The endogenous cannabinoid ligand anandamide is biosynthesized from membrane phospholipid precursors and is believed to reach its sites of action on the CB1 and CB2 receptors through fast lateral diffusion within the cell membrane. To gain a better insight on the stereochemical features of its association with the cell membrane and its interaction with the cannabinoid receptors, we have studied its conformation, location, and dynamic properties in a dipalmitoylphosphatidylcholine multilamellar model membrane bilayer system. By exploiting the bilayer lattice as an internal threedimensional reference grid, the conformation and location of anandamide were determined by measuring selected inter- and intramolecular distances between strategically introduced isotopic labels using the rotational echo double resonance (REDOR) NMR method. A molecular model was proposed to represent the structural features of our anandamide/lipid system and was subsequently used in calculating the multipin dephasing curves. Our results demonstrate that anandamide adopts an extended conformation within the membrane with its headgroup at the level of the phospholipid polar group and its terminal methyl group near the bilayer center. Parallel static $^2$H NMR experiments further confirmed these findings and provided evidence that anandamide experiences dynamic properties similar to those of the membrane phospholipids and produces no perturbation to the bilayer. Our results are congruent with a hypothesis that anandamide approaches its binding site by laterally diffusing within one membrane leaflet in an extended conformation and interacts with a hydrophobic groove formed by helices 3 and 6 of CB1, where its terminal carbon is positioned close to a key cysteine residue in helix 6 leading to receptor activation.

The membrane lipid bilayer is a ubiquitous molecular assembly into which are embedded a variety of proteins, natural hormones, and neurotransmitters. Accumulated evidence indicates that many fatty acid-derived lipophilic neurotransmitters are synthesized, stored, and degraded and also exert their functions within the lipid membrane (1, 2). Therefore, it has been suggested that the conformation, location, and orientation of the ligand in the membrane are critical in determining its ability to reach and interact productively with its site of action (3–5). Exploring the conformational and dynamic properties of these ligands in the membrane can lead to a better understanding of the molecular features involved in their interactions with the target proteins (6).

$N$-Arachidonylethanolamine (anandamide), initially isolated from mammalian brain, has been identified as an endogenous ligand for the two known G protein-coupled cannabinoid receptors (CB1 and CB2) (7). This endocannabinoid exerts its activity by modulating several physiological functions such as pain, cognition, and memory. Site-directed mutagenesis evidence has shown that the anandamide binding sites are embedded in the trans-membrane helices of the receptor (8, 9). This correlates well with studies of other G protein-coupled receptors, such as rhodopsin and the β-adrenergic receptor, which provide evidence that their respective ligands interact within the trans-membrane domains of the receptor (10). Moreover, enzymes that are involved in anandamide biosynthesis and degradation, including a D-type phosphodiesterase (11) and fatty acid amide hydrolase (7), are also membrane-bound, and their respective substrates and products originate from membrane phospholipids (12). Because all of the above mentioned endocannabinoid-related proteins are membrane-bound, the conformation, location, and dynamic behavior of anandamide within the cell membrane would be of particular importance for a better understanding of the nature of receptor or enzyme activation/deactivation and may also enhance our ability to design novel therapeutic medications acting through the endocannabinoid system. Because of the high lipophilicity (clogP = 6.3) of anandamide, this amphipathic ligand is expected to reside nearly exclusively within the membrane bilayer.

