Cholesterol and Lipid Phases Influence the Interactions between Serotonin Receptor Agonists and Lipid Bilayers* 

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The majority of drug targets are membrane proteins. Hence the interaction of a drug with the membrane is crucial for its efficacy. A high location probability in a particular part of a membrane and its orientation with respect to the membrane normal could well be relevant to how the drug is presented to the target protein’s binding site (1, 2). Cholesterol-rich microdomains ("lipid rafts") add another layer of complexity because the lateral organization of the membrane could well affect the concentration of a drug close to its target protein. This possibility is backed up by the observation that many chemicals, including drugs that target serotonin receptors, preferentially localize into these domains (3–5). Meanwhile, the location and activity of some membrane proteins, particularly G-protein-coupled receptors, seem to be affected by lipid interactions, so called “membrane-lipid therapy,” have only relatively recently been explored (7, 9, 10).

Some simple lipid mixtures have been used as models for in vivo microdomains. A particularly well studied mixture consists of 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) and cholesterol (11–16). With 20 mol % cholesterol, Scheidt et al. (14) interpreted the phase behavior as follows. At high temperatures (above 316 K), the whole bilayer is in the liquid-disordered (Ld) phase, characterized by rapid axial rotation and highly disordered aliphatic chains (16). Between 311 and 316 K, a mixed phase of liquid-ordered (Lo) and Ld phase lipids exists. Below 311 K, the membrane starts to coalesce into a mixture of Ld and gel phase (where the rotation of the lipids slows dramatically) before finally forming a homogenous gel phase. It is the Ld domains that are thought to resemble cholesterol-rich microdomains found in biological membranes.

Proton NMR and specifically magic angle spinning-assisted nuclear Overhauser enhancement spectroscopy (MAS-NOESY) experiments have proven to be an excellent tool for investigating, with atomic resolution, the location of small molecules embedded in lipid membranes (17–20). However, these studies have been limited to binary mixtures because anything more complicated results in serious spectral overlap. To circumvent this problem, we have used ternary mixtures of agonists, cholesterol, and chain deuterated DPPC lipids (DPPC-d62), thus removing the signals that would otherwise swamp the cholesterol resonances. The system allows interactions between cholesterol and agonist to be observed. Furthermore, the rapid axial motion of the lipids in the Ld and liquid-ordered (Ld) phases allows high resolution 1H spectra to be collected in both of these phases (14).

The work presented here follows from a study in which it was noticed that several of the agonists induced significant chemical shifts in the cholesterol signals of a brain lipid extract (17). To gain further insight into the interactions between bilayers containing cholesterol and the agonists, we now present a solid state NMR-based approach that utilizes DPPC-d62 to reveal NOESY interactions within the Ld and Ld phases.

*This work was supported by a Royal Society project grant and an Engineering and Physical Sciences Research Council doctoral training account.
‡The on-line version of this article (available at http://www.jbc.org) contains supplemental Fig. 1.
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Printed in the U.S.A.
phases, shedding light on how cholesterol and bilayer phases influence the interactions between bilayer components. Finally, we have also analyzed the effect that agonist have on the phase behavior of the lipid bilayers, and we comment on how this may provide an alternative route of action for drugs. We concentrate on quipazine and LY-165,163 (Fig. 1) because they previously showed strong interactions with cholesterol (17).

EXPERIMENTAL PROCEDURES

Materials—DPPC, DPPC-d_{62}, and cholesterol were purchased from Avanti polar lipids (Alabaster, AL). All other chemicals, including quipazine as a maleate salt and LY-165,163, were purchased from Sigma.

Sample Preparation—Samples of DPPC/cholesterol, DPPC/agonist, and DPPC/cholesterol/agonist were prepared at mole ratios of 8:2, 9:1, and 7:2:1, respectively. Typically, 80 mg of DPPC was used per sample. The lipids and agonists were co-dissolved in chloroform/methanol (1:1, v/v). The solvents were evaporated under vacuum, and the resulting lipid cake was suspended in 1 ml of doubly distilled water, frozen in liquid nitrogen, and then lyophilized overnight under high vacuum. The dry lipid mixtures were hydrated with D_{2}O for proton NMR or H_{2}O for deuterium NMR experiments and then subjected to three freeze/thaw cycles. The mixtures were centrifuged to remove excess water. 4-mm MAS rotors were filled with the resulting pellets. Samples were kept in the rotors for both 1H and 2H measurements.

