STRUCTURE AND LOCATION OF AMIODARONE IN A MEMBRANE BILAYER AS DETERMINED BY MOLECULAR MECHANICS AND QUANTITATIVE X-RAY DIFFRACTION

MARK TRUMBORE,[§‖] DAVID W. CHESTER,[‡‖] JILL MORING,[‖] DAVID RHODES,[*‖] AND LEO G. HERBETTE[*‡§‖]

[*]*Departments of Radiology,* [‡]*Medicine, and* [§]*Biochemistry, and* [‖]*The Biomolecular Structure Analysis Center, University of Connecticut Health Center, Farmington, Connecticut 06032*

ABSTRACT Amiodarone is a drug used in the treatment of cardiac arrhythmias and is believed to have a persistent interaction with cellular membranes. This study sought to examine the structure and location of amiodarone in a membrane bilayer. Amiodarone has a high membrane partition coefficient on the order of 10^6. Small angle x-ray diffraction was used to determine the position of the iodine atoms of amiodarone in dipalmitoylphosphatidylcholine (DPPC) lipid bilayers under conditions of low temperature and hydration where the DPPC bilayer is in the gel state. The time-averaged position of the iodine atoms was determined to be ~6 Å from the center (terminal methyl region) of the lipid bilayer. A dielectric constant of $\kappa = 2$, which approximates that of the bilayer hydrocarbon core region, was used in calculating a minimum energy structure for membrane-bound amiodarone. This calculated structure when compared with the crystal structure of amiodarone demonstrated that amiodarone could assume a conformation in the bilayer significantly different from that in the crystal. The results reported here are an attempt to correlate the position of a membrane-active drug in a lipid bilayer with its time-averaged conformation. This type of analysis promises to be of great use in the design of drugs with greater potency and higher specificity.

INTRODUCTION

Amiodarone is a drug used in the treatment of arrhythmia (1). It has unusual pharmacokinetic properties characterized by an onset time with oral administration of 4–7 d and a persistence of 30–45 d after treatment is discontinued (1). Amiodarone interacts nonspecifically with a variety of membrane sites such as sodium channels (2) and adrenergic receptors (3). This ligand contains two iodine atoms as a part of its covalent structure (see Fig. 1). The high electron density of the iodine atoms, in contrast to the lipid bilayer, allows the time average position of amiodarone in the membrane bilayer to be determined by small angle x-ray diffraction.

In this study, the location of amiodarone in a synthetic lipid bilayer was examined using x-ray diffraction. The location was then used to assign a suitable dielectric environment in which the crystallographic drug conformation could be energy minimized via the molecular mechanics program MMP2 (4). Whereas a number of drug conformation studies have been done, typically these studies have been carried out in vacuo using either the crystallographic conformation of the drug (5), a default conformation based on standard bond lengths, bond angles, and torsion angles (6), or by sampling the conformation space defined by the torsion angles of a structure derived from standard bond lengths and angles (7). Our study differs from these types of studies in that we use a dielectric environment similar to what exists in the lipid bilayer for our analysis. We thereby more closely simulate the in vivo environment of the drug yielding a more accurate picture of the conformation of a drug in the bilayer. Knowledge of both the location and conformation of a drug in the membrane might shed light on the mechanism by which membrane-active drugs such as amiodarone induce their effects.

This report includes (*a*) a comparison of the partition coefficients of amiodarone in different environments, (*b*) the location of amiodarone within the membrane bilayer, and (*c*) a calculated minimum-energy conformation of amiodarone in a dielectric environment approximating the membrane bilayer hydrocarbon core.

MATERIALS AND METHODS

Membrane Preparation and Measurement of Partition Coefficients

The preparation of sarcoplasmic reticulum membranes from rabbit hind leg skeletal muscle has been previously described (8, 9). Either sarcoplasmic reticulum membrane vesicles or octanol/buffer were used in the

Address correspondence to Leo G. Herbette, Biomolecular Structure Analysis Center, University of Connecticut Health Center, Farmington, CT 06032.

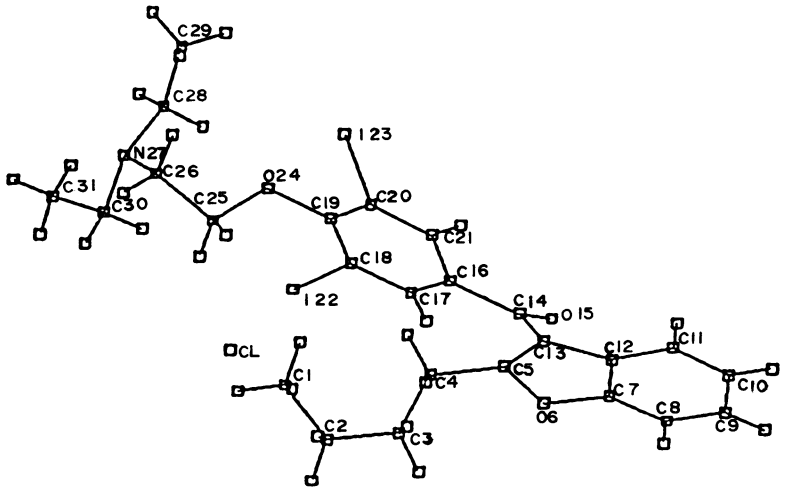

FIGURE 1 Crystal structure of amiodarone showing the two iodine atoms that are part of the drugs covalent structure.

measurement of partition coefficients as described in detail (Herbette, L. G., Y. M. H. Vant Erve, and D. G. Rhodes, submitted for publication). Membrane partition coefficients determined in DPPC vesicles were measured by a filtration technique as previously reported (Herbette, L. G., Y. M. H. Vant Erve, and D. G. Rhodes, submitted for publication).

