Interaction of Electric Dipoles with Phospholipid Head Groups. A ^2H and ^{31}P NMR Study of Phloretin and Phloretin Analogues in Phosphatidylcholine Membranes†

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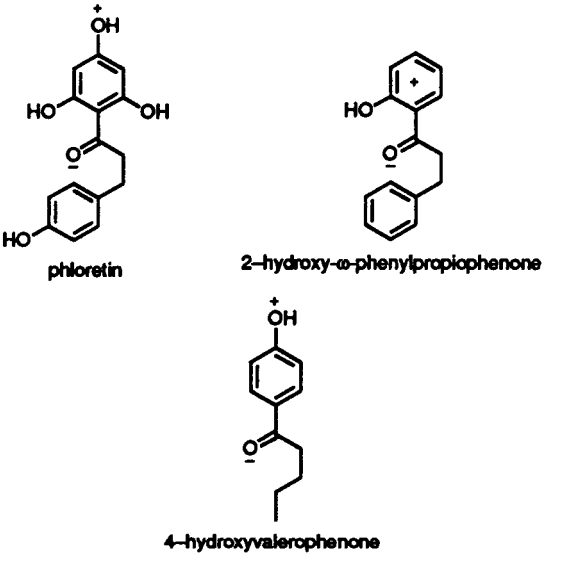
ABSTRACT: Phloretin, 4-hydroxyvalerophenone, and 2-hydroxy-ω-phenylpropiophenone are lipophilic dipolar substances that modify ionic conductances of bilayer membranes. The structural changes at the level of the head groups and the hydrocarbon chains as induced by the incorporation of phloretin and its analogues were investigated with deuterium and phosphorus nuclear magnetic resonance. Membranes composed of 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) were selectively deuterated at the choline head group and at the hydrocarbon chains, and ^2H and ^{31}P NMR spectra were recorded with varying concentrations of dipolar agents. Incorporation of phloretin leaves the bilayer structure intact, induces only a small disordering of the hydrocarbon chains, and has no significant effect on the head-group dynamics. On the other hand, quite distinct structural changes are observed for the phosphocholine head group. While the $^-P–N^+$ dipole is oriented approximately parallel to the membrane surface for pure POPC bilayers, addition of phloretin, and to a lesser extent 4-hydroxyvalerophenone and 2-hydroxy-ω-phenylpropiophenone, rotates the N^+ end of the $^-P–N^+$ dipole closer to the hydrocarbon layer. The resulting normal component of the $^-P–N^+$ dipole partly compensates the electric field of the dipolar agents. In addition to this structural change, phloretin also modifies the hydration layer at the lipid–water interface. Much less ^2H_2O is adsorbed to the membrane surface when the bilayer contains phloretin, 4-hydroxyvalerophenone, or 2-hydroxy-ω-phenylpropiophenone. Moreover, a rather large change in the residual phosphorus chemical shielding anisotropy argues in favor of hydrogen-bond formation between the phosphate segment and the phloretin hydroxyl groups.

The interaction of cell membranes with their aqueous surroundings is mediated, in part, by the phospholipid head groups. Physical–chemical methods have demonstrated that the lipid head groups are not rigidly fixed in a single conformational state but can change their average conformation in response to external stimuli. From neutron diffraction and NMR[1] studies it follows, for example, that the $^-P–N^+$ dipoles of an electrically neutral phosphatidylcholine membrane are extended almost parallel to the membrane surface (Büldt et al., 1979; Seelig et al., 1987). However, adsorption or binding of positively charged molecules such as metal ions (Brown & Seelig, 1977; Akutsu & Seelig, 1981; Altenbach & Seelig, 1984), local anesthetics (Boulanger et al., 1981; Seelig et al., 1988), or peptides (Dempsey & Watts, 1987; Dempsey et al., 1989; Roux et al., 1989; Kuchinka & Seelig, 1989; Beschiaschvili & Seelig, 1990) moves the N^+ end of the phosphocholine dipole toward the water phase. Addition of a negative charge has the opposite effect; i.e., the N^+ end moves toward the hydrocarbon phase (Scherer & Seelig, 1989). The driving force for the reorientation of the head groups is an electrostatic attraction–repulsion mechanism that is largely independent of the chemical nature of the charge carrier. The larger the electric surface charge density, the larger are the angular excursions of the phospholipid head-group dipoles from the membrane surface.

In the present study, the electric charge concept was extended to dipolar molecules with large dipole moments. We have incorporated phloretin (dipole moment μ = 5.6 D; Reyes et al., 1983), 4-hydroxyvalerophenone (μ = 3.8 D), and 2-hydroxy-ω-phenylpropiophenone (μ = 2.9 D) into phospholipid membranes and have investigated the effect of these molecules on the lipid head groups and, in part, on the hydrocarbon chains. Phloretin and its analogues are known to increase the



cationic conductances and to decrease the anionic conductances in artificial membranes containing ion carriers or lipophilic

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[1] Abbreviations: NMR, nuclear magnetic resonance; PC, 1,2-diacyl-*sn*-glycero-3-phosphocholine; DOPC, 1,2-dioleoyl-*sn*-glycero-3-phosphocholine; POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; POPA, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphatidic acid.