NMR Measurements—All NMR experiments were carried out on a Bruker Avance II 500-MHz spectrometer using a 4-mm MAS probe operating at a frequency of 500.1013 MHz (1H) and a wide line probe at 76.7685 MHz (2H). 1H experiments were carried out with an MAS speed of 10 kHz. 2H measurements were conducted without sample spinning. 1H spectra were externally referenced to tetramethylsilane at 0 ppm. 1H experiments were conducted with a typical π/2 pulse length of 7 μs and a relaxation delay of 4 s. Two-dimensional NOESY experiments had 256 or 512 increments and up to 64 scans/increment. NOESY build-up curves were acquired using mixing times between 10 and 800 ms. Samples were heated to 333 K before cooling to 273 K and then heating to the desired measuring temperature. Experiments were conducted at 298, 308, and 316 K (according to the thermocouple in the probe head). MAS at 10 kHz was found to heat the sample by 2 K (derived from the relative positions of methanol peaks (21)). All subsequent references to the sample temperature will take this into account.

2H quadrupole echo experiments (22, 23) were acquired with a spectral width of 100 kHz, a recycle delay of 1 s, 30-μs echo delay, 10-ms acquisition time, π/2 pulses of 5.5 μs, and between 2048 and 4096 scans. All samples were heated to 333 K before cooling down to 273 K prior to temperature scans. Measurements were then taken at 1 K intervals between 273 and 333 K (according to the thermocouple in the probe head). NMR data were processed using Topspin version 1.3 (Bruker Instruments, Karlsruhe, Germany). NOESY peak volumes were obtained by peak fitting and integration using CARA (24).
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Data Analysis—NOESY data were used to calculate the location of a given nucleus in the lipid membrane using the "full matrix rate analysis," described in detail by Huster et al. (25). In short, experimentally measured NOESY peak volumes, represented by the matrix A, at the mixing time $t_m$, and the cross relaxation rates $R$ are linked by the matrix equation as follows.

$$A(t_m) = A(0) \cdot \exp(-Rt_m)$$  \hspace{1cm} (Eq. 1)

The relaxation rate matrix $R$ is calculated by rewriting Equation 1 as follows.

$$R = -\frac{X(lnD)X^{-1}}{t_m}$$  \hspace{1cm} (Eq. 2)

Here, $X$ is the matrix of eigenvectors, and $D$ is the diagonal matrix of eigenvalues of the normalized peak volume matrix $a(t_m) = A(t_m)/(A(0)^{-1})$. The relaxation rates, contained in $R$, were taken as indicators of the relative location probabilities and are plotted to give a location profile of the agonists.

All calculations were carried out with the help of Python (Python Software Foundation, Wolfeboro Falls, NH), specifically with the packages matplotlib (available from the sourceforge Web site) and scipy (26). $^2$H spectra were de-Paked, and first spectral moments were calculated using NMR-Depaker software (available from the Launchpad Web site). The de-Paking procedure was performed according to the fast Fourier transform-based fast deconvolution algorithm (27).

Individual C–$^2$H bond order parameters ($S_{CD}$) were calculated from quadrupolar splittings ($\nu_q$), as described previously (23, 28, 29),

$$S_{CD} = \frac{4}{3} \times \frac{\Delta \nu_q}{A_0}$$  \hspace{1cm} (Eq. 3)

where $A_0$ is the static deuterium quadrupolar constant (167 kHz for C–$^2$H bonds (30)). $S_{CD}$ profiles for the sn-1 chain of DPPC-$d_{62}$ were constructed according to the published assignments (28, 29) and comparisons with specifically labeled phospholipids (31, 32).

First moment ($M_1$) calculation was performed using a sign reversal for negative frequencies to avoid zero values due to $^2$H NMR spectrum symmetrical relative to the origin,

$$M_1 = \int_{0}^{-\infty} -\omega S(\omega) d\omega + \int_{-\infty}^{0} \omega S(\omega) d\omega$$ \hspace{1cm} (Eq. 4)

where $\omega = 0$ is the center of the spectrum.
sign a cholesterol peak to a particular part of the molecule. However, broadly speaking, it is possible to group the peaks into structural categories. The terminal CH₃ groups (C26/27c) are clearly resolved from the CH₃ groups protruding up from the plane of the sterol rings (C18c, C19c, and C21c), whereas the protons on the sterol rings give another set of peaks.