Preparation of Lipid/Drug Samples for X-Ray Diffraction

DPPC was dissolved in a 2:1 mixture of chloroform:methanol (2:1 vol/vol) and dried to a thin film using a stream of nitrogen gas. Buffer (0.5 mM Hepes, 2.0 mM NaCl, 18.5 μM NaN_2, pH 7.3) was then added to provide a final lipid concentration of 5 mg/ml. This solution was sonicated at 48°C in a Branson B-22-4 ultrasonic bath for a brief time to disperse the lipid. The resulting opalescent multilamellar vesicle suspension was stored at 4°C before use. Amiodarone was dissolved in 95% ethanol to a final concentration of 3.1 mM and stored in the dark until use. Oriented multilamellar samples for x-ray diffraction were prepared using the "spin-dry" procedure described previously (11, 12). For control experiments, 50 μl of DPPC lipid vesicle suspension (~250 μg) was introduced into a Lucite sedimentation cell. Amiodarone was incorporated by incubating 50 μl of DPPC vesicles with 0.5 μl or 1.00 μl of the stock amiodarone solution. The equilibrated amiodarone: vesicle suspension was then added to the sedimentation cells. The samples were centrifuged at 3°C for 30 min at 85,000 *g* in a SW28 rotor (Beckman Instruments, Inc., Fullerton, CA) to pellet the lipids onto aluminum foil. These pellets were then dried during centrifugation at 45,000 *g* for 6 h as previously described (12). The resulting dried lipid films were then mounted on curved glass holders and equilibrated at 4°C and 66% relative humidity overnight to form partially dehydrated multilayers before x-ray exposure.

X-Ray Diffraction Procedures

Small Angle Studies. All x-ray diffraction experiments using lipid bilayers were carried out using an RU-3 x-ray generator (Rigaku/USA Inc., Danvers, MA), equipped with a camera utilizing a single vertical Franks' mirror as previously described in detail (13). The diffraction patterns were collected at 8°C on either x-ray film (Kodak NS-5T) or a Braun position-sensitive proportional counting gas flow detector (PSPC) (Innovative Technology Inc., South Hamilton, MA).

Diffraction samples were equilibrated to different relative humidities in a range of 13–66% in sealed containers at 8°C (13). Under these conditions of humidity and temperature the DPPC bilayer remained in the gel state. For x-ray film experiments a vacuum path was used with a specimen to film distance of 105 mm. Detector experiments used a helium path for specimen to detector distances of 120 mm and an air path for specimen to detector distances of 80 mm. For data collected on x-ray film, relative intensities for the diffraction orders were obtained by scanning with a Zeineh model SL-2DUV soft laser scanning densitometer (Biomed Instruments Inc., Fullerton, CA) as previously described (13). Data collected on the PSPC detector was integrated directly from computer plots by computing areas under the reflections. Data reduction (background and other geometrical corrections) for either mode of data collection has been described (13). Structure factors were phased by swelling experiments (14) using the algorithm reported by Stamatoff and Krimm (15).

Small Molecule Crystallographic Studies. The crystal structure of amiodarone has been solved by Luft and Cody (16). To verify amiodarone structural integrity we redetermined the crystal structure of amiodarone. The amiodarone crystallization was performed out of ethanol. The selected crystal was acicular measuring 0.3 × 0.1 × 0.1 mm. All diffraction was collected using a TEXRAY/Rigaku system equipped with an RU-200 generator, AFC-5 diffractometer, and TEXRAY control software. The entire system was obtained from Molecular Structures Corp., College Station, TX. Intensity data were collected to $\sin\theta/\lambda = 0.56$ by measuring counts at peak positions, and background corrected using counts collected at $\Delta\omega \simeq \pm 0.4°$. Cell constants were determined by fitting 20 data points, $20° \leq 2\theta \leq 34°$. Sample degradation and orientation were monitored by measuring the intensity of three standard reflections every 150 data points. The crystal structure was solved by direct methods using the TEXSAN package of programs (Molecular Structures Corp.), including MITHRIL.

Characterization of Amiodarone by UV Spectroscopy

UV spectroscopy was used to quantify the amount of amiodarone incorporated into DPPC samples and to verify that the structure of amiodarone did not change as a result of x-ray exposure. Spectroscopic measurements were carried out on a model 250 spectrophotometer (Gilford Instrument Laboratories Inc., Oberlin, OH) equipped with a model 250 wavelength scanner. All samples were dissolved in redistilled chloroform, which was used as a reference blank. To verify that the membrane incorporated amiodarone was intact after x-ray exposure, we extracted multilamellar membrane samples containing amiodarone with 500 μl of chloroform and scanned from 245 to 360 nm. The resulting absorption spectra were then compared with spectra obtained from stock solutions of amiodarone and lipid as well as spectra of solubilized thin films of lipid with and without amiodarone scanned over the same wavelength range. The amount of amiodarone incorporated into these samples was quantified spectrophotometrically by measuring absorbance at the 310 nm absorbance peak of amiodarone ($\epsilon_{310} = 3{,}030\ M^{-1}\ cm^{-1}$). The measured absorbance was plotted against standard curves obtained from amiodarone and amiodarone + 200 μg DPPC. Both standard curves

were linear in the concentration range measured and gave identical values for ϵ_{310}. The amount of lipid in each x-ray sample was determined by exhaustive acid hydrolysis of the samples followed by measurement of total phosphate (12).

