DIFFUSION OF DIHYDROPYRIDINE CALCIUM CHANNEL ANTAGONISTS IN CARDIAC SARCOLEMMAL LIPID MULTIBILAYERS

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ABSTRACT A membrane bilayer pathway model has been proposed for the interaction of dihydropyridine (DHP) calcium channel antagonists with receptors in cardiac sarcolemma (Rhodes, D. G., J. G. Sarmiento, and L. G. Herbette. 1985. *Mol. Pharmacol.* 27:612–623) involving drug partition into the bilayer with subsequent receptor binding mediated (though probably not rate-limited) by diffusion within the bilayer. Recently, we have characterized the partition step, demonstrating that DHPs reside, on a time-average basis, near the bilayer hydrocarbon core/water interface. Drug distribution about this interface may define a plane of local concentration for lateral diffusion within the membrane. The studies presented herein examine the diffusional dynamics of an active rhodamine-labeled DHP and a fluorescent phospholipid analogue ($DiIC_{16}$) in pure cardiac sarcolemmal lipid multibilayer preparations as a function of bilayer hydration. At maximal bilayer hydration, the drug diffuses over macroscopic distances within the bilayer at a rate identical to that of DiI ($D = 3.8 \times 10^{-8}$ cm^2/s), demonstrating the overall feasibility of the membrane diffusion model. The diffusion coefficients for both drug and lipid decreased substantially as the bilayers were dehydrated. While identical at maximal hydration, drug diffusion was significantly slower than that of $DiIC_{16}$ in partially dehydrated bilayers, probably reflecting differences in mass distribution of these probes in the bilayer.

INTRODUCTION

1,4-Dihydropyridine (DHP) calcium channel antagonists inhibit channel opening of voltage-dependent calcium channels in cardiac sarcolemma and a variety of other membranes (Janis and Triggle, 1984; Hess et al., 1984). The specific binding of these drugs to this membrane receptor has been shown by Belleman and co-workers (1983) to be highly stereoselective, saturable, and reversible (with K_d values ranging from 0.2 to 3.0 nM in cardiac membranes [Janis and Triggle, 1984]).[1] A second low affinity binding site with a K_d of 30–100 nM has also been identified in cardiac sarcolemma (Janis and Triggle, 1984). Receptor–ligand stereoselectivity is highlighted by structure/activity relationships observed for various DHP analogues as well as the demonstration of both positive and negative inotropic activities associated with enantiomeric DHP pairs, e.g., Sandoz 202, 791 and PN 200, 110 (Hof et al., 1985; Kongsamut et al., 1985; Langs and Triggle, 1985).

Recently, Rhodes and co-workers (1985) proposed that a two-step mechanism, the "membrane bilayer pathway" model, is involved in DHP receptor binding. This membrane model (M) involving drug partition and orientation within the membrane and lateral diffusion within a plane of the bilayer on approach to their specific receptor is in contrast to the "aqueous pathway" model (A) involving random diffusion through the bulk water phase and subsequent receptor interaction at an exposed site (see Fig. 1). Theoretical calculations for diffusion-limited rates for drug binding demonstrate the membrane pathway to be some three orders of magnitude faster than the aqueous pathway (Rhodes et al., 1985).

Several lines of evidence support the concept of the membrane pathway being operable for the interaction of DHP calcium channel antagonists (and possibly agonists) with their receptors. Membrane partition coefficients for several DHPs range from K_p of 5,000 to 150,000 (Herbette et al., 1985, 1986). We have also determined the time-averaged position of these drugs at the hydrocarbon core/water interface in both native and model membrane systems.[1,2] Moreover, nimodipine has been shown, by neutron

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[1]Herbette, L. G., Y. V. VantErve, and D. G. Rhodes, manuscript submitted for publication.

[2]Chester, D. W., S. D. Wang, and L. G. Herbette, manuscript in preparation.