1H NMR signals of the agonists’ aromatic moieties (Fig. 2, C and D) are well resolved and downfield of the lipid resonances (>6 ppm). Aliphatic agonist signals overlap with the stronger lipid signals, with the exception of the CH₂ protons in the piperazine ring of quipazine (resonance 1, Fig. 2D) and aliphatic CH₂ protons of LY-165,163 at 3.4 and 2.7 ppm (resonance 1, Fig. 2C).

Phase Behavior—Phase diagrams of binary mixtures of DPPC and cholesterol are well documented. However, the phase behavior of the DPPC/cholesterol/agonist mixtures are unknown. To gain some insight into the properties of these mixtures, 2H NMR measurements were performed at 1 K intervals between 273 and 333 K on DPPC-d₆₂ with and without cholesterol and agonists (Fig. 3 and supplemental Fig. 1). Fig. 3 shows the temperature dependence of the first spectral moment (M₁) for the 2H spectra of DPPC-d₆₂ plus agonist (without cholesterol). M₁ is proportional to the average quadrupolar splitting and thus gives a measure of the width of all spectral components (29). The spectra of pure DPPC-d₆₂ show a small decrease in the M₁ at 301 K as the lipid enters a ripple phase (34). This is followed by a sudden decrease between 309 and 310 K as the lipid goes through the gel to L₄ transition. The addition of agonists to the system appears to abolish the spectral changes characteristic of the gel to ripple phase transition. Furthermore, in the presence of agonist and prior to chain melting, M₁ is significantly lower than for DPPC-d₆₂ alone. The transition from the gel to L₄ phase is also slightly lowered and broadened, now occurring between 304 and 307 K for DPPC-d₆₂/quipazine and between 304 and 309 K for DPPC-d₆₂/LY-165,163.

DPPC-d₆₂/cholesterol mixtures behave as expected (11). There is no sudden phase transition; instead, there is a shallow decrease in line widths and first spectral moments indicative of gradual transitions between gel, L₄, and L₅ phases, all of which may co-exist (Fig. 5). The presence of agonists lowers the transition temperatures but not the overall trend.

However, at low temperature, the samples containing agonist and cholesterol displayed unusual behavior (Fig. 5). Between 278 and 293 K, the first spectral moments increased before peaking prior to the onset of gel phase melting.

Closer inspection of the 2H spectra of DPPC-d₆₂ reveals that at some temperatures two sets of peaks arise from the terminal methyl groups; this can be clearly seen in the insets of Fig. 3. This splitting is the result of the non-equivalency of the two lipid chains (11) and can be used as a reporter for the presence of L₄ phase (11, 35, 36). Methyl peak splittings are observed between 293 and 317 K for DPPC-d₆₂/cholesterol and over a similar temperature range, just shifted down by a few K (between 287 and 313 K) for DPPC-d₆₂/cholesterol and with agonist (Fig. 4). Methyl peak splittings were observed over a much larger range, between 280 and 327 K. Interestingly, methyl peak splitting is also seen at 306 K in the DPPC-d₆₂/LY-165,163 sample (supplemental Fig. 1d). The magnitudes of these splittings are shown in Figs. 4 and 5.

2H spectra can also be used to report on the gel phase where it is manifested as large quadrupole splittings and poorly resolved signals. Using these criteria, it is possible to define the upper limit of the gel phase as being 310 K (in agreement with the literature (11, 14)), 300 K, and 298 K for DPPC-d₆₂ samples containing cholesterol, cholesterol plus quipazine, and cholesterol plus LY-165,163, respectively.

Order Parameters—Individual bond C–H order parameter (S′₂₂) profiles derived from 2H NMR on DPPC-d₆₂ at 323 K are shown in Fig. 6. The non-equivalency of the two lipid chains leads to a separate profile for each chain (29). However, for the sake of clarity, only data from the sn-1 chain are shown.