Absolute Electron Density Scale Determination

The average electron densities for the DPPC headgroups, fatty acyl methylene segments, terminal methyl region, and water were calculated to be 0.430 (0.46 for the phosphate moiety and 0.42 for the glycerol moiety), 0.260, 0.196, and 0.334, respectively, utilizing previously accepted values deemed reasonable (17, 18). A step-function equivalent profile was fitted to the continuous experimental electron density profile structure of DPPC at a resolution of 7.5 Å. An average electron density was assigned to the steps corresponding to the headgroup phosphate and methylene region of the profile. The resulting scale indicated that for the pure water steps outside the headgroup region the average electron density was the same as that calculated, 0.334 e/Å^3. Therefore, the assigned absolute electron density scale for the profile structure of pure DPPC was considered reasonable. The profile structure for DPPC + amiodarone was empirically compared with the DPPC profile structure and assigned an absolute scale based on the two steps within the step-function equivalent profile corresponding to the phospholipid headgroup region (phosphate moiety) and the pure water layer. The additional electron density in the hydrocarbon core region of the membrane was attributed to the amiodarone molecule with two iodine atoms. This additional electron density was calculated from the assigned absolute electron density scale and compared with the electron density determined from the composition, i.e., the measured lipid to amiodarone molar ratio. These two independent approaches gave the same assignment for the absolute electron density of the iodine in the experimental profile structure of DPPC + amiodarone. It is possible to assign absolute electron densities by comparing electron density profiles of varying drug/lipid ratios. Our method of using one drug/lipid ratio that was spectrophotometrically quantified allowed the use of a low drug concentration while insuring sufficient signal to noise that the position of the drug could be determined unambiguously.

Molecular Modeling

The molecular mechanics program MMP2 (4), which assumes a homogenous environment for the molecule, was used to estimate the minimum energy conformation of amiodarone in the bilayer interior. We deemed this to be an acceptable approximation as the position amiodarone assumes in the bilayer is far enough removed from the hydrocarbon core/water interface that the molecule is surrounded by a homogeneous environment of lipid acyl chains. Because several needed parameters (particularly those involving iodine) are not in the MMP2 parameter set, some parameters based on published data were used (19–21). Crystallographic coordinates were used as initial coordinates for the calculation. To determine the minimum energy conformation, energy minima were computed in a dielectric range from $\kappa = 2$ to $\kappa = 80$ using the crystal structure for the initial coordinates. To account for the possibility that the computed minimum energy conformations were not global minima, conformation space for the starting coordinates was sampled using the "dihedral driver" option of MMP2 at $\kappa = 2$ to generate several energy-minimized sets of conformations related to the initial crystallographic conformation. These conformations were generated by torsion in steps of 60° about the C4–C5, C13–C14, C14–C16, C19–024, and 024–C25 bonds (see Fig. 1). The total energies for the energy-minimized structures derived from the crystal structure and the dihedral driver option were compared to determine whether a given structure was a local minimum. The conformation with the lowest total energy was deemed to be the "global" minimum in this study. The structure of lowest energy was compared with the crystal structure as follows. The benzofuran rings of the two structures were mapped onto each other. Because these are planar rigid structures their conformations do not change on energy minimization and can therefore be used to define a common origin for the two structures. Using the common origin defined by the superimposed benzofuran rings, the RMS deviation of nonhydrogen atoms between the two structures was calculated. The change in the angle of orientation of the benzofuran and 3,5-diiodophenyl ring was determined by measuring the torsion angle between atoms C5 and C13 of the benzofuran and atoms C16 and C17 of the iodophenyl moiety.

Materials

All chemicals used were reagent grade with aqueous solutions made using glass distilled deionized water. Unlabeled and ^{125}I-amiodarone were gifts from s.a. Labaz-Sanofi n.v., Brussels, Belgium, made possible by P. Chatelain. Labeled amiodarone was stored at −12°C and unlabeled amiodarone was stored at 4°C. All drug stocks were protected from light at all times. DPPC was obtained from Avanti Polar Lipids, Birmingham, AL, and was used without further purification. TLC analysis of these lipids yielded a single spot.

The molecular graphics package Chem-X (developed and distributed by Chemical Design Limited, Oxford, UK) was used to display the conformation of amiodarone in a membrane and in a crystal and to obtain some geometric parameters.

RESULTS

Partition Coefficients

Membrane partition coefficients for amiodarone were obtained in light sarcoplasmic reticulum membrane preparations and octanol/buffer (Table I). The membrane partition coefficient for DPPC below the thermal phase transition (Tm) was 20,000 ± 5,900, whereas the partition coefficient above the thermal phase transition was at least two orders of magnitude greater, ~2,000,000. Above the Tm, the extremely high amiodarone lipid binding affinity, and the resultant low free ligand concentration, makes determination of the exact partition coefficient value difficult. The partition coefficients in the sarcoplasmic reticulum membrane compare favorably with those determined in DPPC vesicles above the thermal phase transition.

X-Ray Crystallography of Amiodarone

Amiodarone crystallized in a monoclinic system with space group P21/a. The lattice parameters were a = 9.1739, b = 17.104, c = 17.138, and beta = 98.384°. The lattice parameters reported here match those reported by Luft and Cody (16). 1,732 reflections were recorded giving a final structure with an r-fit of 0.053. During the course of the experiment the intensities of the reference reflections changed by <5%. Fig. 1 shows the crystal structure of amiodarone. No solvent was present in the crystal unit cell.

TABLE I
AMIODARONE PARTITION COEFFICIENTS

LSR/buffer	760,000 ± 93,000
Octanol/buffer	350 ± 30
DPPC/buffer (2°C)	20,000 ± 5,900*
DPPC/buffer (>42°C)	>2,000,000

*Measurement done using the filtration technique.

Scattering Profiles and Calculated Electron Density Profiles

Fig. 2 shows the scattering profiles observed for both DPPC and DPPC with amiodarone at 8°C and 13% relative humidity. At this humidity the DPPC multilayer unit cell repeat distance was 57.6 Å whereas the unit cell repeat distance for DPPC with amiodarone was 60.6 Å. The highest order reflection observed in the experiment was $I(h = 12)$. Only reflections through $I(h = 8)$ could be unambiguously phased. The calculated electron density profiles corresponding to $I(h = 8)$ for DPPC and DPPC with amiodarone are shown in Fig. 3 The overall shapes of the two electron density profiles are similar. Both profiles are beginning to resolve the glycerol backbone from the phosphate of the phosphatidylcholine headgroup. The additional electron density, attributed to the amiodarone molecule with two iodine atoms on the 3,5-diiodophenyl ring, is indicated by the area of increased electron density at ±6 Å from the terminal methyl region of the bilayer.