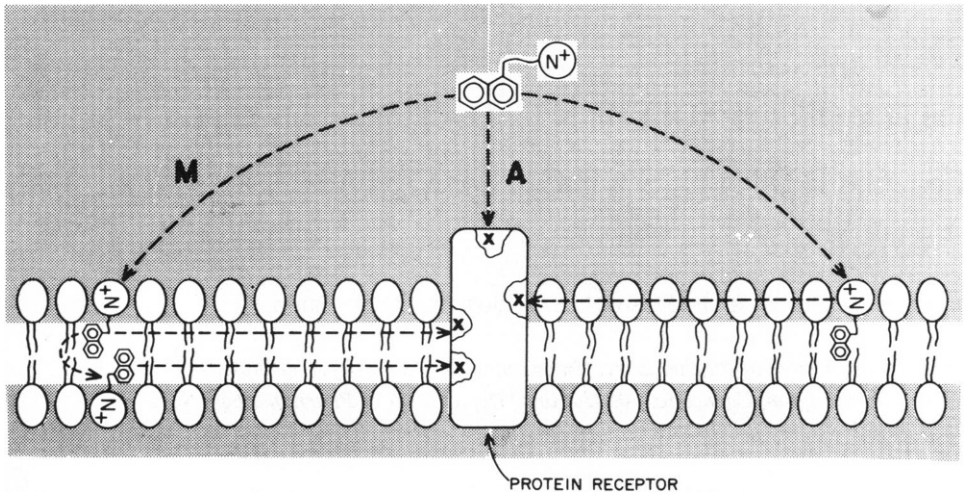

FIGURE 1 Schematic diagram of the possible pathways by which an amphiphilic or lipophilic drug could reach its receptor site: a single step aqueous pathway (A) or a two-step membrane pathway (M). According to the membrane pathway, a drug may enter the bulk lipid phase on the side of the membrane to which it was added and diffuse laterally to a hydrophobic (left side) or hydrophilic (right side) binding site. Alternatively, a drug could diffuse or flip-flop across the bilayer with some characteristic rate to gain access to the opposite side of the membrane. (Reproduced from *Mol. Pharmacol.*, 1985, 27:612–623, by copyright permission of the American Society for Pharmacology and Experimental Therapeutics.)

diffraction studies, to diffuse ("flip-flop") between the monolayers of the membrane bilayer (Herbette, 1986). The ability to diffuse through the membrane is consistent with recent studies by Affolter and Coronado (1985, 1986) demonstrating that both DHP (Bay K 8644) and phenylalkylamines (D-600) readily cross the bilayer to interact with asymmetrically reconstituted calcium channels in a planar lipid bilayer system.

Several physical characteristics of cardiac sarcolemmal membranes (CSL) are also in support of this notion. Because of low receptor site density in CSL membranes (Colvin et al., 1985), two-dimensional diffusion through the bilayer can lead to a significant rate advantage in the diffusion-limited case (McCloskey and Poo, 1986), but only if the ligand can assume the required position and orientation within the bilayer needed for proper interaction with the receptor binding site. Low receptor site density in these membranes is coincident with a relatively high lipid/protein ratio (3,000 nM/mg protein; Colvin et al., 1985), high receptor binding affinities (Janis and Scriabine, 1983), and intrinsic "on rates" for specific binding of $0.6–1 \times 10^7$ $(ms)^{-1}$ reported by Herbette et al.[1]

The multipoint analysis fluorescence recovery after photobleaching (FRAP) technique described by Koppel (1979) has been used to further our analysis of the membrane bilayer pathway model by measurement of the lateral diffusion coefficient (D) of a fluorescently labeled DHP (nisoldipine-lissamine rhodamine [Ns-R]) in cardiac sarcolemmal lipid multibilayers. Such measurements serve two fundamental purposes. First, they put the analysis of the bilayer pathway model on a firmer quantitative basis. Measured values of D put upper limits to the possible values of drug binding rates obtained in a diffusion-limited case. Furthermore, even if binding rates prove not to be diffusion limited (and thus, not particularly sensitive to diffusion rates), the relative values of D obtained in these experiments, compared with those of other well characterized fluorescent probes in the same membrane system, provide a critical assay of the nature of the interaction of these probes with the membrane bilayer. These studies, therefore, constitute an initial approach directed toward determination of the microscopic rate constants associated with DHP receptor binding. The multibilayer samples used for these studies were prepared by a modification of the spin-dry procedure described by Clark et al. (1980). This procedure produces extended multilamellar membrane samples and allows precise control over drug/lipid ratios and relative bilayer hydration. Drug and phospholipid analogue diffusion coefficients were found to be identical at maximal bilayer hydration, but differ significantly at lower hydration.