It is immediately noticeable that the S′₂₂ value of bilayers containing cholesterol is, as expected, appreciably higher than
those without. The addition of agonists has more subtle effects; there is no appreciable difference between the SCD values of pure DPPC-d_{62} and DPPC-d_{62} containing agonists. Nor does the addition of quipazine to the cholesterol-containing bilayers have any affect. However, in the presence of cholesterol, LY-165,163 causes an increase in order parameter throughout the whole of the chain.

**MAS-NOESY Measurements on DPPC Membranes Doped with Agonist**—MAS-NOESY of DPPC/agonist mixtures was performed above the main phase transition at 310 K using 10-, 100-, 200-, and 400-ms mixing times. NOESY measurements on DPPC-d_{62}/cholesterol/agonist were conducted at the lowest temperature at which individual resonances could still be resolved (300 K) with mixing times of 10, 200, 300, and 400 ms. The experiments were repeated at 318 K with mixing times increased to 20, 400, 500, and 600 ms to take into account the decrease in correlation times at the higher temperatures (37). At 318 K, DPPC-d_{62}/cholesterol/quipazine is in a pure L_d phase, but DPPC-d_{62}/cholesterol/LY-165,163 still has traces of L_o phase (Fig. 5). However, increasing the temperature further resulted in correlation times being too short to practically measure cross-peak intensities (data not shown).

A typical MAS-NOESY spectrum of LY-165,163/DPPC-d_{62} and cholesterol membranes is depicted in Fig. 7. Cross-peaks are visible between all aromatic LY-165,163, glycerol and...
headgroup DPPC, and cholesterol resonances. The build-up rates of the cross-peaks with increased mixing times give an indication of the proximity of two protons. Consequently, the location probability of a particular agonist proton in the lipid membrane can be determined relative to other lipid protons (Fig. 8). If multiple agonist proton signals are resolved (see Fig. 2), the orientation of the molecule relative to other components in the bilayer can also be determined.

The locations of quipazine and LY-165,163 in DPPC broadly agree with our previous studies conducted in dioleoyl-sn-glycero-3-phosphocholine (DOPC) (17); the aromatic ring at one end of the quipazine molecule has a maximum location probability in the chain region of the bilayer, whereas the other end locates in the interface region, indicating that it has a net orientation parallel to the lipids. In the case of LY-165,163, the maximum location probability is the same as reported previously in DOPC, but in DPPC, it is much more tightly distributed around the chain/glycerol regions.

It should be noted at this point that the reported quipazine resonances are the same as we used previously (17). However, here, different LY-165,163 peaks are used because they can be more clearly resolved at 300 K. One of these peaks is derived from protons situated in the center of the molecule, whereas the other is at one end of the LY-165,163 molecules (Fig. 1); thus, some spatial resolution has been lost. The NOESY measurements on DPPC-$d_{62}$/cholesterol/agonist samples give
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Information on the interactions between agonist and cholesterol at the expense of information on interactions between the chain region of DPPC and the agonist.

At 318 K (Fig. 8, E and F), both agonists have weak interactions with the terminal methyl groups of the cholesterol chain, consistent with the previous observations that the agonists do not accumulate deep within the chain region of the bilayer. Beyond this similarity, the agonists behave quite differently. Quipazine shows large cross relaxation rates with all of the remaining cholesterol resonances. The aromatic ring of quipazine interacts more strongly with the C18 of the piperazine ring at the other end of the molecule, whereas the piperazine ring has stronger interactions with the phospholipid headgroup. Interestingly, the presence of cholesterol also seems to change the interactions between quipazine and the phospholipid. Cross-relaxation rates between quipazine and DPPC α resonance are larger in the ternary DPPC-d62/cholesterol sample than in the binary DPPC sample.

In the case of LY-165,163, the center of the molecule has strong cross-peaks with C9/19/21 but little contact with anything else. Meanwhile, the aromatic at the end of the molecule has significant interactions with all reported cholesterol peaks with the exception of the C14/17/24 peak. Furthermore, the presence of cholesterol appears to increase the interactions between LY-165,163 and the phospholipid headgroup.