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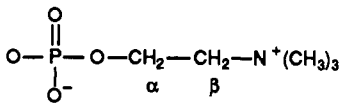
ions (Andersen et al., 1976). The molecular origin of this effect is a change in the membrane dipole potential that for neutral phosphatidylcholine membranes has a value of a few hundred millivolts (membrane interior positive) and that is considerably reduced upon addition of phloretin and its analogues [cf. Perkins and Cafiso (1987)]. Apparently, these agents adopt a membrane orientation such that their electric dipole moment counteracts the intrinsic membrane dipole potential; i.e., the positive end of the phloretin dipole faces the aqueous phase, whereas the negative end is buried in the hydrocarbon layer.

The modulation of the electric behavior of phosphatidylcholine membranes by electric dipoles could be accompanied by a change in the orientation and conformation of the phospholipid head groups or in the ordering of the hydrocarbon chains. We have therefore selectively deuterated POPC membranes at the two methylene segments of the phosphocholine head group and, alternatively, at the cis double bond of the oleic acyl chain and have recorded ^2H and ^{31}P NMR spectra as a function of the concentration of phloretin or phloretin analogues. As a control, we have also performed studies with cholesterol, which has only a small dipole moment (μ = 1.6 D) and has a comparatively small effect on the membrane conductivity but induces a distinct ordering of the lipid hydrocarbon chains (Franks, 1976; Worcester & Franks, 1976).

During the course of these studies it became clear that phloretin not only modifies the membrane dipole potential but simultaneously decreases the hydration layer around the phospholipid head groups. By hydrating POPC with ^2H_2O and measuring the hydration layer with ^2H NMR, we have obtained a semiquantitative estimate of the size of the hydration layer as a function of the phloretin concentration.

Materials and Methods

In order to simplify the discussion, the following notation is used for the phosphocholine head-group structure:



Lipid Synthesis. α- and β-deuterated cholines were synthesized according to Gally et al. (1975) and were coupled to POPA (Harbison & Griffin, 1984) to yield α-C^2H_2-POPC and β-C^2H_2-POPC, respectively. 1-Palmitoyl-2-[9′,10′-$^2H_2$]oleoyl-*sn*-glycero-3-phosphocholine (9′,10′-$^2H_2$-POPC) was synthesized according to Seelig and Seelig (1977). Nondeuterated lipids were purchased from Avanti Polar Lipids. Phloretin was obtained from Sigma, and its analogues 2-hydroxy-ω-phenylpropiophenone and 4-hydroxyvalerophenone were from Aldrich. Cholesterol (Fluka, Switzerland) was recrystallized in ethanol prior to use.

Lipid Samples for NMR Measurements. Approximately 20 μmol of deuterated lipid was mixed with the appropriate amount of phloretin, phloretin analogues, or cholesterol in a solution of dichloromethane/methanol. The solvent was evaporated under a stream of nitrogen, and the mixtures were dried further in high vacuum. Then 100 μL of buffer [150 mM NaCl and 20 mM NaH_2PO_4, pH 5.5, or 150 mM NaCl and 20 mM piperazine, pH 5.5, both made with deuterium-depleted water (CEA, Saclay, France)] was added. At pH 5.5, most phloretin molecules are in an uncharged state (pK $\simeq$ 9.0 when bound to POPC membranes; Bechinger, 1989). All substances investigated exhibit a low solubility in water (e.g., phloretin solubility is $<2.5 \times 10^{-4}$ M; Andersen et al., 1976) and a high affinity to membranes [the partition coefficient of phloretin to small unilamellar vesicles of POPC is $K_p = (26 \pm 8) \times 10^3$ M^{-1}; Bechinger, 1989; Verkman & Solomon, 1980; Reyes et al., 1983]. At pH $\leq$ 7.5, more than 95% of the phloretin partitions into the membranes. The lipid mixtures were dispersed by vortexing until the suspensions appeared to be completely homogeneous. Further equilibration was achieved by several freeze–thaw cycles.

NMR Measurements. ^2H NMR measurements were performed at 46.1 or 61.4 MHz by using the quadrupole echo technique (Davis et al., 1976) with phase cycling. The 90° pulse length was 3.5–4.0 μs and the echo pulse separation 40 μs. The spectra were recorded with quadrature phase detection, a spectral width of 10–100 kHz, and a memory size of 1–4K.

^{31}P NMR spectra were recorded at 121.5 or 162.0 MHz, with a Hahn echo sequence with proton decoupling and phase cycling (Rance & Byrd, 1983). The 90° pulse length was 3.0–4.5 μs, the interpulse delay 40 μs, the recycling delay 1 s, the spectral width 50 kHz, the data size 4K, and the number of acquisitions 5000–7000. Inverse gated decoupling was used.

