Interaction of Charged and Uncharged Calcium Channel Antagonists with Phospholipid Membranes. Binding Equilibrium, Binding Enthalpy, and Membrane Location†

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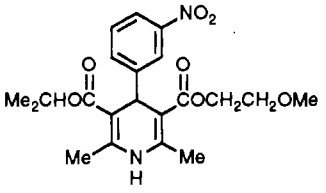
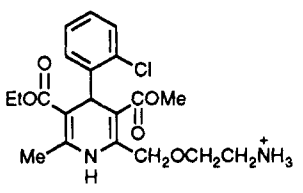
ABSTRACT: The membrane location and the binding mechanism of two Ca^{2+} channel antagonists, amlodipine and nimodipine, in pure lipid membranes were investigated with deuterium and phosphorus-31 nuclear magnetic resonance, with thermodynamic methods such as high-sensitivity titration calorimetry, and by measuring the membrane surface charge via the ζ-potential. The two drugs exhibit quite different physical–chemical properties. The noncharged nimodipine is strongly hydrophobic, and selective deuteration of the lipid membrane reveals a homogeneous distribution of nimodipine across the whole hydrocarbon layer, but no interaction at the lipid headgroup level. The membrane behavior of the amiphiphilic amlodipine (electric charge $z = +1$) is distinctly more complex. Deuterium magnetic resonance demonstrates that amlodipine adopts a well-defined position in the bilayer membrane. In particular, the charged ethanolamine side group of amlodipine is located near the water–lipid interface, interacting with the dipoles of the headgroup region according to a nonspecific, electrostatic mechanism and inducing a reorientation of the phosphocholine dipoles toward the water phase. At the level of the hydrocarbon segment, the nonpolar ring system of amlodipine interacts specifically with the cis double bond of the membrane lipid, forming a weak association complex. With increasing amlodipine concentration the deuterium signal of the cis double bond gradually loses intensity, a phenomenon previously observed only in related studies on protein–lipid interactions. The binding equilibrium of amlodipine to phosphatidylcholine membranes was studied by measuring the electrophoretic mobility of lipid vesicles and with a centrifugation assay. Hydrophobic interactions of the nonpolar ring systems and electrostatic repulsions at the membrane surface contribute to the binding energy. Electrostatic effects were taken into account by means of the Gouy–Chapman theory, and both experimental methods then lead to identical results: the binding of amlodipine to a lipid membrane can be described by a surface partition equilibrium with an intrinsic partition constant $K_p = 15\,500\ M^{-1}$, yielding a Gibbs free energy of binding of $\Delta G = -8.1$ kcal/mol. The enthalpy of amlodipine binding to neutral phosphatidylcholine membranes was measured independently with a high-sensitivity titration calorimeter, yielding $\Delta H = -9.2$ kcal/mol at 27 °C. The partitioning of the amphiphilic drug into the lipid bilayer is thus driven by the binding enthalpy ΔH. The entropy of transfer is negative, which is in contrast to the usual interpretation of the hydrophobic effect.

Amlodipine and nimodipine, two calcium channel antagonists, bind to specific receptors in cardiac and smooth muscle, modulating the transmembrane influx of extracellular calcium involved in the excitation–contraction process (Janis et al., 1987). The positively charged amlodipine has been shown



nimodipine



amlodipine

to be a potent inhibitor of Ca^{2+}-induced contraction of K^+-depolarized rat aorta (Burges et al., 1987). The two drugs have different physical–chemical properties. Nimodipine is electrically neutral and almost insoluble in water, but is highly soluble in lipid. In contrast, amlodipine carries an ethanolamine side group ($pK = 9.02$) and is greater than 95% protonated at physiological pH (Mason et al., 1989). Because of its charge the molecule exhibits distinct amphiphilic properties and is soluble both in water and lipid.

The location of nimodipine and amlodipine with respect to the membrane surface was determined with X-ray diffraction and, for nimodipine, also with neutron diffraction methods (Herbette et al., 1986; Mason et al., 1989). It was suggested that the nonpolar ring systems of both molecules adopt the same orientation in the hydrophobic part of the membrane with the long molecular axis extended parallel to the hydrocarbon chains. The dihydropyridine ring of both drugs was positioned with the NH end pointing toward the aqueous phase while the aromatic ring system was buried in the hydrocarbon core (Mason et al., 1989). The positive charge of amlodipine, though not directly detectable with X-ray diffraction, was positioned in the vicinity of the phosphate moiety of the phosphocholine dipole (Mason et al., 1989; Figure 7).

Diffraction techniques provide a rather coarse picture of the molecules embedded in a membrane, with positional accuracies not better than a few angstroms. We have therefore employed deuterium nuclear magnetic resonance (^2H NMR)[1] in com-

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[1] Abbreviations: ^2H NMR, deuterium nuclear magnetic resonance; POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; DOPC, 1,2-dioleoyl-*sn*-glycero-3-phosphocholine; DEPC, 1,2-dielaidoyl-*sn*-glycero-3-phosphocholine; TNS, 2-*p*-toluidinylnaphthalene-6-sulfonate.

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bination with selectively deuterated lipids to obtain a more detailed insight into the molecular aspects of drug–lipid interaction (Seelig, 1977). Deuterium labels were placed at the headgroup segments of phosphatidylcholine lipids and at different positions of the hydrocarbon chains. Both cis and trans unsaturated fatty acids were employed. By recording the variation of the deuterium quadrupole splittings with the concentration of amlodipine or nimodipine, a segment-by-segment resolution of the effect of these drugs on the membrane organization was obtained.

The quantitative interpretation of the ^2H NMR data is aided by a thermodynamic understanding of the binding process. How much drug is bound to the membrane, how much remains in the aqueous phase, and what is the drug concentration immediately above the plane of binding? Since the physical–chemical aspects of amlodipine and nimodipine binding/adsorption to lipid membranes are only superficially characterized, we have measured the binding by a centrifugation assay and have also recorded the electrophoretic mobility of phospholipid vesicles as a function of amlodipine concentration.

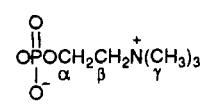
Amlodipine binding to neutral lipid vesicles appears to be anticooperative at first sight, since the binding becomes increasingly more difficult as more and more drug molecules are accommodated at the membrane surface. However, the quantitative analysis of the binding isotherm and the electrophoretic mobility, using the Gouy–Chapman theory, can explain the apparent anticooperativity in terms of electric repulsion effects and leads to a simple *surface partition* equilibrium for amlodipine binding.

The binding enthalpy of amlodipine was determined with a recently developed injection microcalorimeter (Wiseman et al., 1989). Knowledge of the binding constant and the binding enthalpy allows the evaluation of the entropic contributions to the binding process. The results are distinctly different from conventional oil/water partitioning studies, providing new insight into the molecular nature of the so-called *hydrophobic effect*.

Physical–chemical binding studies on nimodipine turned out to be difficult because of the very low solubility of this compound in water. On the other hand, nimodipine appears to be freely miscible with phospholipids up to a concentration of about 10 mol %, and at least a lower limit for the partition equilibrium constant could be estimated.