MATERIALS AND METHODS

Membrane Isolation and Lipid Preparation

Crude canine cardiac sarcolemmal membranes were isolated by the method of Jones et al. (1980). Lipids were extracted from these CSL preparations essentially by the method of Folch et al. (1957). Polar and neutral lipid composition was analyzed by thin layer chromatography (TLC) on 250-μm silica gel 60 TLC plates (EM Reagents, Merck, Darmstadt, FRG) by developing ~15 cm in a single dimension with chloroform/methanol/20% methylamine/water (60:36:10:0.3, vol/vol) to resolve the polar lipids with subsequent development (20 cm) in the same direction with benzene/diethylether/ethanol/28% ammonia (50:40:2:0.1, vol/vol) to resolve the neutral lipids. For two-dimensional TLC, chloroform/methanol/28% ammonia (65:35:5, vol/vol) was used as the primary and chloroform/acetone/acetic acid/water (5:3:1:1:0.5, vol/vol) as secondary solvent. The various lipid classes were visualized under long wave UV light after the fluorescent spray reagent, 1 mM 2-*p*-toluidinyl-6-naphthalenesulfonate (TNS; Eastman Kodak Co., Rochester, NY) in 50 mM Tris/HCl pH 7.5, was applied to the plates. Lipid class composition was assessed by scanning TNS fluorescence with a model SL-2DUV soft laser scanning densiotometer (Zeineh Biomedical Instruments, Inc., Fullerton, CA) or scraping of bands for lipid phosphate analysis. For preparative purposes, polar lipids and endogenous cholesterol were separated from contaminating neutral lipids (triglycerides, cholesterol esters, etc.) by developing the plates in benzene/diethylether/ethanol/28% ammonia (50:40:2:0.1, vol/vol), which resolves all the

neutral lipids while leaving the polar lipids at the origin. Cholesterol and polar lipid bands were identified with distilled water, scraped, eluted with chloroform/methanol (1:1, saturated with water), and dried on a rotary evaporator and resuspended in chloroform/methanol (19:1, saturated with water). Trace amounts of silica and non-lipid contaminants were removed by elution of the lipid preparation from a 10 × 0.5-cm Sephadex G-25 column equilibrated in methanol/water (1:1, vol/vol) and pre-run with chloroform/methanol (19:1, vol/vol, water saturated) just before fractionation. The phospholipid and cholesterol class composition was examined and compared against profiles before purification and the lipids stored under N_2 at −20°C.

Lipid phosphorus was determined by a modification of the method of Chen et al. (1956). Briefly, 50 μl of 72% perchloric acid is added to 1–40 nmol of phospholipid phosphorus (solvents dried off) in borosilicate tubes and hydrolyzed for 1 h at 180°C in a sand block heater. The following reagents were prepared for the phosphomolybdate complex color development: solution A, 1.58% ascorbate (prepared fresh daily); solution B, 5.09 g ammonium molybdate, 33 ml concentrated H_2SO_4, and 84 ml distilled water; solution C, 21.0 ml reagent A, 2.0 ml reagent B. Reagent C (0.9 ml) is added to the cooled perchloric acid digest, color developed at 80°C for 15 min, and absorbance determined at 820 nm. Unexposed silica was used as a blank for phosphate analysis off the TLC plate.

All organic solvents were redistilled before use.