Deuterium T_1 relaxation times were measured at 61.43 MHz by using the inversion–recovery sequence followed by a quadrupole echo.

In order to quantitate the amount of bound ^2H_2O, the free induction decay of a spin–echo or inversion–recovery sequence was processed in the absolute intensity mode, and the spectra were integrated by using the Bruker DISMSL software. Comparison of the membrane signal intensity to an internal ^2H_2O standard yielded a quantitative estimate of the water bound to the membrane surface.

Results

Figure 1 provides a comparison of ^2H NMR spectra of POPC membranes with and without phloretin or cholesterol. The spectra are characteristic of a homogeneous liquid-crystalline bilayer with no evidence for a lateral phase separation (Seelig, 1977; Griffin, 1981). The data thus demonstrate that phloretin and its two analogues (spectra not shown) can be mixed with POPC lipids up to rather large mole fractions without destabilizing the bilayer structure. However, even though the mixing behavior of phloretin parallels that of cholesterol to some extent, the effect of the two agents on the phosphocholine head group is quite different. While phloretin increases the splitting of the α segment and decreases that of the β segment, cholesterol has almost no influence on the α splitting and decreases the β splitting only at cholesterol concentrations >20% [cf. Brown and Seelig (1978)]. The variations of the ^2H NMR spectra in the presence of phloretin are qualitatively similar to those induced by the adsorption of negatively charged molecules (cf. below).

The quantitative evaluation of the ^2H NMR spectra is displayed in Figure 2, which shows the variation of the quadrupole splittings with the mole fraction of added agent. In a limited concentration range the data can be approximated by straight lines according to

$$\Delta\nu_i = \Delta\nu_i^0 + m_i X_b \quad \text{(kHz)} \qquad (1)$$

where $\Delta\nu_i$ is the measured quadrupole splitting, X_b is the mole fraction of added dipolar agent, and $\Delta\nu_i^0$ is the quadrupole splitting of pure POPC membranes. The slopes m_i provide a measure of the efficacy of the dipolar molecules in changing the head-group orientation, and their numerical values are listed in Table I. A comparison of the slopes m_α and m_β reveals that phloretin has the largest effect on the choline segments, followed by 4-hydroxyvalerophenone and lastly

Table I: Phloretin and Phloretin Analogues in POPC Membranes. Correlation of the Quadrupole Splittings and the Phosphorus Chemical Shielding Anisotropy with the Mole Fraction of Added Compound[a]

compound	dipole moment[b] (D)	m_p (ppm)	m_α (kHz)	m_β (kHz)	m_β/m_α	line range (mol %)
phloretin (pH 5.5)	5.6	39	4.9	−12.6	−2.7	0–20
4-hydroxyvalerophenone	3.8[c]	19	1.3	−6.0	−4.5	0–50
2-hydroxy-ω-phenylpropiophenone	2.9	2	0.7	−1.5	−2.0	0–50
cholesterol	1.6	≃5	∼0	∼0		0–20
anionic amphiphiles[d]		18.7	15.1	−13.9	−0.92	
cationic amphiphiles[d]		46.2	−30.0	+20	−0.67	

[a] The slopes m were evaluated according to $\Delta\nu_i = m_i X_b + \Delta\nu_i^0$ for the quadrupole splittings and $\Delta\sigma = m_p X_b + \Delta\sigma^0$ for the phosphorus chemical shielding anisotropy. [b] Reyes et al. (1983). [c] Reyes et al. (1983) determined the dipole moment of 4-hydroxybutyrophenone. [d] Scherer and Seelig (1989).

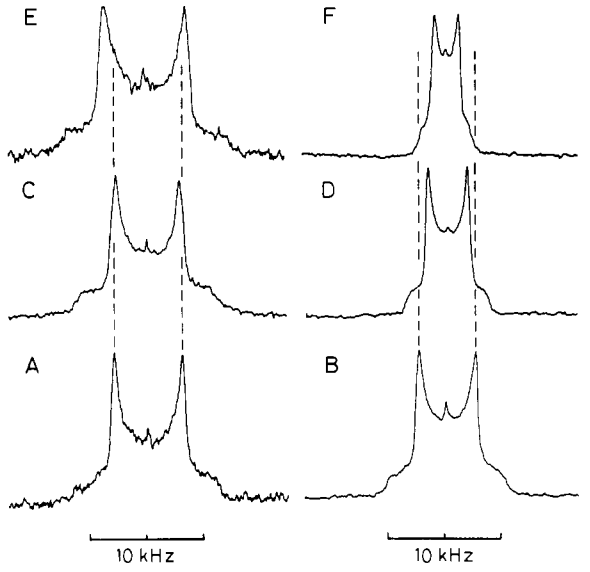


FIGURE 1: ^{2}H NMR spectra of POPC membranes with and without phloretin or cholesterol: (A) pure α-C^2H_2-POPC; (B) pure β-C^2H_2-POPC; (C) α-C^2H_2-POPC/cholesterol (50/50 mol/mol); (D) β-C^2H_2-POPC/cholesterol (50/50 mol/mol); (E) α-C^2H_2-POPC/phloretin (50/50 mol/mol); (F) β-C^2H_2-POPC/phloretin (50/50 mol/mol). All spectra were recorded in buffer at pH 5.5 and 25 °C.