The second set of NOESYs were conducted at 300 K (Fig. 8, C and D). Under these conditions the orientational bias of quipazine is even more apparent. As before the aromatic ring interacts more strongly with C18 but now, unlike in the binary DPPC or DOPC mixtures, the piperazine ring has strong interactions with all three sets of phospholipid headgroup protons. In the case of LY-165,163, cooling the sample causes an increase in the interactions between the center of the LY-165,163 molecule and the various CH₃ groups in cholesterol (with the exception of the chain terminal methyls), whereas the aromatic ring appears to have a preference for the cholesterol ring protons. Furthermore, the interactions between LY-165,163 and the phospholipid headgroups are lost.

DISCUSSION

This work investigates the effect of cholesterol and lipid phase behavior on the interactions between two serotonin receptor agonists and the lipid bilayer components. Our prior work showed that the agonists induced a chemical shift of cholesterol signals in brain lipid bilayers (17). To further investigate this phenomenon, we have simplified the system so that it now contains just phospholipid, cholesterol, and an agonist. This allows the composition of the system to be manipulated to include chain-deuterated lipids, resulting in increased ²H spectral resolution and allowing ²H measurements to be performed on the same sample. Together, this allows NOESY cross-peaks to be observed between all components, giving information on the direct interactions between membrane components, whereas analysis of the ²H spectra provides data on the agonists’ bulk effects.

In the absence of cholesterol, the agonists have no noticeable effect on structural characteristics of the Lₒ phase (derived from ²H data; see Table 1) and a small but significant effect on the lipid phase behavior (Fig. 4). Most notably, they appear to abolish the gel to ripple phase transition while lowering M₁ prior to the onset of chain melting. Together, these data suggest that either the agonists induce formation of the ripple phase at a much lower temperature (so its onset is not seen within the measured temperature range) or the incidence of the ripple phase is much reduced by the presence of agonist. Both agonists also reduce the gel-Lₒ phase transition temperature, which now occurs 3 and 5 K lower in the presence of quipazine and LY-165,163 respectively. The phase transition also occurs over a wider temperature. This implies that there may be a small range in which a mixture of phases exists, a supposition that is backed up by the observation that at 306 K and in the presence of LY-165,163, the ²H terminal methyl peaks are split (supplemental Fig. 1d and Fig. 4). These spectral features are consistent with the presence of Lₒ phase (see below). As far as we are aware, prior to this, Lₒ phases in DPPC have only ever been reported in the presence of sterols.

The combination of agonists and cholesterol generates some unusual phase diagrams. First spectral moments as a function of temperature behave as expected for mixtures of DPPC-d62 and cholesterol (Fig. 5); as temperature increases, M₁ slowly decreases before lipids begin to change phase and M₄ drops more swiftly. At high temperatures, DPPC-d62/cholesterol/agonist mixtures behave in an analogous fashion to DPPC-d62/cholesterol. However, quite different behavior is seen at low temperatures. The limit of the gel phase is a good 10 K lower in the presence of agonist. However, more interestingly, immediately prior to the onset of gel melting M₁ increases, reaching a peak for both agonists at about 293 K and then dropping as expected (Fig. 5, a and b). As far as we are aware, this phenomenon has not been reported previously and may represent an unknown sub-gel phase. However, the scope of this study does not include any further analysis of this part of the phase diagram, and we have not investigated it further.

The methyl peak splitting seen in the ²H spectra of DPPC-d62 is thought to reveal the onset of the Lₒ phase. The terminal methyl group peaks splitting into doublets (Fig. 3 and supplemental Fig. 1) arises from non-equivalence of the two aliphatic chains in the Lₒ phase (11, 35, 36). This occurs between 290 and 313 K for DPPC-d62/cholesterol (published figures 288–315 K) (11), between 286 and 310 K for DPPC-d62/cholesterol/quipazine, and between 280 and 325 K for DPPC-d62/cholesterol/LY-165,163. Clearly, LY-165,163