Earlier computational work (13, 14) on the conformational properties of anandamide has shown that its arachidonoyl component is capable of assuming a variety of conformations in solution, which can be generally characterized as hairpin (U-shaped), J-shaped, and extended (Fig. 1). Because the structure of anandamide is highly flexible, its conformation could be very sensitive to its immediate environment, and the molecule may exhibit different conformational preferences depending on its surrounding media. However, there were no experimental studies on the conformational properties of anandamide in a bilayer membrane environment. From earlier work with (−)-Δ$^2$-tetrahydrocannabinol (−Δ$^2$-THC) we had demonstrated that the orientation of a lipophilic ligand within an anisotropic membrane environment does not always conform with the expected predictions based on optimal packing within the phospholipid amphipathic environment (15). Our experiments showed that this amphipathic cannabinoid does not align as...
expected with the long axis of its tricyclic component parallel to the phospholipid chains. Instead, the molecule assumes an awkward orientation in which the long axis of its tricyclic core is perpendicular to the bilayer chain (Fig. 2). We were thus motivated to design a series of experiments to determine experimentally which of the three computationally identified conformations of anandamide is the dominant one in an anisotropic membrane system and to study its location and dynamic properties within the membrane. Traditionally, this information is obtained with the help of high resolution NMR where the ligand conformation is studied in SDS micellar or other membrane-mimicking environments (16–18). However, such approaches suffer from several limitations, one of which is the highly curved nature of micelles that may not serve as an ideal membrane model (19). In the case of long chain lipid messengers, such spectra are further complicated by the fact that their respective ¹H NMR resonances are unresolved because of severe overlap. In this study, we have employed a novel approach to examine the location and conformation of anandamide in a model membrane system consisting of dipalmitoylphosphatidylcholine (DPPC) multilamellar bilayers using the rotational echo double resonance (REDOR) NMR technique. We have also used static solid-state ²H NMR to obtain information on the dynamic properties of anandamide in the bilayer.

Our experimental strategy was to employ the phospholipid multilamellar bilayer system not only as a model membrane environment but also as an internal reference grid for the incorporated anandamide molecule because this supramolecular lipid assembly is a highly organized lattice (20). The conformation of anandamide can then be obtained by determining the geometric relationships between anandamide and the lipid molecules. This was accomplished by identifying key atom pairs within the anandamide/DPPC bilayer assembly and determining the respective intra- or intermolecular distances using rotational echo double resonance (REDOR), a powerful solid-state NMR method capable of accurately measuring distances between two different nuclei, such as ¹³C–³¹P, ¹³C–¹⁵N, and ¹³C–²H (21, 22). This technique uniquely allows the measurement of mid-range internuclear distances and has been applied successfully to study peptide backbone conformations and ligand-protein binding geometries (23, 24). A distance range of 8.0 Å may be reliably measured for a pair of high γ nuclei, such as ¹³C and ³¹P. Although the measurable distance is shorter for the low γ ²H nucleus, this range is expanded in the experiments described here because of the dipolar contributions of multiple ²H atoms with each of the observed ¹³C nuclei.

In this study, we have introduced ¹³C, ¹⁵N, and ²H isotopic labeling in strategic positions within anandamide and the surrounding DPPC molecules and determined the preferred conformation of anandamide within the bilayer using selected internuclear distances between these labels through a series of REDOR experiments (Fig. 3). The exact location and conformation of anandamide in the lipid bilayer were obtained by simulating the multispin REDOR dephasing data based on our proposed molecular model of the anandamide/DPPC system. Parallel static solid-state ²H NMR experiments with the anandamide/DPPC system in the liquid crystalline phase (Lα) were used to confirm further our findings and obtain information on the dynamic properties of this endocannabinoid within the cell membrane from which it originates and is believed to reside. This approach may also be of general interest for studying the conformational properties of other small molecules, peptides, and integral membrane proteins within a membrane system.
Anandamide Conformation in Lipid Membrane Bilayers

EXPERIMENTAL PROCEDURES

Materials

DPPC was 2H labeled independently at the 2’, 7’, and 16’ positions of both sn-1 and sn-2 acyl chains (Fig. 3) to obtain 1,2-[2-2H2]DPPC, 1,2-[7-2H2]DPPC, 1,2-[16-2H2]DPPC (25), whereas [1-13C]anandamide, [20-13C,15N]anandamide/unlabeled DPPC, [20-13C]anandamide/1,2-[2-2H2]DPPC, and [1-15N]anandamide/1,2-[7-2H2]DPPC, where the anandamide 1-20 and 15N as the dephased nucleus. The remaining five samples: [20-13C,2-2H2]DPPC, unlabeled anandamide/1,2-[7-2H2]DPPC, and unlabeled anandamide/1,2-[16-2H2]DPPC were used in the static solid-state 2H NMR experiments. To invert the deuterium nuclei for sufficient dephasing, a composite XY-4 pulse scheme was employed on the 2H channel, whereas an XY-8 composite pulse scheme was used on the 13C channel to compensate for possible pulse imperfections (27). A sample of [1-13C,2-2H2]sodium acetate (Cambridge Isotope Laboratories, MA) was used as a standard for all 13C observe, 2H dephased REDOR experiments.

