/mol of POPE)	1.03 ± 0.08	1.06 ± 0.11
K_{a1} (M $^{-1}$)	786 ± 49	$3100 \pm 289^{**}$
H_1 (kcal/mol)	413 ± 16	$159 \pm 9^{**}$
n_2 (mol of OA/mol of POPE)	0.75 ± 0.05	$1.66 \pm 0.09^{**}$
K_{a2} (M $^{-1}$)	122 ± 8	124 ± 10
H_2 (kcal/mol)	-1180 ± 58	$2170 \pm 164^{**}$
n_t (mol of OA/mol of POPE)	1.78 ± 0.08	$2.72 \pm 0.10^*$

* $p < 0.05$; ** $p < 0.01$.

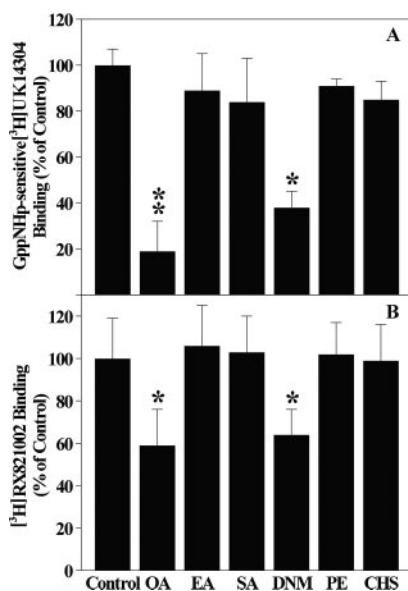


Fig. 2. Specific binding of the α_2 -adrenoceptor agonist [3 H]UK14304 (A) and the antagonist [3 H]RX821002 (B) to 3T3-AR membranes in the presence or absence (control) of OA, ES, SA, DNM, PE, or CHS (300 μ M). Specific [3 H]UK14304 binding was obtained by subtracting the radiolabeled ligand binding in the presence of 100 μ M Gpp(NH)p from that in the absence of Gpp(NH)p. Specific [3 H]RX821002 binding was carried out in the absence of Gpp(NH)p. Data are expressed as percentage values (mean \pm S.E.M.) with respect to the control untreated membranes. Binding of [3 H]UK14304 to control (untreated) membranes was 0.9 ± 0.15 pmol/mg of protein, whereas binding of [3 H]RX821002 to control (untreated) membranes was 5.3 ± 0.5 pmol/mg of protein. For other details, see text. *, $p < 0.01$; **, $p < 0.001$.

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 (H_{II}) 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 H_{II} 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 H_{II} propensity ($\sim T_h$ of -16°C). In POPE, used here, the high T_h value ($\sim 71^\circ\text{C}$; 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 H_{II} propensity. We recently demonstrated that activated G α_i proteins prefer lamellar-prone membranes, whereas heterotrimeric Gi ($\alpha\beta\gamma$) proteins and G $\beta\gamma$ dimers prefer H_{II} phases (Vögler et al., 2004). Here, we show that OA modulated the H_{II} 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

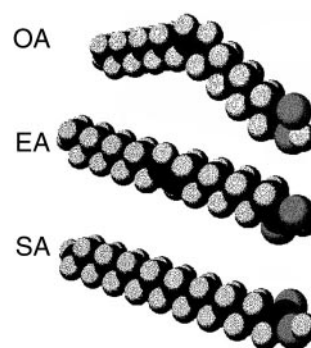


Fig. 3. Chemical structure of OA, *cis*-9-octadecenoic (18:1 c Δ 9), EA, *trans*-9-octadecenoic (18:1 t Δ 9), and SA, octadecanoic (18:0). OA has a double (*cis*) bond that restrains the mobility between C9 and C10, inducing a boomerang-like molecular shape. EA (the *trans*-isomer of OA) and SA have a molecular shape that resembles a rod. Only OA altered GPCR-mediated signaling.

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 [^3H]UK14304 but also [^3H]RX821002 binding [insensitive to Gpp(NH)p] to $\alpha_{2\text{AD}}$ -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 α_2 -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; Gior-

gione 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 $\text{L} \leftrightarrow \text{H}_{\text{II}}$ transition) than on membrane fluidity (defined by the $\text{S} \leftrightarrow \text{L}$ transition) (Tables 1 and 2). Here, we also showed that the lipid structure influenced the binding of fatty acids to model membranes. In a similar manner to OA, DNM has also been shown to interact differentially with model membranes of different composition (Escribá et al., 1990). In addition, it has been observed that resistance to antitumor drugs is related to the membrane lipid properties, which can be modulated by treatments with fatty acids (Escribá et al., 1990; Callaghan et al., 1993).