Materials and Methods

Chemicals. To simplify the discussion we introduce the following notation for the phosphocholine headgroup segments:



1-Palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) was selectively deuterated at the α and β segment of the choline headgroup as described previously (Tamm & Seelig, 1983). 1-Palmitoyl-2-[9′,10′-$^2H_2$]oleoyl-*sn*-glycero-3-phosphocholine, 1,2-di[9′,10′-$^2H_2$]elaidoyl-*sn*-glycero-3-phosphocholine (DEPC), and related compounds were prepared as described by Seelig and Waespe-Sarcevic (1978). Unlabeled POPC was purchased from Avanti Polar-Lipids Inc. (Birmingham, AL), and its purity was controlled by TLC before use.

Amlodipine [2-[(2-aminoethoxy)methyl]-4-(2-chlorophenyl)-3-(ethoxycarbonyl)-5-(methoxycarbonyl)-6-methyl-1,4-dihydropyridine] was kindly provided by Pfizer (Sandwich, U.K.) as the maleate salt. In order to avoid interference from the maleate anion, the latter was exchanged against chloride as follows. Fifty to sixty milligrams of the amlodipine maleate mono salt was dissolved in 20–30 mL of water, and 10 g of a strong base ion exchanger (Merck, FRG) was added for 3 min. The ion exchanger was filtered off, the solution was acidified with hydrochloric acid to pH = 6, the solvent was evaporated, and the sample was lyophilized. The purity of the product was checked with ^1H NMR and ^{13}C NMR spectroscopy. The spectra excluded the presence of maleate and showed no other impurities. The absorption coefficient of amlodipine was determined to be 6280 ± 20 M^{-1} cm^{-1} at 365 nm (average of six measurements). The absorption band at 365 nm corresponds to the characteristic absorption band of the dihydropyridine ring in the range of 325–370 nm (Thoma et al., 1985).

Nimodipine [2,6-dimethyl-3-[(2-methoxyethoxy)-carbonyl]-5-(ethoxycarbonyl)-4-(3-nitrophenyl)-1,4-dihydropyridine] was a gift from Bayer (Wuppertal-Elberfeld, FRG). All experiments with nimodipine were carried out at a wavelength above 500 nm, since exposition to light of $\lambda < 450$ nm induces diverse photoreactions similar to those observed for nifedipine (Thoma et al., 1985; Matsuda et al., 1989).

All other chemicals were purchased from Merck (Darmstadt, FRG).

All experiments were performed in 100 mM NaCl and 10 mM Tris buffer at pH 7.25. For the ^2H NMR experiments deuterium-depleted water was used.

Measurement of Binding Enthalpies. Reaction enthalpies were determined with a MicroCal MC-2 high-sensitivity titration calorimeter (MicroCal, Northampton, MA) as described by Wiseman et al. (1989). The sample cell had a volume of 1.27 mL and contained sonicated lipid vesicles at a lipid concentration of ~4 mM in buffer. Injections (10 μL) of amlodipine solutions (1–3 mM) were made with an injection syringe coupled to a digital stepping motor.

Sample Preparation for Binding Assay. Typically, a solution of 4 mg of unlabeled POPC in dichlormethane/methanol was placed in a glass tube, the solvent removed with a stream of nitrogen, and the sample dried under high vacuum for 12 h. Defined amounts of buffer and amlodipine hydrochloride in buffer were added. The mixture was vortexed periodically and subjected to 8–10 freeze–thaw cycles. The equilibrated sample was then centrifuged at 3×10^5 g at 25 °C for 150 min, resulting in a clear and lipid-free supernatant. The equilibrium concentration of amlodipine in the supernatant (c_{eq}) was determined by UV spectroscopy using the absorption coefficient determined above. The amount of amlodipine bound to lipid could then be calculated from the difference between the starting concentration and the equilibrium concentration (c_{eq}). The molar ratio of bound amlodipine hydrochloride to the molar amount of POPC was denoted X_b.

NMR Measurements. High-resolution measurements were recorded on a Bruker MSL 400 NMR spectrometer operating at 400.13 MHz for protons and 100.62 MHz for ^{13}C. Solid-state NMR measurements (^2H NMR and ^{31}P NMR) of membranes were made on the same spectrometer operating at 61.4 MHz for ^2H and 161 MHz for ^{31}P. For the ^2H NMR measurements a quadrupole echo sequence with a pulse spacing of 40 μs was used (Davis et al., 1976). The $\pi/2$ pulse width was 4–6 μs for a 10-mm solenoid coil, the sweep width 50–100 kHz, and the recycling delay 400 ms. A total of 5000–10000 FIDs were accumulated. ^{31}P NMR measurements were performed by using a Hahn echo sequence with gated decoupling (Rance & Byrd, 1983). The recycling delay was 3 s, the interpulse spacing 40–45 μs, and the $\pi/2$ pulse

width 2.3–2.8 μs. A total of 1000–2500 FIDs were accumulated. The ^{31}P chemical shift anisotropy was determined from the distance between the edges of the spectrum, measured at half-height of the low-field shoulder. Usually 10–20 mg of deuterium-labeled lipid was incubated with amlodipine buffer solution and subjected to several freeze–thaw cycles as described above. The lipid pellets obtained from the centrifugation were used for the solid-state measurements. Samples containing nimodipine were prepared by mixing a defined solution of nimodipine in chloroform with POPC also dissolved in $CHCl_3$. After drying under high vacuum, 100 μL of deuterium-depleted water was added and the sample vortexed several times.

ζ-Potential Measurements. Multilamellar vesicles for measuring the electrophoretic mobility were prepared according to the procedure of Bangham et al. (1974). All experiments were made at 25 °C with a Rank Bros. Mark II microelectrophoresis apparatus (Bottisham, Cambridge, U.K.). The electrophoretic mobility of vesicles was observed in the stationary layer according to Henry (1938). The microelectrophoretic mobility, u, was measured as described by Chung et al. (1985). The ζ-potential was calculated according to Helmholtz–Smoluchoswki equation

$$\zeta = \eta u/\epsilon_r\epsilon_0 \quad (1)$$

where ϵ_0 is the permittivity of free space, ϵ_r is the permittivity of the solution, and η the viscosity of the solution [cf. Aveyard and Haydon (1973)]. Each ζ-potential value was based on the average of fifteen mobility measurements. For low amlodipine concentrations a waiting time of 6 h was included before starting the measurements in order to obtain a better equilibration. In addition, an aliquot of the samples was centrifuged after equilibration with amlodipine, and the equilibrium concentration of amlodipine in the supernatant was determined by UV spectroscopy.