Static Solid-state 2H NMR—All of the deuterium spectra were obtained using a Chemagnetics CMX300 spectrometer operating at 46.05 MHz with a linewidth probe at 42 °C. The quadrupole echo pulse sequence, [(π/2),−(π/2)], was employed with 2.5 μs for the 2H π/2 pulse, 35 μs for τ, and 200 ms for recycle delay. A total of 5,000 echoes were accumulated for each spectrum. Before recording a spectrum, each sample was held at 50 °C in the probe for 15 min to ensure complete equilibration.

RESULTS AND DISCUSSION

Solid-state REDOR NMR experiments were used to elucidate the location and conformation of anandamide within the DPPC multilamellar membrane bilayer by detecting heteronuclear dipolar couplings between anandamide and the phospholipid molecules, as well as between the 20-13C- and 15N-labeled sites of anandamide. Each of our REDOR experiments consists of two parallel parts: the 13C signal is first observed without the dephasing pulse train and is then repeated with the dephasing pulse train applied. The intensity difference ∆S between the two spectra can be directly related to the dipolar coupling of each spin pair, from which the internuclear distance can be deduced (28). The first group of experiments (1-13C-observe) was aimed at determining the location of the anandamide headgroup, and we observed positive effects (∆S ≥ 0) in both the 1-13C-observe, 31P-dephased (1-13C/31P) and the 1-13C-observe, 2-2H2-dephased (1-13C/2-2H2) experiments. However, no effects (∆S = 0) were observed in the 1-13C/15N-2H2 REDOR experiment. The second group (20-13C-observe) was designed to identify the location of the anandamide terminal methyl within the DPPC bilayer. Only in the 20-13C/16-2H2 experiment did we observe positive effects, whereas the 20-13C/7-2H2 and 20-13C/15N experiments showed no effects, evidence that these internuclear distances are beyond the REDOR detection limits.

Location of Anandamide within the DPPC Lipid Bilayer

Anandamide Headgroup (1-13C-Observe REDOR Experiments)—The location of the anandamide headgroup within the lipid bilayer was identified from three REDOR experiments. Fig. 5, left panel, shows four pairs of 1-13C/31P REDOR spectra equipped with a dry ice/acetone bath. The magic angle spinning speed was set to 4,000 Hz and controlled by the MAS controller with a fluctuation of ±2 Hz. The observe nucleus was always 13C resonating at 75.43 MHz, whereas the dephased nucleus was 2H, 31P, or 15N at 46.05, 121.44, or 30.39 MHz, respectively. A contact pulse (1.6-ms duration and 50-kHz power) was applied in the proton channel (299.99 MHz) for cross-polarization, and its power was increased to 82 kHz for proton decoupling (see Fig. 4) The observed 13C resonances in the experiments are well resolved and separated from other chemical shift signals in the 13C spectrum of the sample. To invert the deuterium nuclei for sufficient dephasing, a composite XY-4 pulse scheme was employed on the 2H channel, whereas an XY-8 composite pulse scheme was used on the 13C channel to compensate for possible pulse imperfections (27). A sample of [1-13C,2-2H2]sodium acetate (Cambridge Isotope Laboratories, MA) was used as a standard for all 13C observe, 2H dephased REDOR experiments.