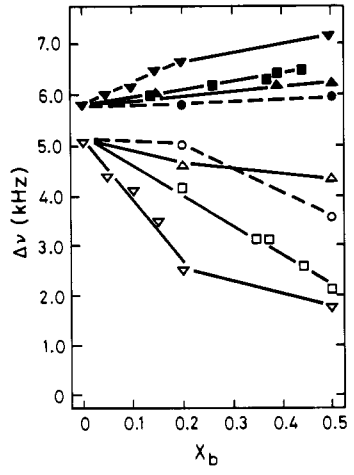


FIGURE 2: Variation of the deuterium quadrupole splittings of POPC membranes with the mole fraction, X_b, of membrane-bound agents. Closed symbols (upper part of figure): α-C^2H_2-POPC. Open symbols (lower part of figure): β-C^2H_2-POPC. (▼, ▽) POPC + phloretin; (□, ■) POPC + 4-hydroxyvalerophenone; (△, ▲) POPC + 2-hydroxy-ω-phenylpropiophenone; (○, ●) POPC + cholesterol. All measurements were made in buffer at pH 5.5 and 25 °C.

2-hydroxy-ω-phenylpropiophenone. Table I further demonstrates that all three agents affect the β splitting more than the α splitting since m_β/m_α ranges from −2 to −4.5. This is a rather unexpected finding. Addition of positively or negatively charged molecules to the membrane surface always

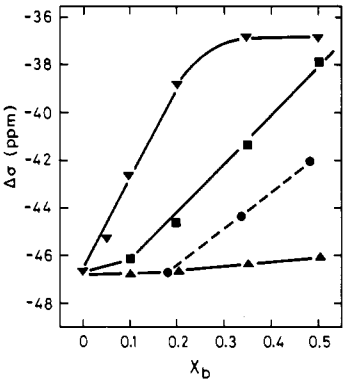


FIGURE 3: Variation of the phosphorus chemical shielding anisotropy with the mole fraction, X_b, of membrane-bound agent: (▼) POPC + phloretin; (■) POPC + 4-hydroxyvalerophenone; (▲) POPC + 2-hydroxy-ω-phenylpropiophenone; (●) POPC + cholesterol. Measurements were made in buffer at pH 5.5 and 25 °C.

yields m_β/m_α ratios of about −0.5 and −1, respectively, regardless of the chemical structure of the compounds involved (cf. Table I, last two rows).[2]

In order to further characterize the interaction of phloretin with the ^-P–N^+ dipole, the motion of the phosphate segment was investigated with ^{31}P NMR. For all POPC membranes containing phloretin or one of its analogues, the ^{31}P NMR spectra were consistent with a liquid-crystalline bilayer phase. From the edges of the spectra the residual chemical shielding anisotropy Δσ could be derived; Δσ is a measure of both the motional restrictions and the average orientation of the phosphate segment [cf. Seelig (1978)]. Figure 3 shows the variation of Δσ with the mole fraction of phloretin or phloretin analogues. We note that phloretin considerably reduces the size of the residual chemical shielding anisotropy, $|\Delta\sigma|$, up to 20 mol % phloretin; at higher concentrations no further change is observed. The 4-hydroxy analogue affects the phosphate segment only at concentrations above 10 mol %, but no saturation level is reached in the concentration interval investigated. The 2-hydroxy analogue induces almost no change. A yet different behavior was observed for cholesterol in POPC membranes: Δσ remained constant up to a mole fraction of 20% and then changed in parallel to the 4-hydroxy analogue.

The linear parts in Figure 3 can be approximated by

$$\Delta\sigma = \Delta\sigma_0 + m_p X_b$$

and the slopes are included in Table I. A comparison of the

[2] The breaks in the $\Delta\nu_Q$ vs X_b plots of phloretin suggest a rather complex lipid–phloretin interaction pattern at high phloretin concentrations. However, at all phloretin concentrations only a single time-averaged quadrupole splitting is observed, indicating a rapid exchange of POPC between different microdomains. It should also be noted that the break point at $X_b \sim 0.2$ is limited to the head-group segments. A continuous variation of $\Delta\nu_Q$ with X_b is found for the hydrocarbon-chain region (cf. Figure 4).