Results

Binding Equilibrium and Electrophoretic Mobility. The binding/adsorption of amlodipine to neutral POPC bilayers was measured in the concentration range of 1 μM < c_{eq} < 35 μM by the centrifugation assay described above. The corresponding binding isotherm (in buffer at pH 7.2) is displayed in Figure 1. The degree of binding X_b (mmol of amlodipine/mol of lipid) is plotted vs the equilibrium concentration of free amlodipine. The figure contains results from vesicles used for ζ-potential measurements (no freeze–thaw cycles involved) and from coarse liposome dispersions subjected to several freeze–thaw cycles. Good agreement of the two sets of measurements was obtained. The dashed line in Figure 1 corresponds to a partition equilibrium in which the amount of bound drug is linearly proportional to the equilibrium concentration c_{eq}, i.e., $X_b = K_p c_{eq}$, calculated with K_p = 15 500 M^{-1}. The experimental data deviate from linearity with X_b being smaller than predicted, indicating increasing electrostatic repulsion of amlodipine as the membrane surface becomes positively charged. The solid line corresponds to a theoretical model which combines a partition equilibrium (using again K_p = 15 500 M^{-1}) with the Gouy–Chapman theory (cf. below).

The electric charge imparted to lipid vesicles by the binding of amlodipine molecules leads to a vesicle movement in the presence of an electric field. By measuring the migration velocity of the lipid vesicles, it is possible to derive the ζ-potential according to eq 1. The variation of the ζ-potential with the amlodipine equilibrium concentration is shown in Figure 2. While pure POPC vesicles are electrically neutral and do not migrate in the electric field, the ζ-potential increases to about +30 mV for POPC vesicles dispersed in a 30 μM amlodipine solution. The measurement of the ζ-potential provides independent physical–chemical information on the binding/adsorption process. The solid line in Figure 2 corresponds to the same theoretical model (with the same partition constant K_p = 15 500 M^{-1}) as used for Figure 1.

Taken together, the binding isotherm and the ζ-potential provide a consistent picture of the binding process and allow the evaluation of the partition constant K_p with high accuracy. By repeating the experiments at different temperatures, the enthalpy of the partition equilibrium, ΔH, can be determined. However, since such measurements are rather tedious and

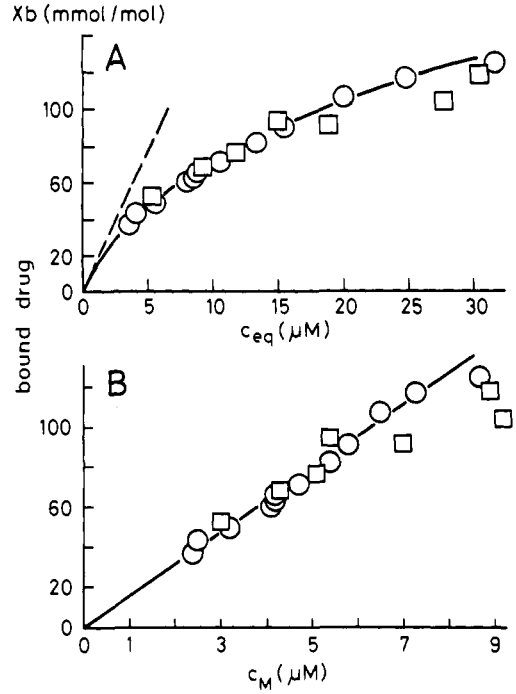


FIGURE 1: Binding of amlodipine (electric charge z = +1) to bilayer membranes composed of 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC). (A) The degree of binding (millimoles of amlodipine per mole of lipid) is plotted vs the equilibrium concentration of amlodipine in solution. The dashed line corresponds to a hypothetical partition equilibrium $X_b = K_p C_{eq}$, with K_p = 15 500 M^{-1}. The experimental data (○, centrifugation assay; □, microelectrophoretic measurement) deviate from the hypothetical partition equilibrium due to electrostatic repulsion. The solid line is the theoretical binding curve calculated with a partition equilibrium (K_p = 15 500 M^{-1}) taking into account electrostatic effects by means of the Gouy–Chapman theory (buffer: 0.1 M NaCl, 10 mM Tris, pH 7.2; 23 °C). (B) The same data as in (A) but plotted against the interfacial concentration $C_M = C_{eq}\exp(-F_0\psi_0/RT)$. The solid line corresponds to $X_b = K_p C_M$, with K_p = 15 500 M^{-1}.

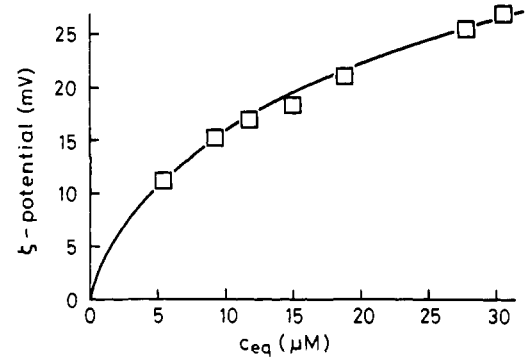


FIGURE 2: ζ-Potential measurements of POPC multilamellar vesicles at various concentrations of amlodipine. The theoretical curve was calculated with K_p = 15 500 M^{-1} and z = +1 by use of the same model as in Figure 1 (0.1 M NaCl, 10 mM Tris-HCl, pH 7.2).

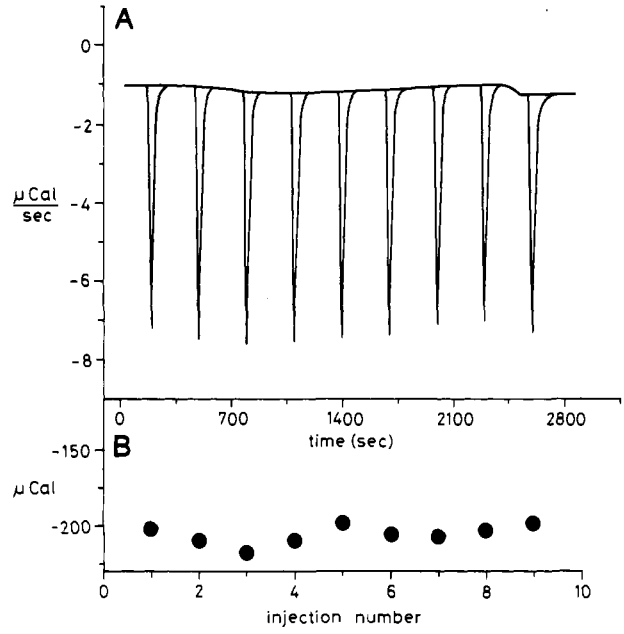


FIGURE 3: Measurement of the reaction enthalpy of amlodipine binding to POPC membranes. (A) Raw data obtained for 10 automatic injections, each 10 μL, of amlodipine solution (2.47 mM) into the sample cell containing sonicated lipid vesicles (4 mM). Temperature 27 °C. Buffer composition: 0.1 M NaCl and 10 mM Tris-HCl, pH 7.25. (B) Plot of the reaction enthalpies obtained by integrating the raw data of panel A.