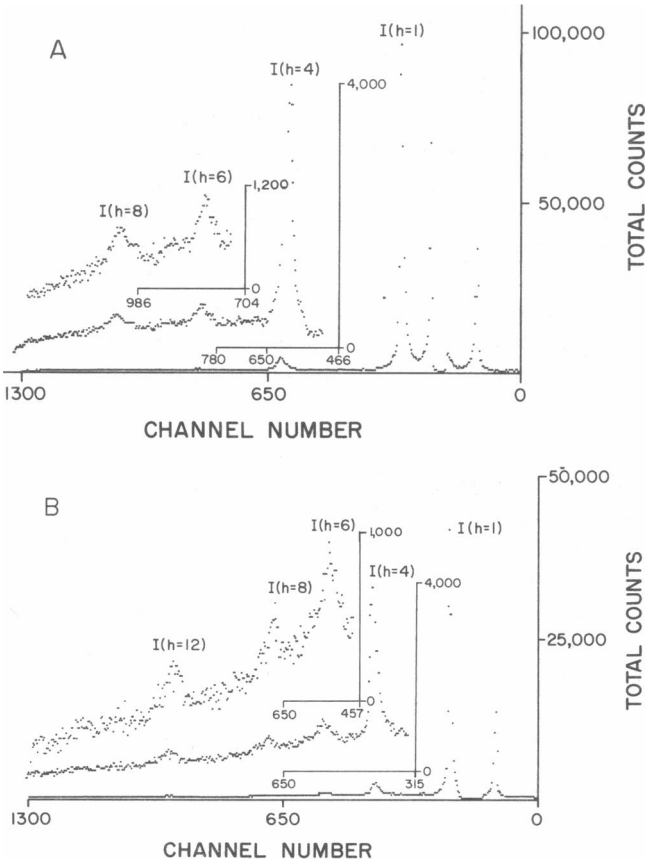

FIGURE 2 X-ray diffraction patterns from (*A*) DPPC at 13% relative humidity and (*B*) DPPC + amiodarone at 13% relative humidity taken on a one-dimensional position sensitive x-ray detector. Up to $I(h = 12)$ was observed. Unit cell repeat distances were 57.60 and 60.56 Å, respectively.

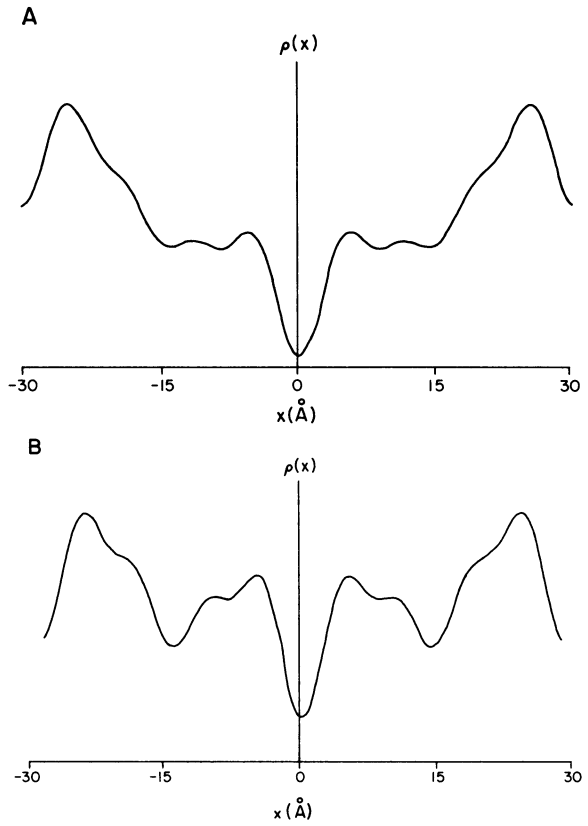

FIGURE 3 Electron density profile structures for (*A*) DPPC and (*B*) DPPC with amiodarone for diffraction patterns corresponding to Fig. 2, *A* and *B*, respectively.

Model Calculations of Absolute Electron Densities

The electron density profiles were placed on an absolute electron density scale as shown in Fig. 4. This scale was consistent with the measured composition of the DPPC/amiodarone samples (1 amiodarone [2 iodine atoms] per 115 lipids). Thus, the assignment of the additional electron density (centered at ~±6 Å from the terminal methyl region of the hydrocarbon core) to the iodine atoms of the amiodarone must be correct. The size of the molecule is

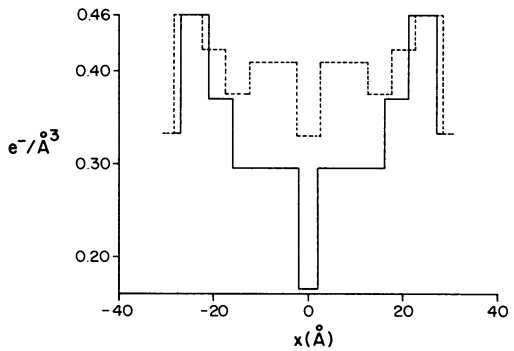

FIGURE 4 Step function equivalent profiles to the experimental profile structures of Fig. 3 placed on an absolute electron density scale.

such that two molecules can span the hydrocarbon core region of the bilayer (Fig. 5 *B*). Therefore, the overall increase of electron density in the hydrocarbon core region of the membrane is probably due to the presence of amiodarone.