| Lipid mixture | Plateau order parameter | Hydrophobic thickness | Average area per lipid |
|---------------|-------------------------|-----------------------|------------------------|
| DPPC-d62      | 0.201                   | 14.2                  | 63.4                   |
| DPPC-d62 + quipazine | 0.205                   | 14.3                  | 63.0                   |
| DPPC-d62 + LY-165,163 | 0.205                  | 14.3                  | 63.0                   |
| DPPC-d62 + cholesterol | 0.304                 | 16.3                  | 55.3                   |
| DPPC-d62 + cholesterol + quipazine | 0.302               | 16.3                  | 55.4                   |
| DPPC-d62 + cholesterol + LY-165,163 | 0.339               | 17.0                  | 53.0                   |
greatly extends the temperature range in which the methyl
peak splittings are observed. However, at low temperatures
(e.g. Fig. 3, 283 K), these splittings are not accompanied by any
other sharp spectra features that would be expected for lipids
undergoing rapid axial motion associated within a L_α phase.
These sharp features do not begin to emerge until 299 K
(DPPC-d_{62}/cholesterol), 286 K (DPPC-d_{62}/cholesterol/LY-
165,163), and 293 K (DPPC-d_{62}/cholesterol/quipazine). Nev-
ertheless, wherever the exact onset of the L_α phase is, the con-
clusions remain the same. Together, the presence of the
unknown sub-gel phase, the extended range of the L_α phase,
and the order parameter measurements all seem to suggest
that LY-165,163 has a marked effect on the bulk bilayer prop-
erties when in the presence of cholesterol. By contrast, quip-
azine induces the same sub-gel phase, but at higher tempera-
tures it does little more than shift the phase transitions by a
few degrees. These differences between the agonists effects on
the phase behavior are mirrored in their effect on bilayer or-
der parameters. Neither agonist causes any change in the or-
der parameters when mixed with DPPC alone. However,
when cholesterol is added into the mix the further addition of
LY-165,163 then causes a significant increase in order param-
eter, whereas once again quipazine elicits no change. These
changes may be more intuitive when expressed in terms of
structural characteristics (Table 1), derived from the order
parameter measurements as described by Petrache et al. (38).
Now it becomes apparent that, as expected, cholesterol causes
an increase in the hydrophobic thickness by ~2 Å.
LY-165,163 has no effect by itself but together with chole-
sterol causes a further thickening of 0.7 Å.
NOESY interactions can be used to shed light on specific
contacts made between agonists and lipids (Fig. 8). The mag-
nitude of the NOESY cross-peaks may be a function of not
just the internuclear distances but also the mobility of inter-
acting nuclei and spin diffusion. Huster and Gawrisch (25, 39,
40) have conducted several studies to better understand the
contribution that all of these factors have on the interpreta-
tion of cross-relaxation rates. They have concluded that spin
diffusion can be ruled out in lipid bilayer systems under MAS
conditions and that “cross relaxation rates report true statis-
tics of nearest-neighbor contacts in the bilayer” (25, 39, 40).
Correlation times certainly influence observed cross-peak
intensities however, these have been shown (at least for etha-

nol/lipid interactions) (37) to be consistent throughout an L_d
bilayer. But in mixed phase systems the lipids will be in at
least two different motional regimes, with the lipids in the L_α
phase likely to experience longer correlation times than the
same lipids in an L_d phase. This could lead to the NOESY data
overrepresenting interactions that occur in the L_α phase. The
aim of the NOESY measurements conducted at 300 K was to
investigate interactions in the L_α phase; therefore, the results
may be skewed toward L_α phase interactions. However, this
does not affect the interpretation of the results. At the higher
temperature (318 K), the bilayer is almost entirely L_d phase,
and therefore interactions in the L_α phase will represent a tiny
fraction of the overall signal.

Given these factors, it is then reasonable to interpret the
cross-relaxation rates in Fig. 8 as an indication of the distribu-
tion of an agonist in the bilayer. Therefore, the locations of
the agonists in the DPPC lipid bilayer are in broad agreement
with those published on DOPC; the agonists tend to locate in
the interface region, and quipazine is oriented parallel with
the lipid axis, although the distribution of LY-165,163 does
seem to be narrower than in DOPC (17). Similar differences
between drug interactions with DPPC and DOPC have been
reported before with β2-adrenoreceptor agonists (5). Possibly,
interactions between the π-electrons of the alkene groups in
the DOPC chain and the agonist’s aromatics may have served
to pull the agonist deeper into the DOPC bilayer. Alterna-
tively, here we have used lower concentrations of agonist as
compared with our previous MAS-NOESY study (17); conse-
quently, the bilayer may not be saturated with agonist and
thus is able to accommodate the majority of the agonist in its
preferred location.