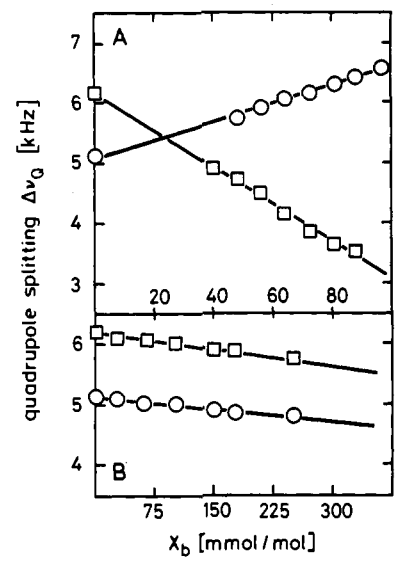


FIGURE 4: Variation of the phosphocholine headgroup with bound amlodipine and nimodipine. The deuterium quadrupole splitting is plotted vs X_b, the mole fraction of bound drug: (□) [α-CD_2]POPC; (○) [β-CD_2]POPC. (A) Binding of amlodipine induces counterdirectional changes at the α- and β-segment of the choline moiety in accordance with the "molecular electrometer" concept. (B) Incorporation of nimodipine into the POPC membranes causes a simultaneous disordering of both choline segments.

prone to error we have measured ΔH directly with a high-sensitivity titration calorimeter (Wiseman et al., 1989). The calorimeter cell, equilibrated at 27 °C, contained sonicated lipid vesicles (lipid concentration 4–8 mM) in buffer, and 10-μL injections (15-s duration) of a 2.47 mM amlodipine solution (in buffer) were made at 5-min intervals. Figure 3A shows a trace of 10 such injections as recorded by the instrument. The reaction is exothermic and the reaction enthalpy is constant since the lipid is in large excess over the added drug. This is demonstrated by evaluating the area underneath each calorimeter trace (Figure 3B). The reaction enthalpy is $\Delta h = -(206 \pm 6)$ μcal per injection. The heat of dilution, Δh_{dil}, was determined separately by injecting the same drug solution into buffer without lipid, leading to a slightly endothermic reaction with $\Delta h_{dil} \simeq 12.7 \pm 0.4$ μcal per injection. All binding enthalpies were hence corrected for Δh_{dil}. In Figure 3, 24.7 nmol of amlodipine was injected into the calorimeter cell in each experiment,[2] leading to a (corrected) reaction enthalpy of −219 μcal. If we assume that *all* drug binds to the lipid, then the *minimum* molar reaction enthalpy is $\Delta H_{min} = -8.87 \pm 0.4$ kcal/mol. However, the detailed analysis shows that with a partition constant of $K_p = 15\,500$ M^{-1} about 96.8% of the injected drug is bound to lipid under the present experimental conditions. The reaction enthalpy per mole of *bound* amlodipine is thus $\Delta H \simeq -9.2$ kcal/mol. The calorimetric measurements were also performed at 8 and 39.7 °C, yielding minimum reaction enthalpies ΔH_{min} (all drug bound) of −8.4 and −9.4 kcal/mol, respectively. Correcting for unbound amlodipine leads to reaction enthalpies of −8.7 kcal/mol (8 °C) and −9.7 kcal/mol (39.7 °C). A plot of these enthalpies against the temperature yields a straight line with a slope of $C_p \approx -32$ cal/(mol·K).

For nimodipine an approximate partition coefficient of 5000 M^{-1} has been reported (Mason et al., 1989). We found nimodipine too little soluble in buffer to detect even traces with UV, indicating a nimodipine solubility of $\leq 10^{-6}$ M. On the other hand, nimodipine could be easily dissolved in membranes up to a mole fraction of 10%. Provided that kinetic hindrance can be excluded, the lipid/water surface partition coefficient of nimodipine could thus be larger than 10^5 M^{-1} for the POPC membrane system.

Deuterium and Phosphorus-31 NMR Studies of the Phospholipid Headgroup. The effect of amlodipine and nimodipine on the phosphocholine headgroup was studied with deuterium and phosphorus NMR. All spectra were characteristic of liquid-crystalline bilayers, and only a single quadrupole splitting and a single phosphorus chemical shielding anisotropy were seen at all drug concentrations, indicating a time-averaged headgroup conformation. The residence time of amlodipine or nimodipine at an individual lipid segment must be shorter than the time scale of deuterium NMR, i.e., shorter than 10^{-5} s. Apparently, the mobility of the two drugs in the lipid membrane is quite fast, and even at low drug concentrations their presence is sensed by all lipids [cf. Mason and Chester (1989)].

Amlodipine and nimodipine have different effects on the headgroup parameters. Binding of charged amlodipine induces a counterdirectional response of the two choline segments, with the quadrupole splitting of the α-segment, $\Delta\nu_\alpha$, decreasing and that of the β-segment, $\Delta\nu_\beta$, increasing (spectra not shown). In contrast, uncharged nimodipine decreases the quadrupole splittings of *both* choline segments simultaneously. The variation of the headgroup splittings with the amount of bound drug, X_b, is summarized in Figure 4. For both compounds the quadrupole splittings depend linearly on X_b, and linear regression analysis of the data of Figure 4 yields for *amlodipine binding*

[2] The degree of binding, X_b, varies between 4.8 mmol of amlodipine/mol of lipid (1 injection) and 48 mmol/mol after 10 injections. This calculation assumes (i) complete binding of drug and (ii) availability of all lipid for drug binding. Under the present experimental conditions 97% of the added amlodipine is membrane bound. However, charged amlodipine cannot penetrate the hydrophobic core of the membrane; only the outer monolayer, comprising ~70% of the total lipid, is available for drug binding. Hence the effective X_b (considering only the outer monolayer) is about 30% larger than calculated above.

$$\Delta\nu_\alpha = 6.15 - 30.5X_b \text{ (kHz)} \quad (2)$$

$$\Delta\nu_\beta = 5.1 + 15.25X_b \text{ (kHz)} \quad (3)$$

and for *nimodipine binding*

$$\Delta\nu_\alpha = 6.15 - 1.9X_b \text{ (kHz)} \quad (4)$$

$$\Delta\nu_\beta = 5.15 - 1.5X_b \text{ (kHz)} \quad (5)$$

where X_b is measured in moles of drug bound per mole of lipid. The 10–20-fold larger slopes induced by amlodipine clearly demonstrate the much higher efficacy of the charged drug in altering the lipid headgroup conformation.

The influence of the two drugs on the phosphate moiety was investigated with ^{31}P NMR. All spectra were consistent with the typical bilayer shape (spectra not shown). The chemical shielding anisotropy was −49 ppm and remained approximately constant in the concentration range investigated.

Drug Binding and Hydrocarbon Chain Ordering. The influence of amlodipine and nimodipine on the hydrophobic part of the bilayer membrane was investigated with bilayers composed of 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC; T_c = −22 °C) and the corresponding trans-unsaturated lipid, 1,2-dielaidyl-*sn*-glycero-3-phosphocholine (DEPC; T_c = 9.5 °C). Deuterium labels were attached either at the C-2 segment or at the cis or trans double bond (C-9′,10′ segment). Additional measurements were made with 1-palmitoyl-2-[9′,10′-$^2H_2$]oleoyl-*sn*-glycero-3-phosphocholine ([9′,10′-$^2H_2$]-POPC).