Synthesis of the (2-*N*-Sulfonamidolissamine rhodamine)ethyl methyl-2,6-dimethyl-4-(2-nitrophenyl)-1,4-dihydropyridine-3,5-dicarboxylate Fluorescent DHP Probe

Full details of the synthetic methodology will be published separately. In brief, to (2-aminoethyl) methyl-4-(2-nitrophenyl)-1,4-dihydropyridine-3,5-dicarboxylate (0.68 g, 0.001 mol) in 10 ml of methanol containing 0.15 ml triethylamine was added dropwise lissamine rhodamine sulfonylchloride (0.25 g, 0.0004 mol; Molecular Probes, Inc., Junction City, OR) dissolved in 25 ml acetone. The reaction stood overnight, was concentrated in vacuo, diluted with 200 ml methylene chloride, and the organic layer washed with 100 ml of 5% hydrochloric acid and aliquots of water totaling 2,500 ml. The washed organic layer was then dried and concentrated to a deep red oil that crystallized from ethanol. The fluorescent DHP was examined for purity by TLC yielding R_f values of 0.5 and 0.44 after developing in ethanol (ETOH) or ethyl acetate/ETOH/chloroform (30:25:15, vol/vol), respectively. Yield was 0.036 g, melting point 217–220°C, and spectral assignments (infrared, nuclear magnetic resonance) were in accord with the structural assignment.

Specific Nitrendipine Displacement Studies with Ns-R

Ns-R displacement of specifically bound [^{3}H]nitrendipine (New England Nuclear, Boston, MA; specific activity 85 Ci/mmol) to a microsomal fraction of guinea pig ileal longitudinal smooth muscle was determined according to our previously established methods (Bolger et al., 1983).

Multilamellar Vesicle Preparation

Multilamellar vesicles were prepared in the presence or absence of known amounts of fluorescent probe essentially by the method of Bangham et al. (1965). CSL lipids were dried as a thin film on glass tubes under N_2 and residual solvent removed en vacuo (<10 μm vacuum) for 2–4 h. A specified volume of 0.5 mM Hepes, pH 7.27, 2 mM NaCl containing Ns-R was added to the dried lipid preparation yielding a final lipid phosphorus concentration of 3.62 μM/ml and drug/lipid ratios ranging from 1:10^3 to 1:10^5 (3.62 nM/ml–36.2 pM/ml). The 1,1'-dihexadecyl-3,3,3',3'-tetramethylindocarbanocyanine ($DiIC_{16}$) probe (Molecular Probes Inc.) used for the determination of phospholipid diffusion coefficients in this study was co-dried with the lipid with a constant probe/lipid ratio of 1:10^4. Multilamellar vesicles were prepared and equilibrated with the drug (or $DiIC_{16}$) overnight at 4°C before formation of multilayers (see below).

Preparation of Multibilayer Samples for FRAP and X-ray Diffraction

The spin dry technique of Clark et al. (1980) has been adapted to our specific needs. This procedure takes advantage of the centrifuge vacuum for dehydration of the membrane sample during centrifugation producing highly ordered multibilayer pellets. Precise control can be exerted over drug (or probe)/lipid ratios since these ratios can be preset during vesicle preparation. Further, in excess of 95% of the added lipid phosphate is typically recovered in the membrane pellet. Quantitative and reproducible recovery is important for these FRAP studies as well as diffraction studies to determine bilayer drug location which use similar sample preparation conditions (Trumbore, M., D. W. Chester, and L. G. Herbette, manuscript submitted for publication).

The major modification to the Clark et al. (1980) spin dry method involves the use of lucite sedimentation cells (University of Pennsylvania Biomedical Instrumentation Shop; design previously described by Herbette et al., 1977) which provide a flat surface onto which the membranes may be pelleted. These cells, routinely used for diffraction sample preparation, are adapted to fit the rotor SW-28 series (Beckman Instruments Inc., Palo Alto, CA). A second set of caps to these rotor buckets (spin dry caps), for use during the spin dry process, have been tapped with a 1-mm hole which is overlaid with a mylar window containing a 100-μm diam hole. The size and condition of each hole is readily assessed and cleaned (with a microsyringe wire, <100-μm diam) on a dissecting microscope. For multibilayer sample formation, 50 μl of the multilamellar vesicle preparation described above was added to the sedimentation cells with either a nonfluorescent Aclar (Dacron; Allied Chemical Corp., Morristown, NJ) or aluminum foil substrate used respectively for either FRAP or diffraction measurements. The vesicles were sedimented onto the substrate at 85,000 *g* for 30 min in an SW-28 rotor. The normal bucket caps were then replaced with the spin dry caps and the pelleted vesicles spin dried at 65,000 *g* for 5 h. On completion of the spin dry process, the samples were mounted and rehydrated over saturated salt solutions for FRAP and diffraction methods as described below.