Model Calculations of the Membrane Minimum Energy Conformation of Amiodarone

Minimum energy conformations for amiodarone in a DPPC membrane bilayer were calculated for several different values of dielectric constant, using MMP2, which assumes a homogeneous environment for the molecule. Crystal structure data were used for starting coordinates, and calculations were carried out for dielectric constants ranging from $\kappa = 2$, which corresponds to the hydrocarbon region of the membrane, to $\kappa = 80$, which corresponds to water. Because electrostatic interactions made only minor contributions to the total energy of the structure, increasing the dielectric constant from $\kappa = 2$ to $\kappa = 80$ had a negligible effect on the calculated conformation. The dihedral driver option of MMP2 was used to generate 30 sets of starting coordinates, which sampled a wide range of conformation space. Minimum energy structures were calculated for all of these starting coordinants at a dielectric constant of $\kappa = 2$. The computed minimum energy structures of several of these were nearly identical, with calculated total energies that differed by <1.5 kcal/mol. The conformation taken as the "global" minimum by virtue of having the lowest total energy was generated by a 60° torsion about the 024–C25 bond.

Minimization produced pronounced changes in the bilayer structure of the drug as compared with the crystallographic structure. The lowest energy structure showed an overall RMS shift of 1.36 Å from the initial crystallographic structure. The largest change was a reorientation of the benzofuran ring with respect to the central 3,5-diiodophenyl ring. In the crystal structure, the torsion angle between these two rings was 81.84°. In the minimized bilayer structure of the drug, the benzofuran ring was more nearly parallel to the 3,5-diiodophenyl ring with a torsion angle of 40.85° as shown in Fig. 5 *A*. Shifts in the conformation of other regions of the molecule were not as

TABLE II
ATOMIC COORDINATES FOR THE CRYSTAL STRUCTURE AND MEMBRANE MINIMUM ENERGY STRUCTURE OF AMIODARONE (Å)

	Amiodarone crystal coordinates				Amiodarone minimum energy coordinates		
	X	Y	Z		X	Y	Z
C1	1.24808	4.34290	2.60845	C1	1.34247	5.87277	2.55609
C2	0.58147	5.55359	3.30018	C2	0.38936	6.01078	3.75125
C3	−0.72720	5.92698	2.69747	C3	−1.07323	6.25415	3.34011
C4	−1.84786	4.93349	3.03188	C4	−1.75415	5.01382	2.73082
C5	−3.13561	5.29959	2.30601	C5	−3.15614	5.34503	2.26823
06	−3.91560	6.13211	3.11397	06	−3.92815	6.09455	3.10279
C7	−4.98860	6.47492	2.28955	C7	−4.97543	6.46247	2.33646
C8	−6.08052	7.24260	2.68044	C8	−6.05802	7.26572	2.69404
C9	−7.02991	7.51878	1.78078	C9	−7.04516	7.51582	1.73987
C10	−6.92669	6.97826	0.48887	C10	−6.94251	6.96420	0.45734
C11	−5.87481	6.17363	0.09614	C11	−5.85493	6.15955	0.10738
C12	−4.87362	5.92967	1.02954	C12	−4.86096	5.91407	1.05889
C13	−3.65609	5.17315	1.05551	C13	−3.71961	5.18840	1.04544
C14	−3.00956	4.61877	−0.10876	C14	−3.26196	4.52185	−0.05206
015	−3.24248	5.04463	−1.21740	015	−3.63144	4.88677	−1.14740
C16	−1.91353	3.54909	0.00353	C16	−2.44250	3.43047	0.00542
C17	−0.79552	3.65432	−0.80687	C17	−1.83017	2.94466	−1.15868
C18	0.21753	2.70956	−0.75431	C18	−1.01997	1.80646	−1.15427
C19	0.04377	1.59461	0.11455	C19	−0.74107	1.10754	0.03614
C20	−1.10220	1.44408	0.87077	C20	−1.48973	1.49293	1.15760
C21	−2.10981	2.44405	0.81385	C21	−2.29820	2.63588	1.14420
I22	1.90042	3.03591	−1.93275	I22	−0.27547	1.12585	−3.01273
I23	−1.41687	−0.25227	2.02483	I23	−1.50810	0.29212	2.89006
024	1.03264	0.60918	0.14680	024	−0.01028	−0.04870	0.04625
C25	2.01750	0.82920	1.16040	C25	1.40413	0.07717	0.03059
C26	2.77725	−0.48963	1.39033	C26	1.99572	0.84630	1.22369
N27	3.66120	−0.91803	0.23475	N27	1.81081	0.24054	2.54558
C28	2.89793	−1.50838	−0.89863	C28	2.43030	1.01666	3.62901
C29	2.00395	−2.62657	−0.50469	C29	1.96976	2.47954	3.70898
C30	4.59476	0.16888	−0.18014	C30	2.14719	−1.18573	2.62790
C31	5.74397	−0.35563	−0.98583	C31	3.60188	−1.55047	2.29818

pronounced but were significant. Table II lists the crystallographic coordinates of amiodarone along with those of the energy-minimized conformation of amiodarone in the DPPC lipid bilayer.

DISCUSSION

Location of Amiodarone in a Lipid Bilayer

The electron density profile structures in Fig. 3 indicate additional electron density centered at $x = \pm 6$ Å from the central region of the lipid bilayer in the presence of amiodarone. Model calculations indicate that this additional electron density arises from the amiodarone molecule with two iodine atoms covalently bonded to the phenyl ring of amiodarone. From these data, amiodarone is located within the hydrocarbon core of the membrane bilayer near the terminal methyl region, as schematically portrayed in Fig. 5 *B*. As previously discussed (22), the appearance of additional electron density in a profile structure seen in an x-ray diffraction experiment using a drug with a single electron dense atom substitution, cannot distinguish between the following cases. (*a*) Perturbations of the electron density profile structure of the drug/lipid complex due solely to the presence of the drug's electron dense atom. (*b*) Perturbations of the electron density profile structure due to changes in the lipid bilayer structure resulting from the drug's presence.