The simplest spectral pattern is observed for DEPC membranes with the deuterium label at the trans double bond. Both the *sn*-1 chain and the *sn*-2 chain carry a trans double bond, but the four deuterons give rise to the same averaged quadrupole splitting [cf. Seelig and Waespe-Sarcevic (1978)]. Figure 5A summarizes the variation of this splitting with the mole fraction of bound amlodipine or nimodipine. Amlodipine induces almost no change in $\Delta\nu_Q$, whereas nimodipine causes an increase in $\Delta\nu_Q$ of about 10% at $X_b \simeq 0.15$, which then decreases again at higher nimodipine concentrations. The behavior of the trans double bond is representative also for the C-2 chain segments of DEPC and DOPC. As a second example Figure 5B summarizes the results for the C-2 segment of the *sn*-1 chain of DEPC. Even though this segment is close to the headgroup region, where amlodipine has a large effect, almost no variation of the quadrupole splitting is seen at this position. For nimodipine, which can be added to a 3-fold higher concentration, ^2H NMR indicates a larger variation of $\Delta\nu_Q$ with a maximum effect around 10% nimodipine. For the C-2 segment of the *sn*-2 chain of DEPC and DOPC, two quadrupole splittings can be resolved, indicating an orientational inequivalence of the two deuterons (Seelig & Seelig, 1975). The influence of amlodipine and nimodipine is similar as discussed above, and the numerical data of all segments are listed in Table I.

Quite unexpected effects of nimodipine and amlodipine were observed at the position of the cis double bond. The two deuterons at the cis double bond of POPC give rise to two separate quadrupole splittings of 13.5 and 2.3 kHz for the C-9 and C-10 deuteron, respectively, as shown in Figure 6A [cf. Seelig and Waespe-Sarcevic (1978)]. The molecular origin of this effect is a tilting of the cis double bond with respect to the bilayer normal. Addition of nimodipine first *increases* the C-9 splitting and *decreases* the C-10 splitting continuously up to ~15 mol % nimodipine (~25% change of $\Delta\nu_Q$) as shown in Figure 7. At higher nimodipine concentrations the quadrupole splittings start to return to their original values. The counterdirectional change of the two deuterons attached to the same C=C double bond rules out a more disordered motion of the cis double bond and can only be explained by a change in the tilt angle.

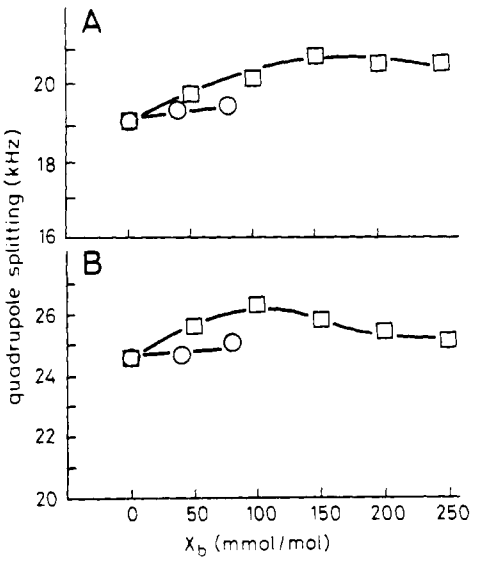


FIGURE 5: Influence of amlodipine (○) and nimodipine (□) on the hydrocarbon chain ordering of membranes composed of 1,2-dielaidoyl-*sn*-glycero-3-phosphocholine (DEPC). The observed quadrupole splitting is plotted vs X_b, the amount of bound drug. (A) Deuterium labels attached at the trans double bonds of both fatty acyl chains. The 4 deuterons give rise to a single quadrupole splitting. (B) Deuterium label attached at C-2 segment of the *sn*-1 fatty acyl chain of DEPC. All measurements were made in excess buffer (0.1 M NaCl, 10 mM Tris-HCl, pH = 7.25, 25 °C).

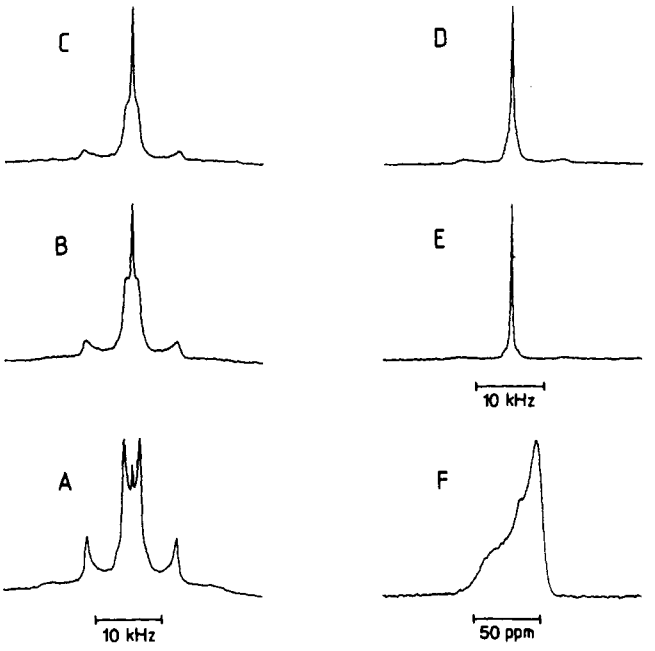


FIGURE 6: Deuterium and phosphorus NMR spectra of 1-palmitoyl-2-[9′,10′-$^2H_2$]oleoyl-*sn*-glycero-3-phosphocholine membranes containing increasing mole fractions of amlodipine. The quadrupole splittings of the C-9′ deuteron (13.4 kHz) and the C-10′ deuteron (2.3 kHz) remain approximately constant, but the signal intensity decreases considerably with increasing amlodipine concentration. All samples contain approximately the same amount of lipid (~20 mg) and buffer and were recorded under identical experimental conditions (number of scans: ~10000 per spectrum). Panel F displays the phosphorus-31 NMR spectrum corresponding to the ^2H NMR spectrum shown in panel E. (A) X_b = 0; (B) X_b = 40 mmol/mol; (C) X_b = 80 mmol/mmol; (D) X_b = 100 mmol/mol; (E) X_b = 120 mmol/mol.