Salt solutions used for the various humidities were as follows: $Mg(NO_3)_2$, 55%; $NaNO_2$, 66%; $(NH_4)_2SO_4$, 81%; KCl, 88%; $ZnSO_4$, 93%; K_2SO_4, 98%.

Microscopy

Light photomicroscopy was performed on a photomicroscope (Zeiss, Oberkochen, FRG) equipped with Nomarski differential interference contrast optics. For transmission electron microscopy, the multilayer samples were exposed to 2% OsO_4 vapor for 5–10 min at 4°C. On completion of fixation, the samples were maintained in a humidified atmosphere until processed for embedment and sectioning. Thin sections were cut, stained for 15 min in 2% uranyl acetate, and viewed on a Hitachi II electron microscope.

Diffraction

In the diffraction studies, the multilayer samples were mounted on a curved glass support and rehydrated initially over saturated $NaNO_2$ (66% relative humidity) at 4°C. The curved multilayer specimens were exposed at 15°C to a collimated, monochromatic x-ray beam (Cuk_α x-rays, $\lambda = 1.54$ A) from a rotating anode x-ray generator (Rigaku Co. Dencki, Ltd., Tokyo, Japan). The experimental method uses a single Franks' mirror defining a line source where $K_{\alpha 1}$ and $K_{\alpha 2}$ are unresolved. The data were recorded on Kodak DEF-5 film. These studies were performed over the same humidity range used in FRAP measurements as a means of verifying changes in the unit cell repeat as a function of bilayer hydration.

Fluorescence Analyses

FRAP measurements of $DiIC_{16}$ (phospholipid) and Ns-R diffusion coefficients were performed with an excitation wavelength of 5,145 Å on an Ortholux II fluorescence microscope (E. Leitz, Inc., Rockleigh, NJ) equipped with a vertical illuminator, water-cooled argon laser light source (Cooper LaserSonics, Fremont, CA), and a galvanometric scanning mirror. The basic design and geometry of the optical system has been previously described (Koppel, 1979). For these studies, it was determined empirically that 180 nM of phospholipid phosphorus should yield a pellet $\leq$10-μm thick. The pellet thickness is of strong consideration in these photobleaching experiments as it is important that the bleach/monitoring beam be focused at the multibilayer sample plane. Under these conditions, sufficient fluorescence signal is detected from the 180–1.8 pmol of drug (Ns-R) used to obtain the desired range of drug/lipid ratios ($1:10^3$ to 10^5). The multibilayer samples were rehydrated over a well (plexiglass/glass slide assembly) containing a small bead of saturated salt solution defining a relative humidity range from 55 to 98% (see above for details). All FRAP studies were carried out at 21.5°C. Samples were maintained at 4°C during equilibration intervals between analyses. The initially uniform sample fluorescence was locally depleted (or bleached) by short (40 ms) exposure to a laser beam focused by a 10× objective to a small, circularly symmetric spot. The fluorescence redistribution after photobleaching was followed with a series of 12-point scans with an attenuated monitoring beam.

The percent recovery and recovery rate were determined with a three parameter nonlinear least squares analysis of the time decay of the fluorescence depletion monitored coincident with the position of the bleaching pulse. To determine the bleach spot size, w, used to calculate the diffusion coefficients from recovery rates, the immediate post-bleach intensity profile was reconstructed (by empirically extrapolating the fluorescence traces measured at each position back to time zero) and fit to an assumed Gaussian profile (Koppel, 1979).