FIG. 3. Structure of DPPC and anandamide. Positions of specific isotopic labeling in both molecules are shown. DPPC is 2H labeled independently in each of the 2’, 7’, or 16’ positions. The arrows indicate the atom pairs for which the dipolar couplings were measured in the REDOR experiments. The solid line represents an observable REDOR effect, and the dotted line reflects an internuclear distance beyond the detection limit.
in which the $^{13}$C resonance was detected at 46.0 ppm. The experiment revealed an intensity difference ($\Delta S$) in each pair of resonances which became progressively pronounced as the rotor cycle progressed from 16 to 64. The $^{13}$C signal was almost completely eliminated because of the $^{31}$P dephasing at rotor cycle 64, indicating a strong coupling between the $^{13}$C of anandamide and $^{31}$P of the DPPC headgroup. In the second experiment, pairs of $^{13}$C/2$^{2}$H$_2$ REDOR spectra were collected, four of which are shown in Fig. 5, right panel. Here again, the signal intensity difference $\Delta S$ for each pair increased noticeably as the rotor cycle progressed from 16 to 64, indicating that the $^{13}$C label of the anandamide headgroup is located in the proximity of the $2^{2}$H labels of DPPC. Conversely, no differences in the signal intensity were observed in the $^{13}$C/7$^{2}$H$_2$ experiments (data not shown), indicating that the dipolar coupling between $^{13}$C of anandamide and the deuterons at the $7$ position of DPPC is beyond the REDOR detection limit.

Collectively, the results from the above three sets of experiments provide evidence that the headgroup of anandamide is located at the water/lipid interface. Such a location also allows for possible intermolecular hydrogen bonding between the anandamide headgroup hydroxyl and the phosphate of DPPC.

**Anandamide Terminal Methyl Group (20-13C-Observe REDOR Experiments)—**To determine the location of the anandamide terminal methyl group within the lipid bilayer, we used $^{20-13}$C anandamide in $^{20}$-13C/16$^{2}$H$_{3}$, $^{20}$-13C/7$^{2}$H$_{2}$, and 20-13C/15N REDOR experiments. Pairs of $^{20}$-13C/16$^{2}$H$_{3}$ REDOR spectra exhibited large $\Delta S$ values (Fig. 6, left panel), providing evidence for strong coupling and close proximity between the 20-13C label on anandamide and the terminal methyl deuterons of the DPPC acyl chains. Conversely, the 20-13C/7$^{2}$H$_{2}$ REDOR spectral pairs showed no discernible intensity differences (Fig. 6, right panel), indicating that the terminal methyl group of anandamide is beyond the measurable range from the middle of the DPPC acyl chains. The 20-13C/15N REDOR experiments using doubly labeled anandamide with $^{15}$N in its headgroup and $^{13}$C in the terminal methyl showed essentially no difference in their respective intensities (data not shown), thus providing evidence that the anandamide terminal methyl group is also not in close proximity with its own headgroup.
Numerical Analysis of the Multispin REDOR Dephasing—
For a multispin system, the REDOR intensity difference $\Delta S/S_0$ can be calculated by (30)

$$\frac{\Delta S}{S_0} = 1 - \frac{1}{8\pi^2} \int_0^{2\pi} \int_\phi^\pi \int_\theta^\pi \prod \cos \left[ N c \frac{\omega_0}{\nu_R} 4 \sqrt{2} \tilde{\alpha}^i(i) \cdot \tilde{\gamma}^i(i) \cdot \tilde{\delta}^i(i) \cdot \tilde{\epsilon}^i(i) \right]$$

where $\nu_R$ is the rotor frequency, $N c$ is the number of rotor cycles,

$$\omega_0 = \gamma_1 \gamma_2 H_i$$

(Eq. 1)

and $\tilde{\alpha}^i(i)$ is the Euler rotation of the unit vector from the $i$th $^2$H or $^{31}$P to $^{13}$C, to simulate the experimental REDOR dephasing curve ($\Delta S/S_0$ versus $N_c$), we developed a computer integration program using the Gaussian quadrature numerical algorithm under a Microsoft FORTRAN PowerStation.