NOESY measurements on the samples containing DPPC-
d_{62} cholesterol and agonists were collected at 318 K where the
mixtures are dominated by the L_d phase, and at 300 K, where
the bilayers are a potential mix of gel, L_d, and L_α phases. Any
gel phase components will give very broad lines, rendering the
individual resonances unresolvable. Therefore, the interac-
tions being observed will be taking place in the L_d and/or L_α
phases.

At 318 K quipazine interacts strongly with all of the re-
solved cholesterol peaks except the terminal methyls (Fig. 8F).
The piperazine ring of quipazine interacts more strongly with the
phospholipid headgroups, whereas the opposite end of the
agonist has stronger interactions with C18c at the bottom of the
cholesterol ring structure. These observations demon-
strate that quipazine’s orientational preference is maintained
in the presence of cholesterol. In fact, cholesterol seems to
increase the interaction between the piperazine group and the
protons in the DPPC headgroup. This trend is even more ap-
parent at 300 K (Fig. 8D), where the piperazine ring now inter-
acts with all of the headgroup protons. This seems to imply
that the ordering of the chains that occurs due to the presence
of cholesterol, and then again in the L_d phase, causes the qui-
pazine to be displaced and pushed up toward the surface of
the bilayer. It should be noted that differences between actual
cross-relaxation rates at different temperatures and samples
are difficult to interpret due to the drastically different motili-
ties experienced in each case. The increase in temperature is
expected to lead to shorter correlation times and a concomi-
tant reduction in cross-peak intensity and cross-relaxation
rate. This is observed for all lipid/agonist interactions with the
exception of the piperazine resonances on the quipazine (Fig.
8, D and F). Here the cross-relaxation rates are higher at the
elevated temperature. This suggests that either the nuclei are
in very close proximity to the lipids or, at the higher tempera-
ture, the piperazine ring is in a motional regime that leads to
an enhanced NOE. We can speculate that this may be a con-
sequence of a strong interaction between cholesterol and the
piperazine ring, leading to a longer correlation time and stron-
ger NOE. Meanwhile, the other end of the quipazine ring
would be left free to rotate around the single bond in the cen-
ter of the molecule and hence is less affected by piperazine/
cholesterol interaction.
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In contrast, to quipazine, LY-165,163 does not show the same degree of interactions with cholesterol (Fig. 8, C and D). There are only very weak interactions with the bottom of the cholesterol rings (C14/17/24c), which seems surprising given that the rest of the ring structure is accessible to LY-165,163. Possibly, the C18\(^\circ\) and C21\(^\circ\) methyl groups that protrude out from the plane of the cholesterol rings may shield this region of the sterol, but more interestingly, and in contrast to quipazine, LY-165,163 appears to be pushed deeper into the bilayer at the cooler temperature. This is evidenced by the fact that interactions between LY-165,163 and the phospholipid headgroup are weaker at lower temperatures, whereas there is a concomitant increase in interactions with C18\(^\circ\).

The behavior of the agonists with respect to cholesterol can be easily reconciled with the agonists’ effects on the bulk lipid properties. Quipazine is pushed out of the chain region by cholesterol and the increase in order associated with the L\(_{\alpha}\) phase. Consequently, it has a relatively small effect on the order parameters and phase transition barriers. However, LY-165,163 appears to locate deeper in the membrane as the fraction of L\(_{\beta}\) increases, so it is in a position where it can have a greater influence on the phase behavior and cause the observed increase in hydrophobic thickness.

Drug/lipid interactions have the potential to modulate the efficacy of the drug in several ways. First, the drug must be able to negotiate membranes on the way to its target site. This may involve crossing the gut epithelium and the blood brain barrier. If this occurs passively then the drug’s ability to partition into cellular membranes is vital. There is plenty of evidence to show that the composition of a membrane alters the partitioning coefficient of drugs (10), but until now, the systems used to study these effects have not been accessed with MAS-NOEY measurements. Hence, although we can be sure that, for example, the addition of cholesterol to a lipid bilayer can have dramatic affects on a drug’s ability to partition into a membrane (5), we have not until now been able to see how this behavior is manifested on an atomic level.