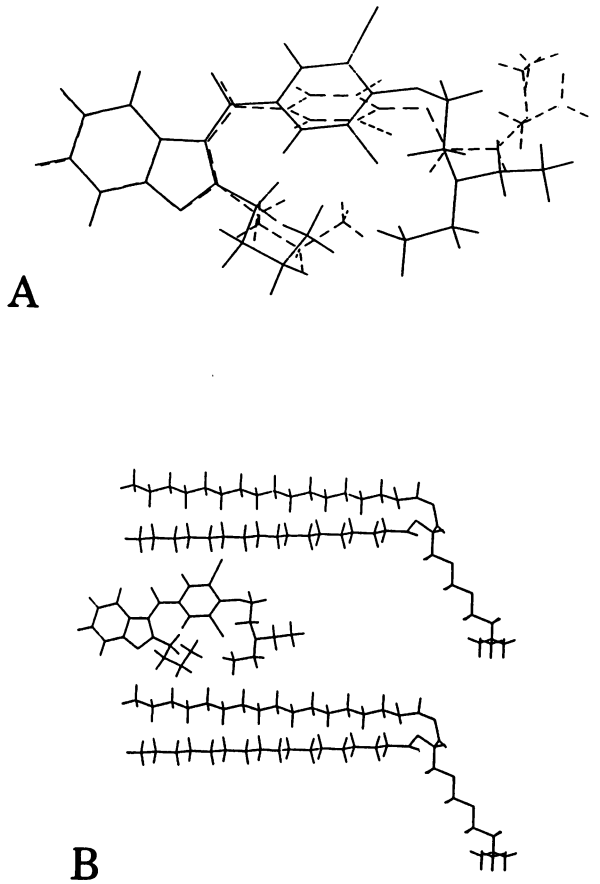

FIGURE 5 (*A*) The minimum energy structure of amiodarone calculated in a low dielectric medium with the program MMP2 superimposed on the crystal structure of the drug. The solid bonds indicate the energy-minimized structure. The crystal structure is indicated by dashed bonds. (*B*) Amiodarone incorporated into a membrane bilayer showing the position of the iodine atoms relative to the acyl chains and the size of the molecule relative to the hydrocarbon core region.

A more definitive experiment using two different halogenated analogues of the same drug (e.g., bromine vs. iodine) could distinguish between the above two situations (22). However, in our studies, the DPPC profile structures in the presence and absence of amiodarone have similar unit cell repeats, and similar phospholipid headgroup separations. With model calculations that indicate a consistency between the absolute electron densities within the profile structure and its composition, the conclusions regarding the location of amiodarone appear to be correct.

The conclusion that amiodarone is located near the center of the hydrocarbon core region of the bilayer below the thermal phase transition and at relatively low hydration is in accord with that reported by Chatelain et al. (23). Chatelain et al. (23) reported that amiodarone serves to decrease membrane fluidity as would be expected for a molecule which specifically interacts with the lipid acyl chains. Jendrasiak et al. (24) have proposed, on the basis of multinuclear NMR studies, that amiodarone assumes a location in the bilayer near the phospholipid headgroup region of a membrane bilayer. This study differed from our study in that Jendrasiak et al. (24) used the charged lipid species phosphatidic acid and stearylamine in fully hydrated liposomes with a lipid to amiodarone molar ratio of 3:2.

Recent theoretical studies indicate the possibility that drugs partitioning into a membrane bilayer will assume discrete preferred locations (25). The theory predicts that a molecule will assume a location either near the hydrocarbon core/water interface or near the terminal methyl region of the membrane bilayer. The molecule's lipophilicity, in part, determines which of these two positions the molecule will assume. This theoretical model appears to hold true for the drug examined in this study. Amiodarone has a high lipophilicity and as predicted is located near the terminal methyl groups of the fatty acyl chains. Lipophilicity is, however, probably only one of several parameters responsible for a drug's location, orientation, and conformation in a membrane bilayer. The physical state of the lipid chains and the degree of hydration of the membrane bilayer may also be important parameters influencing the location of a drug in a membrane bilayer.

Correlation of Location and Partition Coefficient

Intuitively one would predict that a drug with a membrane partition coefficient as large as amiodarone's would be located deep within the membrane bilayer. The position of amiodarone as determined by small angle x-ray scattering

is in accord with this idea. The high membrane partition coefficient for amiodarone is not consistent with its octanol/buffer partition coefficient. This is similar to the results in Herbette et al. (26) in which the authors demonstrated that the octanol/buffer (Table I) partition coefficient is not a good indicator of the partitioning of drug into sarcoplasmic reticulum membranes.

Chatelain and Laruel (27) have previously determined the partition coefficient of both neutral and protonated amiodarone. Whereas the value they observed for the partitioning of neutral amiodarone is in accord with our values, at the pH used in our studies (pH 7.0) amiodarone should be fully protonated. They determined a partition coefficient of 16,500 ± 1,400 for protonated amiodarone in total lipids extracted from erythrocyte ghosts. We, on the other hand, determined a value of 760,000 ± 93,000 for amiodarone partitioning into light sarcoplasmic reticulum (LSR) membranes. We believe the discrepancy between the two sets of partition coefficients to result from two factors. First, in the Chatelain and Laruel study a high lipid amount was used. This can lead to an excessive depletion of the amount of drug in the aqueous pool causing nonequilibrium conditions. Such a condition could then lead to an underestimate of the partition coefficient for the drug being studied. Secondly, we determined our partition coefficients in a different membrane system. Because we used both a concentration of lipid much lower than that used by Chatelain and Laruel and different lipid systems, we feel that the partition coefficients reported by us for amiodarone are more valid.