Based on our model, the $1^\prime$-$^{13}$C/$^{31}$P rhomboidal arrangement requires two distinct $^{13}$C–$^{31}$P distances $r_1$ and $r_2$. In our numerical simulation, a series of REDOR dephasing curves are generated by systematically varying these two distances. Fig. 9a shows three of the simulated curves where the best fit is for values of $r_1 = 5.7$ Å and $r_2 = 8.4$ Å, each with an uncertainty of about $\pm 0.2$ Å. According to our model, the $^2$H labels contributing to the $^{13}$C–$^2$H dephasing are equidistant for the observed $1^\prime$- or $2^\prime$-$^{13}$C of anandamide. For this reason, only one $r$ value was required in our calculations for the $1^\prime$-$^{13}$C and DPPC sn-2-$^{2}$H interactions within the triangular arrangement shown in Fig. 9b, with an optimal distance of $6.0$ Å $\pm 0.5$ Å. For the $20^\prime$-$^{13}$C/$^{16}$-$^{2}$H interactions within the hexagonal arrangement, the simulated REDOR curves are plotted in Fig. 9c. The best fit for the experimental data corresponds to a distance of $r = 6.5$ Å, with an upper limit of $7.0$ Å and a lower limit of $6.0$ Å.
The obtained distances, in conjunction with the structural coordinates of the DPPC supramolecular assembly, unequivocally establish that anandamide adopts an extended conformation with an estimated total “length” of 22 Å in the membrane bilayer.

Dynamic Properties of Anandamide in the DPPC Liquid Crystalline Bilayer

The above REDOR experiments were carried out at a low temperature (−40 °C) by necessity to minimize molecular motions within the bilayer system and allow for the measurement of dipolar interactions between specific atoms within the anandamide/DPPC assembly. To obtain parallel structural information on the anandamide/DPPC system under more “physiological” conditions, we carried out a series of static solid-state 2H NMR experiments using DPPC 3H-labeled at the 2′ and 16′ positions as well as anandamide carrying 2H labels at its terminal methyl group.

The spectra in Fig. 10 were obtained at 42 °C, a temperature at which the fully hydrated DPPC bilayer exists in the liquid crystalline $L_n$ phase. The 2H spectrum from 1,2-[2′-2H]DPPC confirmed that our membrane preparation exists in the liquid crystalline $L_n$ phase and showed a set of three superimposed Pake patterns with quadrupolar splittings of $\Delta v_Q = 12.0, 17.8,$ and 26.7 kHz, caused by 2-[2′S-2H], 2-[2′R-2H], and 1-[2′-2H], respectively, based on our earlier assignments (25). The 2H spectrum caused by 1,2-16′-2H2DPPC from the terminal methyl groups has a $\Delta v_Q$ value of 3.1 kHz. This narrower splitting reflects a considerably freer movement of this segment of the acyl chain. Addition of 10% (molar) anandamide to each of the two DPPC preparations leads only to very small increases in the $\Delta v_Q$ values, indicating that this endocannabinoid ligand has only a minor effect on the dynamic properties of the phospholipid bilayer.

The above data suggest an anandamide/DPPC model in which the lipophilic ligand is well integrated into the bilayer membrane with its long axis parallel to the phospholipid acyl chains. Further support for this model is provided by the 2H experiment from the terminal methyl group of anandamide in which the quadrupolar splitting ($\Delta v_Q = 3.0$ kHz) is very similar to the respective spectrum from the 16′ methyl groups of DPPC, suggesting that the terminal methyl groups from anandamide and DPPC exhibit similar motional properties and arguably occupy a similar space within the bilayer.