A related factor is the propensity of agonists to preferentially partition into particular membrane domains. Serotonin receptors have been shown to be activated by and accumulate in lipid raft domains (3–5). Therefore, for maximum bioefficiency it would clearly be advantageous for a drug to accumulate in the same domain as its target. Because we have shown that quipazine and LY-165,163 both interact strongly with cholesterol in the L\(_{\alpha}\) phase, it would be reasonable to assume that these agonists accumulate in this phase and hence also partition into cholesterol-rich raft domains where they can more readily access their target protein.

Furthermore, the recent structure of the β2-adrenergocceptor suggests that the protein binds cholesterol via a 4-amino acid cholesterol binding motif (41). Most serotonin receptors have the same motif, which appears to be an evolutionarily conserved cholesterol binding site (42), so it is easy to imagine how an interaction between cholesterol and a drug may affect its efficacy, either by interfering with the cholesterol/protein contacts or by the cholesterol “delivering” the drug to the protein.

This then leads to a further factor; the ability of an agonist to access the binding sites on its target protein. To do this most efficiently the drug must accumulate in the membrane close to the binding site and be presented to the protein in the correct orientation. This is of particular importance for serotonin receptors given that its binding sites are thought to be accessible via the membrane as well as the solvent (1). Our data show that the tightening of the bilayer associated with the presence of cholesterol and L\(_{\beta}\) phases results in a change in the location of the agonists. Quipazine is pushed up and out of the hydrophobic region, whereas LY-165,163 is pushed deeper into the membrane. Therefore, it is quite possible that cholesterol and the L\(_{\beta}\) phase serve to retard quipazine binding to some serotonin receptors. Interestingly, quipazine binds relatively weakly to serotonin 1a receptors (43) (with its membrane-embedded binding site) compared with LY-165,163 (44), but it binds much more strongly to the serotonin 3 receptor, which is a ligand-gated ion channel and not a G-protein-coupled receptor and so is thought to have a more exposed binding site.

Finally, it is apparent from our data that the agonists can affect the phase behavior of the lipid bilayer. This is particularly true for LY-165,163, which promotes the L\(_{\beta}\) phase both in the presence and absence of cholesterol and also decreases the fluidity of the bilayer. If this phenomenon holds true at physiological concentrations (and other G-protein-coupled receptor agonists have been shown to alter the bulk bilayer properties at very low concentrations (5)), then the drug may cause condensation of L\(_{\beta}\) phase in vivo. This in turn could serve to modulate the activity of the target protein. There is plenty of evidence that drug molecules can affect the bulk properties of membranes (reviewed by Lucio et al. (10) and references therein). However, the possibility of designing drugs to modulate protein activity via drug/lipid interactions is only just beginning to be explored.

Acknowledgments—We thank Prof. Clemens Glaubitz and Dr. Rob Law for helpful and constructive comments on the manuscript.

REFERENCES
1. Vauquelin, G., and Packeu, A. (2009) Mol. Cell. Endocrinol. 311, 1–10
2. Castanho, M. A., and Fernandes, M. X. (2006) Eur. Biophys. J. 35, 92–103
3. Eisensamer, B., Uhr, M., Meyr, S., Gimpl, G., Deiml, T., Rammes, G., Lambert, J. J., Ziegglänsberger, W., Holsboer, F., and Rupprecht, R. (2005) J. Neurosci. 25, 10198–10206
4. Allen, J. A., Halverson-Tamboli, R. A., and Rasenick, M. M. (2007) Nat. Rev. Neurosci. 8, 128–140
5. Lombardi, D., Cuenoud, B., and Krämer, S. D. (2009) Eur. J. Pharm. Sci. 38, 533–547
6. Björk, K., Sjögren, B., and Svenningsson, P. (2010) Exp. Cell Res. 316, 1351–1356
7. Escribá, P. V., González-Ros, J. M., Gotzi, F. M., Kinnunen, P. K., Vigh, L., Sánchez-Magraner, L., Fernández, A. M., Busquets, X., Horváth, I., and Barceló-Coblijn, G. (2008) J. Cell Mol. Med. 12, 829–875
8. Meyer, H. H. (1899) Arch. Exp. Pathol. Pharmacol. 42, 109–118
9. Escribá, P. V. (2006) Trends Mol. Med. 12, 34–43
10. Lucio, M., Lima, J. L., and Reis, S. (2010) Curr. Med. Chem. 17, 1795–1809
11. Vist, M. R., and Davis, J. H. (1990) Biochemistry 29, 451–464