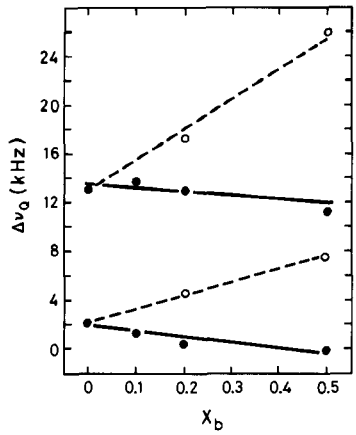


FIGURE 4: Influence of phloretin and cholesterol on the ordering of the hydrocarbon chains. Membranes were composed of 1-palmitoyl-2-(9′,10′-$^2H_2$)oleoyl-*sn*-glycero-3-phosphocholine and (●) phloretin or (○) cholesterol. The large quadrupole splitting is due to the C-9′ deuteron; the small splitting comes from the C-10′ deuteron. X_b is the mole fraction of added agent. Measurements were made in buffer at pH 5.5 and 25 °C.

slopes m_p yields the following order of head-group efficacy: phloretin > 4-hydroxyvalerophenone > 2-hydroxy-ω-phenylpropiophenone, which is consistent with the data obtained by ^2H NMR.

Changes of the lipid head-group conformation could be caused, in part, by structural perturbations at the level of the hydrocarbon chains. We have therefore recorded ^2H NMR spectra of POPC bilayers where the deuterons were attached at the cis double bond of the oleic acyl chains (Seelig & Waespe-Sarcevic, 1978). Figure 4 shows a plot of the quadrupole splittings of the C-9 and C-10 deuteron as a function of the phloretin or cholesterol concentration. The addition of 50 mol % cholesterol leads to the well-known stiffening of the hydrocarbon chains as reflected in an increase of the quadrupole splittings by more than 100%; in contrast, incorporation of phloretin reduces the quadrupole splittings, which can be explained by a small disordering of the hydrocarbon chains.

Phloretin carries three hydroxyl groups in the same ring system that could be involved in hydrogen bonds to the lipid head groups, the phosphate segment probably acting as a proton acceptor. This hypothesis was investigated by measuring the size of the lipid hydration layer in the presence and absence of phloretin. To this purpose, dry phospholipid films were hydrated in a ^2H_2O atmosphere of controlled humidity. The size of the hydration layer was then estimated with ^2H NMR. The experiments were performed as follows. Well-defined amounts of phloretin (or phloretin analogues) and POPC were dissolved in methanol/dichloromethane, the solution was spread on a cover glass, and the solvent was evaporated. The dry lipid films were then transferred into a ^2H_2O atmosphere (90% relative humidity) and were left to equilibrate for several days at room temperature. Two cover slides were clamped together and were oriented in the NMR spectrometer such that the normal to the lipid surface was perpendicular to the magnetic field. ^2H NMR spectra obtained as a function of phloretin/POPC ratio are displayed in Figure 5. At low phloretin concentrations, the ^2H NMR spectra exhibit two sharp lines (Figure 5A,B). This can be explained by the adsorption of ^2H_2O molecules to the membrane surface, forming a rather mobile but ordered hydration layer. The motion of the hydration water is anisotropic, and the quadrupole splitting of a few kilohertz is consistent with earlier hydration studies (Finer, 1973; Finer & Darke, 1974). The line shape of the ^2H NMR spectra deviates from the usual

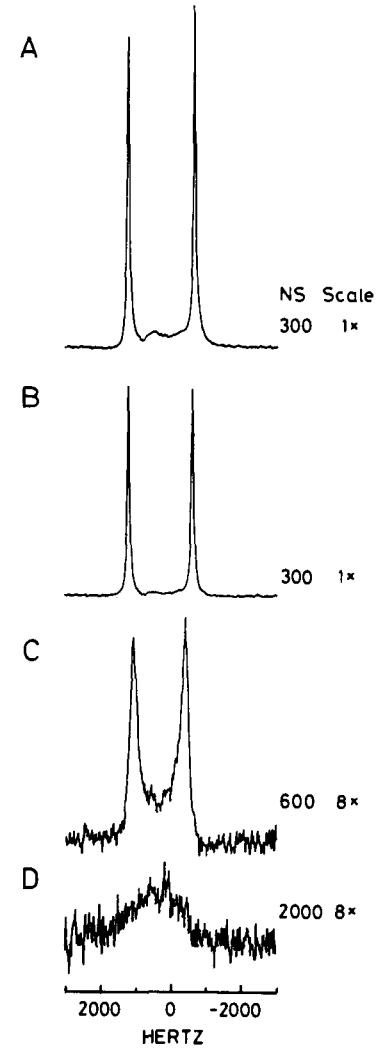


FIGURE 5: ^2H NMR spectra of oriented POPC membranes containing varying amounts of phloretin and equilibrated in a ^2H_2O atmosphere (90% relative humidity; 3 days of equilibration at room temperature): (A) pure POPC; (B) POPC + 10 mol % phloretin; (C) POPC + 35 mol % phloretin; (D) POPC + 50 mol % phloretin. NS denotes the number of scans per spectrum.

powder pattern since the experiments were made with homogeneously oriented membrane layers. The unexpected and new result, however, is the modulation of the ^2H_2O NMR intensity upon addition of phloretin. The lower two traces in Figure 5 were recorded with POPC membranes containing 35 and 50 mol % phloretin, respectively. As is obvious from the dramatic decrease in the signal-to-noise ratio, the number of ^2H_2O molecules adsorbed from the gas phase decreases as more phloretin is incorporated into the membrane. At the highest phloretin concentration, the adsorption of ^2H_2O molecules is almost undetectable. By comparing the integrated spectral intensity with a ^2H_2O standard, it was possible to quantitate the variation of the adsorbed ^2H_2O (Figure 6). Defining the ^2H_2O content of the pure POPC membrane as 100%, the addition of phloretin reduces the ^2H_2O content to almost 5% at 50 mol % phloretin. Figure 6 further demonstrates that phloretin is more efficient in blocking water adsorption than are the two phloretin analogues with only one hydroxyl group.