Fig. 9. Theoretical fittings (solid curves) to the REDOR experimental data (solid triangles) from the 1′-13C/31P (a), 1′-13C/2′-2H (b), and 20-13C/16′-2H (c) experiments. Each curve was calculated based on the indicated distances and the specific geometric arrangement.
It is interesting to compare our findings with work related to the structural properties of unsaturated membrane lipids because anandamide is derived from polyunsaturated phospholipids. Recent studies involving unsaturated diaryl phospholipids such as 1-palmitoyl-2-docosahexaenoyl-sn-glycero-3-phosphocholine (PDPC) and 1-stearoyl-2-docosahexaenoyl-sn-glycero-3-phosphocholine (SDPC) show that in the gel L_{gel} phase, both the saturated and the polyunsaturated chains adopt the all-trans and extended (angle iron or helical) conformations, respectively (31, 32). A similar result was obtained from studies on PDPC and 1-stearoyl-2-arachidonoyl-sn-glycero-3-phosphocholine (SAPC) molecules in the liquid crystal L_{c} phase using either computational or \textsuperscript{2}H NMR approaches (33, 34). Our results now indicate that anandamide, which represents a single chain polyunsaturated lipid and is devoid of a phospholipid headgroup, also assumes such a preferred extended conformation when incorporated in a phospholipid bilayer.

**How Anandamide Reaches Its Active Site at the CB1 Receptor**

The findings reported here serve to expand our current understanding on the structural features of the interaction of anandamide with cell membrane and allow us to speculate on the manner with which this endogenous ligand interacts with its respective receptors. Based on substantial experimental evidence, it has been proposed that lipophilic ligands interact with their respective target proteins by first partitioning into the membrane bilayer where they assume a preferred location and orientation (5). We can thus postulate that the lipophilic anandamide resides predominantly within the membrane bilayer either following its release within a neural synapse or after its enzymatic synthesis within the cell membrane by membrane-bound enzymes (7). While in the bilayer, the endocannabinoid ligand engages in fast lateral diffusion within the bilayer leaflet before undergoing a productive interaction with the receptor. Our results supporting an extended conformation for anandamide with its headgroup at the level of the bilayer phosphate and a length of \( \sim 22.0 \) Å suggest that the CB1 cannabinoid receptor may have a ligand entry port of equivalent size. Our results also demonstrate that such an alignment does not lead to any unfavorable membrane perturbations. Such an interaction allows anandamide to access helices 3 and 6, which are believed to be involved in CB1 receptor activation (Fig. 11). This hypothesis is congruent with data\(^2\) suggesting that the terminal five-carbon chain of the anandamide arachi-
andoyl moiety interacts with the CB1 receptor through a hydrophobic groove situated at the level of the bilayer center and involved with the participation of hydrophobic residues V6.43 and I6.46 (35). We therefore propose that such an extended conformation of anandamide in the lipid bilayer may facilitate a productive interaction between this endocannabinoid and its CB1 receptor. The same hydrophobic groove has been shown to be involved in the interaction of the five carbon side chain of the plant derived cannabinergic ligand, Δ⁶-THC, and some of its more potent longer chain classical cannabinoid analogs. Work from our laboratory has confirmed that a cysteine residue in helix 6 (Cys-47) reacts covalently with an electrophilic isothiocyanate group at its terminal C-20 carbon.³

³ A. Makriyannis and I. Chen, unpublished results.

**CONCLUSIONS**

Our solid-state REDOR experiments provide evidence for anandamide existing in an extended conformation within DPPC bilayers in the subgel phase. Parallel experiments using static solid-state ²H NMR support a similar conformation for anandamide in the Lₙ liquid crystalline bilayer system. In the phospholipid bilayer environment, the arachidonyl chain of anandamide assumes an orientation parallel to the lipid acyl chains with its terminal methyl group near the bilayer center. The anandamide headgroup is located near the bilayer lipid/water interface and may be engaged in hydrogen bonding interactions at the water interface and may be engaged in hydrogen bonding interactions with the lipid phosphate groups. Furthermore, our ²H NMR experiments provide evidence that in the liquid crystalline phase, the dynamic properties of anandamide are generally similar to those of the bilayer phospholipids and indicate that while in the above location and conformation, the endocannabinoid does not lead to unfavorable perturbation of the membrane bilayer. The results are congruent with a hypothesis that anandamide approaches its binding site by laterally diffusing within one membrane leaflet in an extended conformation and activates the cannabinoid receptors by interacting with a hydrophobic groove formed by helices 3 and 6 while its terminal carbon is very closely positioned to a key cysteine residue in helix 6.

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