An again different result was obtained with amlodipine. Even at moderate concentrations, amlodipine induces a line

Table I: Nimodipine and Amlodipine in Unsaturated Lipid Membranes. Hydrocarbon Chain Ordering as a Function of Drug Concentration (0.1 M NaCl, 10 mM Tris-HCl, pH 7.25; 23 °C)

X_b (mmol/mol)	$\Delta\nu$ (kHz)						
	[2′,2′-$^2H_2$]DOPC[a]			[2′,2′-$^2H_2$]DEPC[b]			[9′,10′-$^2H_2$]-DEPC[c]
nimodipine							
0	9.6	14.5	24.0	12.6	16.1	24.5	19.0
50	10.6	15.6	25.0	13.1	17.0	25.4	19.8
100	11.6	16.4	25.7	14.1	17.8	26.2	20.2
150	12.3	17.4	25.8	13.4	17.1	25.7	19.6
200	11.3	16.0	24.8	13.1	17.1	25.3	19.8
250	10.8	15.2	24.2	13.1	17.0	25.0	19.8
amlodipine							
40	10.2	15.3	24.2	12.7	16.3	24.5	19.4
80	10.4	15.3	24.7	12.6	16.3	25.0	19.5

[a] [2′,2′-$^2H_2$]DOPC = 1,2-di[2′,2′-$^2H_2$]oleoyl-*sn*-glycero-3-phosphocholine. The two deuterons at the *sn*-1 chain are equivalent and give rise to the large quadrupole splitting of ~24 kHz. The two deuterons at the *sn*-2 chain are inequivalent and produce the two smaller quadrupole splittings (Seelig & Seelig 1975; Seelig & Waespe-Sarcevic, 1978). [b] [2′,2′-$^2H_2$]DEPC = 1,2-di[2′,2′-$^2H_2$]elaidoyl-*sn*-glycero-3-phosphocholine. Assignment of the resonances as described for [2′,2′-$^2H_2$]DOPC. [c] [9′,10′-$^2H_2$]DEPC = 1,2-di[9′,10′-$^2H_2$]elaidoyl-*sn*-glycero-3-phosphocholine. The two deuterons attached to the same double bond give rise to the same quadrupole splittings for symmetry reasons (Seeling & Waespe-Sarcevic, 1978). Accidentally, the fluctuations of the trans double bonds in the two fatty acyl chains are identical so that only a single quadrupole splitting is observed for the two chains.

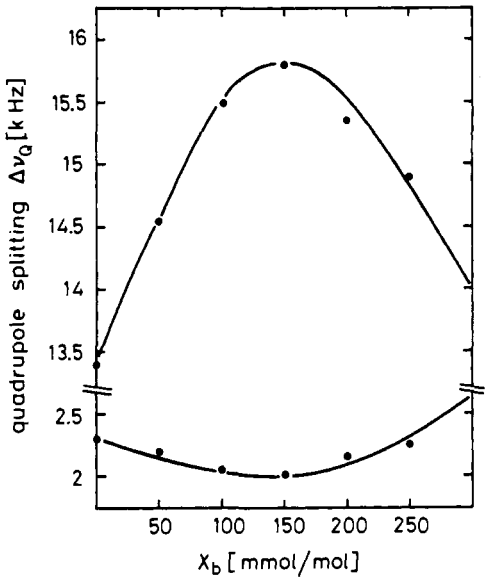


FIGURE 7: Effect of nimodipine on the orientation of the cis double bond in membranes composed of 1-palmitoyl-2-[9′,10′-$^2H_2$]oleoyl-*sn*-glycero-3-phosphocholine. The signal intensity remains constant over the whole concentration range. The counterdirectional variation of the quadrupole splittings (upper panel C-9′ deuteron, lower panel C-10′ deuteron) provides evidence for a change in the tilt angle of the cis double bond with respect to the bilayer normal.

broadening and a decrease in the ^2H NMR signal intensity as demonstrated by the spectra shown in Figure 6. The size of the two quadrupole splittings remains approximately constant (cf. the outer splittings in Figure 6A,D), but the loss in signal intensity is obvious as more and more amlodipine is bound to the membrane. All ^2H NMR spectra were recorded with approximately the same amount of lipid and the same number of scans. The integrated intensity thus decreases distinctly from spectrum A to spectrum E (Figure 6). ^{31}P NMR spectra were recorded for the same samples, and Figure 6F shows the ^{31}P NMR spectrum corresponding to the ^2H NMR spectrum in Figure 6E. All ^{31}P NMR spectra showed the full intensity and were characteristic of a liquid-crystalline bilayer. Only at the highest amlodipine concentration could a trace of an isotropic lipid phase be detected (Figure 6F). The intensity of this isotropic signal is however too small to account for the loss in ^2H NMR intensity. The most likely explanation of the latter effect is therefore a reduction in the rate of motion of the cis double bond to such an extent that the refocusing of the quadrupole echo becomes difficult. Similar, but less pronounced effects have been described for reconstituted lipid–protein systems such as cytochrome *c* oxidase in POPC membranes (Seelig & Seelig, 1978; Tamm & Seelig, 1983) or rhodopsin in 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine membranes (Bienvenue et al., 1981).

Discussion

Thermodynamic Analysis of the Binding Equilibrium. The analysis of amlodipine binding follows the approach described for the binding of local anesthetics (Seelig et al., 1988) and small peptides (Kuchinka & Seelig, 1989; Beschiaschvili & Seelig, 1990a,b) to neutral and charged lipid bilayers. At a given equilibrium concentration, C_{eq}, n_D drug molecules are bound to a total of n_L lipid molecules. The mole fraction of bound drug defined as

$$X_b = n_D/n_L \tag{6}$$

is experimentally accessible as described above. The drug molecules intercalate between the lipids, expanding the surface area (Seelig, 1987). The total surface area, A_T, is given by

$$A_T = n_D A_D + n_L A_L$$

where A_D and A_L are the effective surface areas of amlodipine and lipid, respectively. The surface area of POPC is well-known from a number of different methods (A_L = 68 Å^2; cf. Altenbach & Seelig, 1984). For amlodipine we estimate A_D ~ 50 Å^2 from molecular models. Since we restrict the analysis to a rather low loading of the membrane with drug ($X_b \leq 0.12$) the correct value of A_D is not too critical in the following. The binding of a single drug molecule to the membrane increases the electric surface charge by $+e_0$. The total charge is then given by

$$Q = n_D e_0 \tag{7}$$

leading to a surface charge density, σ, of

$$\sigma = Q/A_T = n_D e_0/(n_D A_D + n_L A_L) \tag{8}$$

$$\sigma = (e_0/A_L)\frac{X_b}{1 + X_b(A_D/A_L)} \tag{9}$$

Since X_b, A_D, and A_L are known, the evaluation of σ is straightforward. The corresponding data are summarized in Table II. The surface charge density σ gives rise to a membrane surface potential $\psi(x)$ which assumes a value ψ_0 at the membrane surface and decays to zero at large distances from the membrane. The surface potential at 2-Å distance is defined as the ζ-potential since the plane of hydrodynamic shear is about 2 Å away from the membrane surface [cf. Eisenberg et al. (1979)]. The surface potential ψ_0 and the ζ-potential can be calculated from the Gouy–Chapman equation [cf. Aveyard and Haydon (1973) and McLaughlin (1977, 1989)]

$$\sigma^2 = 2000\epsilon_0\epsilon_r RT\sum C_{i,eq}(e^{-z_i F_0\psi_0/RT} - 1) \tag{10}$$

where $C_{i,eq}$ is the concentration of the ith electrolyte in the bulk aqueous phase (in moles per liter), z_i the signed valency of the ith species, ϵ_0 the electric permittivity of free space, ϵ_r the dielectric constant of water, F_0 the Faraday constant, and T the absolute temperature. The calculated surface potentials

Table II: Binding of Amlodipine to POPC Membranes (100 mM NaCl, 10 mM Tris Buffer, pH 7.25)