We have also investigated the phospholipid head-group dynamics by measuring the deuterium spin-lattice relaxation times of membranes with and without phloretin. T_1 is particularly sensitive to motions close to the Larmor frequency (64 MHz in the present studies), and T_1 measurements thus shed light on the fast segmental motions. The spin-lattice

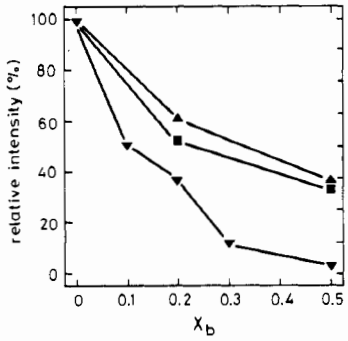


FIGURE 6: Relative intensity of the integrated ^2H_2O resonance (Figure 5) as a function of the mole fraction of phloretin or phloretin analogues: (▼) POPC + phloretin (■) POPC + 4-hydroxyvalerophenone; (▲) POPC + 2-hydroxy-ω-phenylpropiophenone.

relaxation times of pure α- and β-C^2H_2-POPC membranes were 15 ± 1 and 16.5 ± 1 ms, respectively, in agreement with previous studies (Tamm & Seelig, 1983). Incorporation of 20 or 50 mol % phloretin resulted in a small T_1 decrease ($T_1 \simeq 14$ ms for both segments); incorporation of 50 mol % cholesterol increased T_1 to 16.5 ms (α-C^2H_2-POPC) and 18 ms (β-C^2H_2-POPC). The T_1 relaxation time of membrane-bound ^2H_2O was in the range of 25–43 ms, depending on water content and temperature. Phloretin incorporation led to only a small reduction in the water T_1. For all samples T_1 increased with increasing temperature, indicating that the motion is in the fast correlation time regime with correlation times τ_c of 0.15 and 0.05 ns for the choline segments and ^2H_2O, respectively.

Discussion

The NMR results provide evidence that phloretin (μ = 5.8 D) and 4-hydroxyvalerophenone (μ = 3.8 D) induce a distinct change in the head-group orientation of POPC membranes; at the same time, the ordering of the hydrocarbon chains remains almost unaltered. This is in contrast to the influence of cholesterol (μ = 1.8 D), which causes a dramatic increase in hydrocarbon chain order but has a relatively small effect on the head groups. From the deuterium T_1 relaxation times, it further follows that the structural changes are not accompanied by dynamic changes, at least not as far as the fast segmental motions are concerned, which have correlation times in the nanosecond regime.

A comparison of phloretin with other dipolar substances has led to the conclusion that the variation of the membrane dipole potential is not a simple function of the dipole moment but depends also on the location, orientation, and surface density of the dipolar molecules (Andersen et al., 1976; Melnik et al., 1977; Reyes et al., 1983; Arviszus & Stark, 1988). For the three agents investigated here, the capacity to decrease the intrinsic membrane dipole potential was found to follow the sequence phloretin ≃ 4-hydroxybutyrophenone ≫ 2-hydroxy-ω-phenylpropiophenone (Reyes et al., 1983). The NMR results are consistent with this finding since the efficacy of changing the $^-P–N^+$ orientation follows the same order. In contrast, cholesterol has only a negligible effect on the anionic and cationic conductances of phosphatidylcholine membranes (Benz & Cros, 1978; Pickar & Benz, 1978), and its effect on the PC head group is also small.

In order to relate the structural changes observed with NMR to the variations of the membrane dipole potential we (1) summarize the present knowledge on the orientation of the phosphocholine dipoles and their contribution to the membrane dipole potential and (2) discuss a molecular model for the reorientation of the $^-P–N^+$ head group in the presence of phloretin and other dipolar molecules.

Head-Group Orientation and Dipole Potential of Pure Phosphatidylcholine Membranes. Neutron diffraction (Büldt et al., 1979) and ^2H and ^{31}P NMR studies (Seelig et al., 1977) have demonstrated that the $^-P–N^+$ dipoles of phosphatidylcholine membranes are extended essentially parallel to the membrane surface. However, even a small deviation from an exactly parallel orientation is sufficient to produce a considerable normal component of the electric field since the total dipole moment of the $^-P–N^+$ dipole is approximately 20 D (Shepherd & Büldt, 1978). For example, a deviation of ±20° from the plane of the membrane will yield a normal component of ±6.8 D. The resulting dipole potential can be approximated by $\psi_{DIP} \simeq n\mu/\epsilon_0\epsilon_r$ [cf. Adamson (1976)], where n is the surface density of the molecular dipoles, ϵ_0 is the permittivity of free space, and ϵ_r is the dielectric constant. With a lipid surface area of 68 Å^2 and ϵ_r = 40, a dipole component of ±6.8 D generates a potential of ±90 mV. This is quite significant as compared to an estimated total dipole potential of 400–500 mV (membrane interior positive).