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-

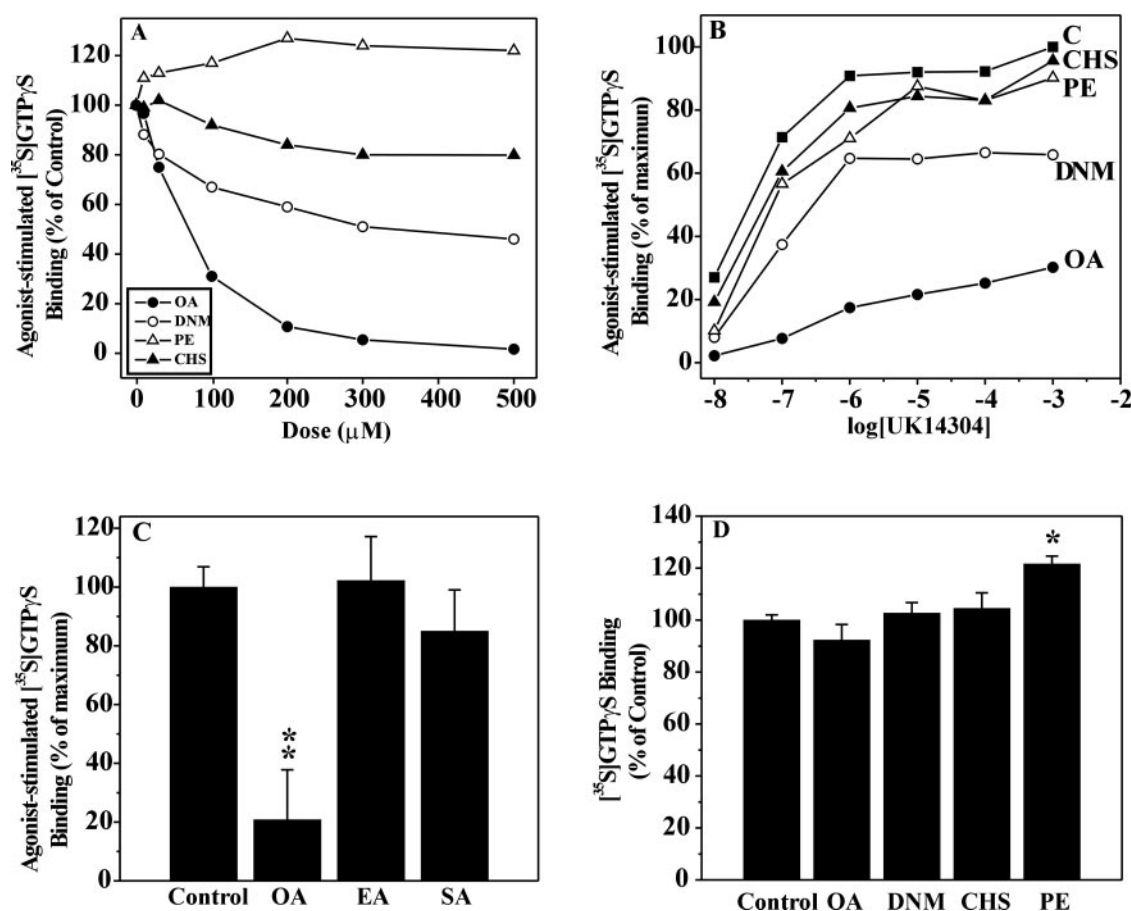


Fig. 4. Influence of agents that modify the lipid phase on agonist-induced binding of [^3S]GTP γ S to 3T3-AR cell membranes and to purified brain G proteins. A, specific agonist-stimulated binding of [^3S]GTP γ S (mean \pm S.E.M.) was measured in the presence or absence (control) of increasing concentrations of OA, DNM, PE, or CHS. B, specific agonist-stimulated binding of [^3S]GTP γ S to 3T3-AR membranes in the absence (C, control) or presence of 100 μM OA, DNM, PE, or CHS at increasing concentrations of the α_2 -AR agonist UK14304. C, effect of 100 μM OA, EA, and SA on the specific agonist-stimulated binding of [^3S]GTP γ S to 3T3-AR membranes. D, specific binding of [^3S]GTP γ S to G proteins purified from bovine brain, in the absence of membranes and in the presence or absence (control) of 100 μM OA, DNM, PE, or CHS. Basal [^3S]GTP γ S (control) binding to 3T3-AR membranes was 182.9 ± 26.9 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. *, $p < 0.05$; **, $p < 0.001$.

acades 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 eukaryotic cell division (Emoto et al., 1996), and in *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 (Escribá et al., 1995). Using this molecular mechanism, we have designed new compounds (2-hydroxyoleic acid) with a great anticancer activity (Martínez et al., 2005). 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 (Escribá 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 (Martín-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.

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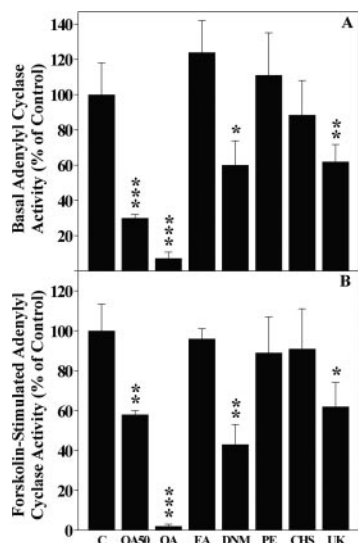


Fig. 5. AC activity. Basal (A) and forskolin-stimulated (B) AC activity measured in 3T3-AR cells, in the presence or absence (C, control) of 50 μ M OA (OA50), 100 μ M OA (OA), 100 μ M EA (EA), 100 μ M DN (DNM), 100 μ M CHS (CHS), or 10 μ M UK14304 (UK). The level of control basal AC activity was 15.37 ± 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 \pm S.E.M. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

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