C_{eq} (μM)	X_b (mmol/mol)[a]	σ (mC/M^2)[b]	ψ_0 (mV)[c]	C_M (μM)[d]	X_b/C_M (mol^{-1})
Freeze–Thaw Cycles + Centrifugation Assay					
3.65	38.0	8.7	11.2	2.4	16080
4.20	44.0	10.0	12.8	2.5	17280
5.60	51.0	11.4	14.5	3.2	15760
8.10	61.8	13.9	17.7	4.1	15210
8.60	64.0	14.4	18.3	4.2	15175
8.92	67.0	15.0	19.1	4.2	15795
10.51	72.3	16.1	20.5	4.7	15260
13.38	83.2	18.5	23.2	5.4	15350
15.45	92.0	20.3	25.4	5.8	15990
20.05	108.0	23.6	29.1	6.5	16720
24.81	118.0	25.6	31.3	7.3	16110
31.70	126.3	27.2	33.1	8.7	14460
Vesicles Used for ζ-Potential Measurements					
5.42	53.0	12.0	15.3	3.0	17780
9.24	69.0	15.5	19.6	4.3	16030
11.80	77.0	17.2	21.7	5.1	15170
15.00	95.0	20.9	26.1	5.4	17480
18.80	92.0	20.3	25.3	7.0	13100
27.70	104.7	22.9	28.3	9.2	11400
30.43	119.0	25.8	31.5	8.9	13350

[a] Bound amlodipine per mole of lipid. [b] Membrane surface charge density σ calculated according to eq 9 by using a lipid area A_L = 68 Å^2 and a drug area A_D = 50 Å^2. [c] Calculated by means of the Gouy–Chapman theory. [d] Calculated according to eq 11.

ψ_0 are also summarized in Table II. In the present case the surface potential is positive and leads to a repulsion of amlodipine in solution; the concentration of drug ions in the solution immediately adjacent to the membrane surface (denoted C_M) is thus smaller than the equilibrium concentration:

$$C_M = C_{eq} \exp(-F_0\psi_0/RT) \quad (11)$$

The calculated C_M values are given in the penultimate column of Table II.

Taking into account the electrostatic repulsion, the drug binding can be dissected into two different steps, namely, (i) an approach from bulk solution (C_{eq}) to interfacial concentration (C_M) and (ii) a penetration of the drug molecule into the membrane. The second step may correspond to a simple surface partition equilibrium with

$$X_b = K_p C_M \quad (12)$$

where K_p is the partition coefficient. In Figure 1 the mole fraction of bound drug, X_b, is plotted versus (A) the measured equilibrium concentration, C_{eq}, and (B) the calculated surface concentration, C_M. The latter representation of the data indeed yields a straight line with a slope of K_p = 15 500 ± 1000 M^{-1}. Using this partition model and the Gouy–Chapman theory, we have then calculated the binding isotherm (Figure 1A) and the ζ-potential (Figure 2) as a function of the equilibrium concentration. The figures show an excellent agreement between theory (solid lines) and the experimental observations.

The nonspecific binding of amlodipine to light sarcoplasmic reticulum (LSR) membranes was determined by centrifugation at various concentrations of [^{3}H]amlodipine from 1 × 10^{-5} to 5 × 10^{-9} M (Herbette et al., 1989). The binding was described by a partition equilibrium with the (dimensionless) partition constant $K_{membrane/water} \simeq$ 19 000 ± 2400. Electric charge effects were not taken into account. The numerical agreement between this previous study and our results is physically meaningful only for a noncharged membrane. If the LSR carries a negative surface charge, the partition coefficient of Mason et al. (1989) should vary considerably even in the low concentration range of 1 nM–1 μM.

Binding Enthalpy as Driving Force of the Hydrophobic Effect? Knowledge of the surface partition coefficient allows the calculation of the free energy of amlodipine binding according to $\Delta G = -RT \ln(55.5K_p) \simeq -8.1$ kcal/mol (at 25 °C), where the factor 55.5 corrects for the cratic contribution [cf. Cantor and Schimmel (1980)]. Since the binding enthalpy was measured as $\Delta H = -9.2$ kcal/mol, the entropic contribution to the Gibbs free energy is $T\Delta S = -1.1$ kcal/mol. The enthalpic term thus provides the driving force for the binding of the amlodipine molecule whereas the binding entropy is negative and is unfavorable for binding.

Partitioning studies of small organic molecules between water and oil have demonstrated that the entropy is the dominant force which drives these nonpolar compounds into the oil phase whereas the reaction enthalpy is close to zero [for details of the so-called "hydrophobic effect" see Tanford (1980)]. In contrast, the partitioning of amlodipine, which is also partially hydrophobic, is enthalpy driven. The negative ΔH for amlodipine is not an isolated observation. We have measured a reaction enthalpy of $\Delta H_{min} = -9.2$ kcal/mol for the partitioning of the hydrophobic anion tetraphenylborate between water and POPC membranes and $\Delta H_{min} = -2.1$ kcal/mol for the charged local anesthetic dibucaine (at 26 °C in 0.1 M NaCl) (J. Seelig and P. Ganz, manuscript in preparation). In addition, Huang and Charlton (1972) measured the binding of the anionic potential sensitive dye 2-*p*-toluidinylnaphthalene-6-sulfonate (TNS) to egg yolk lecithin vesicles using a gel filtration method and derived a binding enthalpy of $\Delta H = -9.5$ kcal/mol from the temperature dependence of the binding constant. We have remeasured the TNS binding enthalpy directly using titration microcalorimetry and found $\Delta H = -9.65$ kcal/mol at 27 °C under comparable experimental conditions (J. Seelig and P. Ganz, manuscript in preparation). The binding constant of TNS was first determined by Huang and Charlton (1972) and later refined by McLaughlin and Harary (1976) using the Gouy–Chapman theory. Since K = 5000 M^{-1}, the total free energy ΔG is fully accounted for by ΔH, and Huang and Charlton (1972) described the binding of TNS with membranes as a "nonclassical" hydrophobic type of interaction. Taken together, these findings appear to be consistent with a recent suggestion of Privalov and Gill (1989) that the interaction between two nonpolar substances is mainly enthalpic in nature, i.e., that it is essentially a van der Waals interaction. In a typical "hydrophobic" partition equilibrium this van der Waals interaction is masked by large hydration energies. However, for membrane equilibria the loss of hydration energy in the water phase appears to be compensated by a restructuring of the water layer at the membrane surface.