The molecular origin of the membrane dipole potential is still a matter of debate. Previous studies have singled out the ester carbonyls (μ = 1.8 D) as the predominant source of the dipole potential [cf. Flewelling and Hubbell (1986)]. However, this approach assumes an exactly parallel orientation of the phospholipid head groups, neglects the role of hydration in water, and also ignores the dipole moment of the terminal methyl groups in the hydrocarbon layer. Measurements on phospholipid monolayers have led to the alternative suggestion that the terminal methyl groups (μ = 0.35 D) make a large contribution to the surface potential of DOPC monolayers and, in turn, also to the dipole potential of DOPC bilayers (Vogel & Möbius, 1988; Beitinger et al., 1989). According to the latter measurements, the CH_3-induced dipole potential of PC bilayers is further enhanced by about 100 mV by the dipole layer at the lipid–water interface [cf. Vogel and Möbius (1988), Table II]. This leads to the interesting conclusion that the N^+ end of the hydrated $^-P–N^+$ dipole must be closer to the membrane than the phosphate group in order to produce a dipole moment of the same polarity as the CH_3 groups.

A small "backward" orientation of the $^-P–N^+$ dipole (which is within the accuracy of the neutron diffraction studies) is further supported by a theoretical analysis of head-group interactions in monolayer and bilayer systems (Stigter & Dill, 1988; Dill & Stigter, 1988). In this model the effective orientation of the $^-P–N^+$ dipole is determined by a balance of the electrostatic energy that anchors the N^+ end in the water phase and the hydrophobic effect that drives the CH_2 and CH_3 groups around the N^+ charge toward the hydrocarbon phase. The model predicts that at 5 °C the $^-P–N^+$ dipole of PC is exactly parallel to the membrane surface and that at higher temperatures the N^+ end moves closer to the hydrocarbon environment. At 25 °C an average out-of-plane angle of the $^-P–N^+$ vector into the hydrocarbon phase of θ = 6° is estimated.

Finally, the head-group conformation of DOPC bilayers at 66% relative humidity has been investigated with X-ray and neutron scattering (Wiener & White, 1990). The authors conclude that "the choline, much more mobile than the phosphate, has a significant probability of venturing into the hydrophobic region of the bilayer".

Head-Group Orientation in the Presence of Phloretin and Other Dipolar Molecules. The variations of the NMR parameters in the presence of phloretin parallel those observed for molecules with a net negative charge; i.e., $|\Delta\sigma|$ is reduced,

$\Delta\nu_\alpha$ is increased, and $\Delta\nu_\beta$ is decreased. Qualitatively similar results have been reported for PC membranes with negatively charged lipids (Sixl & Watts, 1982; Scherer & Seelig, 1987), anionic amphiphiles (Seelig et al., 1987; Scherer & Seelig, 1989), and membrane-bound anions such as SCN^- (Macdonald & Seelig, 1988) and tetraphenylborate (Seelig et al., 1987). The similarity of the NMR parameters suggests that phloretin and negatively charged agents produce closely related conformational changes. By analogy with negatively charged amphiphiles (Scherer & Seelig, 1989), we conclude that phloretin and 4-hydroxyvalerophenone rotate the N^+ end of the PC head group toward the hydrocarbon phase. This backward rotation of the $^-P–N^+$ dipole compensates, in part, the dipole moment of phloretin. Without this response of the PC head group, the conductance changes induced by phloretin would be even larger.

The large positive variation of $\Delta\sigma$ with phloretin ($m_p \simeq +39$ ppm) cannot be explained by a simple electrostatic model since even molecules with a net negative charge are less effective. For example, the anionic amphiphile didodecyl phosphate is characterized by a slope of only +18.7 ppm, which nevertheless is the largest slope encountered in all studies with negative surface charges (Scherer & Seelig, 1989). However, an alternative mechanism is suggested by the hydration studies of Figures 5 and 6, demonstrating the replacement of lipid hydration water by phloretin, at least at low water contents. The large phosphorus slope of $m_p \simeq 39$ ppm suggests that this mechanism also holds true for fully hydrated membranes. The formation of hydrogen bonds between the phosphate segment (Wong & Mantsch, 1988) and the phloretin hydroxyl groups could lead to a specific reorientation of the phosphate group. As a corollary of this model, it also follows that the effective dipole moment at the membrane surface is altered not only by a reorientation of the $^-P–N^+$ dipole but also by a replacement of water dipoles and perhaps by a reorganization of the lipid hydration layer. A reorganization of the hydration layer of POPC membranes has also been observed upon addition of water-soluble polyhydroxyl compounds (Bechinger et al., 1988). The changes in the PC head-group conformation were modest compared to those induced by phloretin but were consistent with the presence of a small negative surface charge. The latter was explained by dipolar interactions due to a reorganization of the hydration layer.