Correlation of Location and Structure

Only part of the total energy that specifies a molecular conformation stems from implicit molecular parameters (bond stretch, bend, etc.), the remainder arises from packing constraints, solvent effects, and other environmental variables. In the crystal form, a molecule's minimum energy conformation is determined by an equilibrium balance between packing constraints and molecular energies such as drug–drug interactions, which may perturb the molecule's "implicit (in vacuo) conformation." In addition, although the environment of a given molecule in a crystal is exactly known by the symmetry of the unit cell, this is not necessarily a uniform environment such as the molecule would sense in a vacuum or (in the time average) in a homogeneous environment such as a solution. Because the bilayer center is thought to be a relatively uniform environment, it should be possible to estimate the conformation of amiodarone partitioned into the center of a bilayer. This calculation necessarily ignores potential packing constraints, essentially assuming the bilayer interior to be a homogeneous solution of "long chain alkane." It is not surprising, therefore, that there are some differences between the computed minimum energy structure of amiodarone in a bilayer and the "experimentally determined minimum energy structure" of amiodarone obtained by crystallography (Fig. 5 *A*). Whereas the effects of acyl chain packing on the conformation of the amiodarone were not examined, we believe that the effect of packing in a bilayer to be smaller than the effects of packing experienced in a crystal. Molecular mechanics calculations indicate that the minimum energy structure of amiodarone in a lipid bilayer is significantly different from the crystal structure having an overall RMS difference of 1.36 Å due chiefly to the reorientation of the two rings of amiodarone to become more planar. Although the absolute (global) minimum energy conformation might be determined by refinement of this computation, this is a reasonable structure for use in further discussions of the membrane structure of amiodarone.

In a study by Ferreira et al (7) the conformation of amiodarone at an air-water interface was examined. In this study, an initial structure for amiodarone was created and other structures generated by torsion about specific bonds. These structures were then minimized in a dielectric gradient and fitted to the air-water interface. They reported that the preferred structures had the benzofuran and iodophenyl rings roughly orthogonal. The MMP2 energy-minimized structure of amiodarone in a lipid bilayer, in contrast, has the two rings in a more planar orientation. The differences observed between our structures and the structures reported by Ferreira et al. are most likely due to differences in the energy minimization methods by which these structures were obtained.

The resolution of the small angle x-ray diffraction experiments was not high enough to determine whether the iodine atoms were lying in the same plane. Therefore, it was not possible to experimentally determine the orientation of the drug in the membrane. However, orienting the whole amiodarone molecule such that the long axis of the amiodarone is parallel to the acyl chain could explain the generally elevated electron density observed in the acyl chain region of the bilayer. If the long axis of the amiodarone were perpendicular to the acyl chains, the drug would not span enough of the hydrocarbon core to account for the elevated electron density seen if the drug is localized to a discrete position in the bilayer as indicated by the diffraction results. Makriyannis et al. (28) have demonstrated that it is possible to determine the orientation of a deuterated molecule in a lipid membrane. An NMR or neutron diffraction approach could potentially be utilized here to determine the orientation of amiodarone in the membrane bilayer. Coupled with knowledge of the location and conformation of a molecule, knowledge of the orientation of a molecule in a lipid bilayer would allow a complete understanding of the molecular interactions of a drug and a bilayer.

SUMMARY

Clearly, if one wishes to understand the pharmacology of a membrane-active drug, a determination of a number of molecular parameters is necessary. One should know the

location and orientation of the drug in the membrane as well as the conformation the drug assumes in this environment. This study sought to address two of these questions, "what is the location of the drug"? and "what is it's predicted energy minimized membrane conformation"? We have been able to localize the position of the iodine atoms of amiodarone to 6 Å from the center of a membrane bilayer. It is much more difficult to determine the conformation of the drug in the bilayer. Based on the crystal structure of the drug and knowledge of its location in a membrane bilayer, a minimum energy structure in a dielectric environment similar to the interior of the bilayer was determined. This established that, for amiodarone, the crystal and energy-minimized membrane bilayer structures were significantly different. The large scale changes were due almost solely to changes in the orientation of the two ring structures. The effects of the acyl chain environment on the conformation of amiodarone was beyond the scope of this analysis but undoubtedly is of importance for a complete understanding of the drug's pharmacology, as is an accurate determination of the orientation of the drug in the membrane bilayer. These results are a first step towards an understanding of the complete interaction between lipid membranes and a membrane-active drug. As such they may provide valuable insight and direction for future studies in this area.

We would like to thank Dr. A. Katz for discussions regarding drug-membrane interactions which allowed us to relate these results to clinical questions. We also thank Dr. P. Chatelain for graciously providing us with experimental drug samples. We would like to also thank Ms. T. Wojtusik for her dedication in preparing this manuscript for submission. The work was carried out in the Biomolecular Structure Analysis Center at the University of Connecticut Health Center. We would like to thank the staff of the Structure Center for their dedication in keeping the facilities in optimal running condition.

This work was supported by research grants HL-33026 from the National Institutes of Health, by a grant from the Whitaker Foundation, and by a gift from the Patterson Trust Foundation and R. J. R. Nabisco, Inc. Dr. D. Rhodes was supported by the Defense Advanced Research Projects Agency. Dr. D. Chester was funded by a National Institutes of Health training grant HL-07420. M. Trumbore was supported by a Health Center Research Advisory Committee graduate student fellowship. Dr. L. Herbette acknowledges his current affiliation as an Established Investigator of the American Heart Association. The Structure Center acknowledges support from the State of Connecticut Department of Higher Education's High Technology Project and Grant Program.

Received for publication 8 March 1988 and in final form 5 May 1988.