Location of Nimodipine and Amlodipine in POPC Membranes. The "Molecular Electrometer" Concept. Neutron diffraction studies on deuterated nimodipine (pyridine: 2,6-CD_3) in sarcoplasmic reticulum membranes have led to the conclusion that nimodipine is "located in the protein knob region and at both water/hydrocarbon core interfaces of the bilayer, with more drug appearing in the inner monolayer" (Herbette et al., 1986). In the present case, where the membrane is composed of a single lipid, the drug distribution is necessarily symmetrical and it is not possible to distinguish between the two halves of the bilayer. For nimodipine all segments in the hydrocarbon layer show the same variation of the quadrupole splittings with increasing drug concentration, suggesting a rather homogeneous distribution of nimodipine in the whole volume of the hydrocarbon layer. Both the NO_2

and the dihydropyridine end of the molecule will probably see the water/hydrocarbon edge intermittently. The phospholipid headgroup itself cannot be reached by nimodipine. Nevertheless, as a consequence of nimodipine incorporation the average surface area per lipid headgroup will increase, allowing a slightly enhanced wobbling motion of the lipid headgroup. This is reflected in the small decrease in both headgroup splittings. At the same time the tilt angle between the C=C double bond and the bilayer normal is also changed, explaining the variation of the C-9 and C-10 deuteron in the opposite sense.

A first conspicous difference between nimodipine and amlodipine is the interaction of the latter with the lipid headgroup. The pronounced variations of the α- and β-quadrupole splittings cannot be explained by a decrease or increase of the average order of the choline moiety; instead, they require a conformational change of the lipid headgroup. The molecular origin of this effect appears to be the positive electric charge at the membrane surface since the same type of headgroup reorientation can be induced by very different compounds such as multivalent metal ions, local anesthetics, peptides, and charged amphiphiles [for a summary of the systems investigated see Beschiaschvili and Seelig (1991)]. The orientation of the phosphocholine dipole is sensitive to the electric surface charge and can thus be considered as a "molecular electrometer" (Seelig et al., 1987). Eliminating X_b from eqs 2 and 3 leads to $\Delta\nu_\beta = +8.17 - 0.5\Delta\nu_\alpha$. Virtually identical slopes have been obtained for the $\Delta\nu_\beta$ vs $\Delta\nu_\alpha$ plots (Akutsu & Seelig, 1981) of all other positively charged membranes investigated so far. The efficacy of amlodipine in changing the headgroup conformation is comparable to that of the charged local anesthetics dibucaine and tetracaine (Boulanger et al., 1981; Seelig et al., 1988). The phosphorus-31 NMR data reveal no change in the ^{31}P chemical shielding tensor, and a direct interaction between the positively charged amino group of amlodipine and the phospholipid phosphate moiety can be excluded. In fact, as suggested by the ^2H NMR data discussed below, amlodipine penetrates into the hydrophobic region at least up to the level of the cis double bond. The positively charged end of the molecule is thus positioned *below* the layer of the $^-P–N^+$ dipoles.

The second unusual effect of amlodipine is the quite dramatic line broadening of the ^2H NMR spectra of the cis double bond. This result suggests a specific van der Waals interaction between the cis double bond and one of the nonpolar ring systems, probably the aromatic chlorphenyl ring. The lifetime of this "complex" can be expected to be in the range of 10^{-7}–10^{-5} s, which is still short enough to remain in the "fast-exchange" limit (no change in $\Delta\nu_Q$) but sufficiently slow to lose echo intensity. As noted above, similar intensity losses have been observed for lipid–protein systems but amlodipine appears to be the first small molecule where such an effect is observable.

The specific interaction of amlodipine with the cis double bond may be used to define the position of the drug molecule with respect to the plane of the membrane. The maximum separation between the amino function and the nonpolar chlorophenyl ring is 8–9 Å, on the basis of the crystal structure (Mason et al., 1989). Next, the distance between the cis double and the *sn*-2 segment of the glycerol backbone can be calculated as follows. The glycerol backbone is oriented perpendicular to the surface of the membrane, whereas the *sn*-2 chain (which carries the cis double bond) starts out parallel to the membrane surface and bends off toward the hydrocarbon interior only at the C-2 segment of the hydrocarbon chain [cf. Seelig and Waespe Sarcevic (1978)]. Assuming an extended, all-trans methylene chain the distance between the cis double bond and the C-2 segment is $7 \times 1.25 = 8.75$ Å. However, due to trans–gauche isomerizations around carbon–carbon single bonds, the average end-to-end distance is shorter by at least 30% (Seelig & Seelig, 1974). The distance between the plane containing the cis double bond and that of the glycerol *sn*-2 segment is only 6–7 Å. Positioning the chlorphenyl ring of amlodipine near the cis double bond, the positively charged amino function is located in the plane containing the glycerol *sn*-3 segment, i.e., below the layer of the $^-P–N^+$ dipoles.

The interaction of amlodipine with the cis double bond of POPC may be contrasted with that of cholesterol (Bechinger & Seelig, 1991). The addition of cholesterol to POPC membranes produces a distinct increase in the spectral splitting but has absolutely no effect on the line width. The addition of 20 molar % cholesterol to POPC membranes increases the average quadrupolar splitting at both the C-9 and the C-10 deuteron of the cis double bond by more than 30%. At the same time the spectrum remains characteristic of the fast-exchange limit with no evidence of phase separation or complex formation.

Conclusions. The nonpolar ring systems of amlodipine and nimodipine are both located in the hydrophobic part of the lipid membrane, but the two molecules induce different perturbations of the membrane structure. Upon incorporation of amlodipine the POPC lipid headgroup exhibits the same response as established for other positive surface charges: the $^-P–N^+$ dipole reacts according to the "molecular electrometer" model, with the N^+ end moving into the water phase (Seelig et al., 1987; Scherer & Seelig, 1989; Beschiaschvili & Seelig, 1991). At the level of the hydrocarbon chains amlodipine incorporation is characterized by a unique association phenomenon involving the cis double bond which—though weak and still in the fast-exchange limit—leads to a gradual loss of the ^2H NMR signal. The intensity loss bears a distinct resemblance to ^2H NMR studies of lipid–protein interactions.

The charge effect at the lipid headgroup and the specific interaction with the cis double bond provide evidence for a well-defined localization of amlodipine with respect to the bilayer surface. In contrast, nimodipine appears to be dissolved randomly in the hydrocarbon layer, and no significant interaction with the lipid headgroup was observed. A specific effect of nimodipine is the change of the tilt angle of the cis double bond.

Amlodipine appears to be a molecule ideally suited to the study of the thermodynamics of amphiphile–membrane interactions. Though the equilibrium is vastly in favor of the membrane phase, the solubility of drug in the aqueous phase is still sufficient to allow a precise evaluation of the binding equilibrium. Electrostatic interactions at the membrane surface are significant and must be taken into account by means of the Gouy–Chapman theory. However, two different types of experiments (equilibrium centrifugation, microelectrophoresis) lead to the same binding model: the binding of amlodipine can be described by a surface partition equilibrium according to $X_b = K_p C_M$, where the interfacial concentration C_M is different from the bulk concentration C_{eq}. Finally, the binding enthalpy ΔH is the driving force for amlodipine incorporation into the lipid membrane whereas the binding entropy is even negative. This result is in agreement with the "nonclassical" hydrophobic type interaction observed for TNS binding by Huang and Charlton (1972) and appears to be a rather general phenomenon as far as membrane equilibria of

charged amphiphiles are concerned.

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Registry No. POPC, 26853-31-6; nimodipine, 66085-59-4; amlodipine, 88150-42-9.

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