In conclusion, ^2H and ^{31}P NMR have demonstrated that the incorporation of phloretin and phloretin analogues into POPC membranes changes the head-group orientation such that the N^+ end of the $^-P–N^+$ dipole moves toward the hydrocarbon layer. This rotation from a dipole orientation almost parallel to the membrane surface to a backward inclination compensates, in part, the effect of the phloretin dipole. In addition, the three hydroxyl groups of phloretin compete with the hydration water and may induce a specific reorientation of the phosphate segment.

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Registry No. POPC, 26853-31-6; phloretin, 60-82-2; 4-hydroxyvalerophenone, 2589-71-1; 2-hydroxy-ω-phenylpropiophenone, 3516-95-8.

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G-Protein $\beta\gamma$ Forms: Identity of β and Diversity of γ Subunits†

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ABSTRACT: Signal-transducing G-proteins are heterotrimers composed of GTP-binding α subunits in association with a tightly bound complex of β and γ subunits. While the α subunits are recognized as a family of diverse structures, β and γ subunits have also been found as heterogeneous isoforms. To investigate the diversity and tissue specificity of the $\beta\gamma$ complexes, we have examined homogeneous oligomeric G-proteins from a variety of sources. The β and γ subunits isolated from the major-abundance G-proteins from bovine brain, bovine retina, rabbit liver, human placenta, and human platelets were purified and subjected to biochemical and immunological analysis. Protease mapping and immune recognition revealed an identical profile for each of the two distinctly migrating β isoforms (β_{36} and β_{35}) regardless of tissue or G-protein origin. Digestion with V8 protease revealed four distinct, clearly resolved terminal fragments for β_{36} and two for β_{35}. Trypsin and chymotrypsin digestion yielded numerous bands, but again each form had a unique profile with no tissue specificity. Tryptic digestion was found to be conformationally specific with the most resistant structure being the native $\beta\gamma$ complex. With increasing trypsin, the complex was digested but in a pattern distinct from that for denatured β. In contrast to the two highly homologous β structures, examination of this set of proteins revealed at least six distinct γ peptides. Two unique γ peptides were found in bovine retinal G_t and three γ peptides in samples of bovine brain derived G_o/G_i. Human placental and platelet G_i samples each contained a unique γ. Finally, rabbit liver G_i preparations contained three electrophoretically resolvable γ peptides. Antisera raised to the retinal $\beta\gamma$ structure recognize both retinal γ forms and no other forms. The larger γ peptide from liver G_i is recognized by antiserum β-8 which specifically recognizes the human placental γ, the most rapidly migrating γ from liver corresponds with the mobility of the human platelet γ, and the third liver γ was found in our previous study to be recognized by a sequence-specific antiserum which identifies one of the two closely migrating G_o/G_i γ's as the product of the γ_2 gene [Gautam, N., Northup, J. K., Tamir, H., & Simon, M. I. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 7973–7977]. An additional γ peptide found in some, but not all, G_o/G_i preparations was identified as γ_3. These studies show a far greater diversity of the γ-subunit structure than had previously been recognized. Our results suggest that the function of the β subunit in G-proteins is highly conserved and that biochemical differences among the $G\beta\gamma$ forms are likely to be due to the diversity in γ structures.

The family of guanine nucleotide binding proteins (G-proteins)[1] plays a major role in transducing extracellular signals to cellular targets, thus transmitting a message from an active cell-surface receptor to cellular effectors. The regulation of adenylyl cyclase activity, visual excitation, activation of specific ion channels, and phosphoinositide turnover are mediated by G-proteins [for a review, see Gilman (1987), Casey and Gilman (1988), and Lochrie and Simon (1988)]. All identified signal-transducing members of this family, purified from a variety of sources, are heterotrimeric membrane proteins composed of three subunits: α, β, and γ (Northup et al., 1980; Fung et al., 1981; Hanski et al., 1981; Sternweis et al., 1981; Baehr et al., 1982; Bokoch et al., 1983; Codina et al., 1984; Sternweis & Robishaw, 1984). The α subunits all contain a high-affinity guanine nucleotide binding site, but they differ in size, sequence, their ability to serve as substrates

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[1] Abbreviations: G-protein, member of the family of signal-transducing GTP-binding regulatory proteins; G_t, vertebrate retinal G-protein (transducin); G_s and G_i, stimulatory and inhibitory G-proteins of the adenylyl cyclase system; G_o, 39-kDa G-protein of high abundance in the brain; GTPγS, guanosine 5′-*O*-(3-thiotriphosphate); DTT, dithiothreitol; Tris, tris(hydroxymethyl)aminomethane; SDS, sodium dodecyl sulfate.

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