REFERENCES

1. Rosenbourn, M. B., P. A. Chiale, M. S. Halpern, G. J. Nau, J. Przybylski, R. J. Levi, J. O. Lazzari, and M. V. Elizari. 1976. Clinical efficacy of amiodarone as an antiarrhthymic agent. *Am. J. Cardiol.* 38:934–944.
2. Mason, J. W., L. M. Hondeghem, and B. G. Katzung. Amiodarone blocks inactivated cardiac sodium channels. *Pfluegers Arch. Eur. J. Physiol.* 396:79–81.
3. Polster, P., and J. Broekhuyser. 1976. The adrenergic antagonism of amiodarone. *Biochem. Pharmacol.* 25:131–134.
4. Allinger, N. L., and H. L. Flanagan. 1983. Isotope effects in molecular mechanics (MM2) calculations on deuterium compounds. *J. Comput. Chem.* 4:399–403.
5. Johansson, A., A. Karlen, C. Grol, S. Sundell, L. Kenne, and U. Hacksell. 1986. Dopaminergic 2-aminotetralins: affinities for dopamine D_2-receptors, molecular structures, and conformational preferences. *Mol. Pharmacol.* 30:258–269.
6. Liljefors, T., and H. Wikstrom. 1986. A molecular mechanics approach to the understanding of presynaptic selectivity for centrally acting dopamine receptor agonists of the phenylpiperidine series. *J. Med. Chem.* 29:1896–1904.
7. Ferreira, J., R. Brasseur, P. Chatelain, and J. M. Ruysschaert. 1986. Properties of amiodarone monolayer spread at the air-water interface. *J. Pharm. Pharmacol.* 38:561–566.
8. Harigaya, S., and A. Schwartz. 1969. Rate of calcium binding and uptake in normal animal and failing human cardiac muscle. *Circ. Res.* 35:781–794.
9. Watras, J., F. C. Messineo, and L. G. Herbette. 1984. Mechanisms of fatty acid effects on sarcoplasmic reticulum. I. Calcium-fatty acid interaction. *J. Biol. Chem.* 259:1319–1324.
10. Deleted in press.
11. Clark, N. A., K. J. Rothschold, D. A. Luipold, and M. A. Simon. 1980. Surface-induced lamellar orientation of multilayer membrane arrays. Theoretical analysis and a new method with application to purple membrane fragments. *Biophys. J.* 31:65–96.
12. Chester, D. W., L. G. Herbette, R. P. Mason, A. F. Joslyn, P. J. Triggle, and D. E. Koppel. 1987. Diffusion of dihydropyridine calcium channel antagonists in cardiac sarcolemmal lipid multibilayers. *Biophys. J.* 52:1021–1030.
13. Herbette, L. G., T. MacAlister, T. F. Ashovaid, and R. A. Colvin. 1985. Structure-function studies of canine cardiac sarcolemmal membranes. II. Structural organization of the sarcolemmal membranes as determined by electron microscopy and lamellar x-ray diffraction. *Biochim. Biophys. Acta.* 812:609–623.
14. Moody, M. F. 1963. X-ray diffraction pattern of nerve myelin: a method for determining the phases. *Science (Wash. DC).* 142:1173–1174.
15. Stamatoff, J. B. and S. Krimm. 1976. Phase determination of x-ray reflections for membrane-type systems with constant fluid density. *Biophys. J.* 16:503–516.
16. Luft, J., and V. Cody. 1986. Iodothyronine deiodinase inhibitors: molecular conformation of amiodarone HCl. American Crystallographic Association Annual Meeting. Series 2. 14:67a.
17. Herbette, L., A. Scarpa, J. K. Blasie, C. T. Wang, L. Hymel, J. Seelig, and S. Fleisher. 1983. Determination of the separate Ca^{2+}, pump protein and the phospholipid profile structures within reconstituted sarcoplasmic reticulum membranes via x-ray and neutron diffraction. *Biochim. Biophys. Acta.* 730:369–378.
18. Pachence, J. M., P. L. Dutton, and J. K. Blasie. 1979. X-ray diffraction of reconstituted reaction center/lipid membranes. *Biochim. Biophys. Acta.* 548:348–373.
19. Suzuki, M. 1977. Vibrational spectra of hexabromobenzene and hexaiodobenzene. *Spectrochim. Acta. Part A Mol. Spectrosc.* 33:921–927.
20. Scott, D. W. 1971. A valence force field for furan and pyrrole and their deuterium and methyl derivatives. *J. Mol. Spectrosc.* 37:77–91.
21. Meyer, A. Y. 1983. Molecular mechanics of organic halides. Part VII. Iodides. *Theochem.* 94:95–106.
22. Herbette, L. G. 1985. X-ray and neutron diffraction for probing the interactions of small molecules with membrane structures. *Curr. Top. Bioenerg.* 14:21–52.
23. Chatelain, P., R. Laruel, and M. Gillard. 1985. Effect of amiodarone on membrane fluidity and Na^+/K^+ ATPase activity in rat-brain synaptic membranes. *Biochim. Biophys. Res. Commun.* 129:148–154.

24. Jendrasiak, G. L., C. Glisson, and R. S. Porter. 1988. A multinuclear NMR study of liposomes containing the antiarrhythmic drug amiodarone. *Biophys. J.* 53:77a.

25. Marqusee, J. A., and K. A. Dill. 1986. Solute partitioning into chain molecule interphases: monolayers, bilayer membranes and micelles. *J. Chem. Phys.* 85:434–444.

26. Herbette, L. G., D. W. Chester, and D. G. Rhodes. 1986. Structural analysis of drug molecules in biological membranes. *Biophys. J.* 49:91–93.

27. Chatelain, P., and R. Laruel. 1985. Amiodarone partitioning with phospholipid bilayers and erythrocyte membranes. *J. Pharm. Sci.* 74:783–784.

28. Makriyannis, A., A. Banijamali, C. Van Der Schyf, and H. Jarrell. 1987. Interactions of cannabinoids with membranes. The role of cannabinoid stereochemistry and absolute configuration and the orientation of Delta-9-THC in the membrane bilayer. *In* Structure-Activity Relationships of the Cannabinoids. A. Makriyannis and R. S. Rapuka, editors. Dept. of Health and Human Services, DHHS Publication No. (ADM)87-1534.