RESULTS

Characterization of Multibilayer Samples Used in FRAP Experiments

The class composition of the CSL lipid preparation used in these studies is provided in Fig. 2 highlighting the phospholipid class heterogeneity of the cardiac sarcolemma. CSL lipids were chosen for these studies since they represent those associated with the DHP receptor in the native membrane and, as such, more closely represent the receptor environment. The sterol present in this preparation is endogenous and represents ~13% of the total lipid consistent with previously reported values (Colvin et al., 1985). All of the non-bilayer neutral lipid contaminants (triglycerides, cholesterolesters, etc.) have been removed by the TLC purification procedure. In the lipid profile shown in Fig. 2, one unidentified band (?) is consistently present in these lipid preparations. Since the CSL lipids were extracted from a crude preparation of cardiac sarcolemma, contaminating membrane subfractions could be responsible for this additional band. Interestingly, while it appears to be a major component as indicated by TNS fluorescence intensity, no phosphate is associated with this band.

Multibilayer sample integrity for FRAP studies was prescreened light microscopically to assess the gross morphological characteristics of the pellets at the various degrees of bilayer hydration. Characteristic multilayer

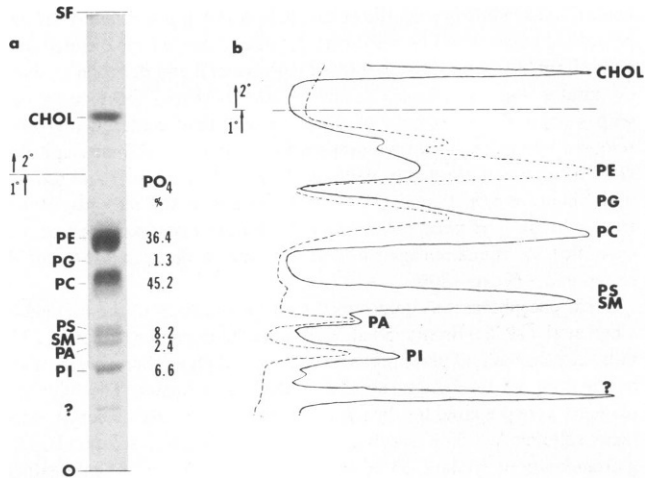

FIGURE 2 Cardiac sarcolemmal lipid class composition. (*a*) The lipids were developed as described in Materials and Methods and charred with 3% cupric acetate, 8% phosphoric acid, and compared against known phospholipid standards. The percent phospholipid phosphate, as determined for each band by phosphate assay off TLC plate, is also indicated. *b* represents fluorescence scans of the phospholipid profile before (----) and after (—) TNS staining to illustrate bands of autofluorescent (----) intensity. For the purpose of comparison, the auto- and TNS fluorescence scans are scaled to approximately equivalent intensity.

samples at several relative humidities appear to be texturally uniform (Fig. 3). This uniformity is maintained for the experimental life of the sample and does not differ significantly for different bilayer hydration states. There is some surface contour to the multilayer (Fig. 3 *C*) consistent with *trans*-axial fluorescence scans of the pellets (data not shown), indicating long-range fluorescence intensity differences. The local sample uniformity was monitored in the normal course of each FRAP experiment (see upper scan stacks in Fig. 5 *A*). Typically, 10 equally spaced scanning positions, covering a scan length of 18 μm, are centered about the bleached spot and flanked on either side by two additional positions separated by ~100 μm. The outermost points provide useful control data sensitive to systematic drifts such as probe bleaching during monitoring.

The extent of multilamellar vesicle fusion into extended multibilayer sheets was observed as shown in Fig. 4. In Fig. 4 *A*, a multilamellar vesicle can be seen with the outer leaflets contiguous with the adjacent membrane stacks while the inner leaflets do not appear to have fused. In these samples, relatively few vesicle ends can be identified, indicating that the in-plane probe diffusion would not be limited by poor vesicle fusion. This is demonstrated by nearly complete fluorescence recovery after the photobleaching event (see Fig. 5). Fig. 4 *B* represents a multilayer that was fixed in a near maximal hydrated state (98% relative humidity). While maintaining the multilamellar structure, the sample appears to be more swollen than samples fixed at 81% relative humidity (Fig. 4 *A*) in agreement with the x-ray diffraction data.

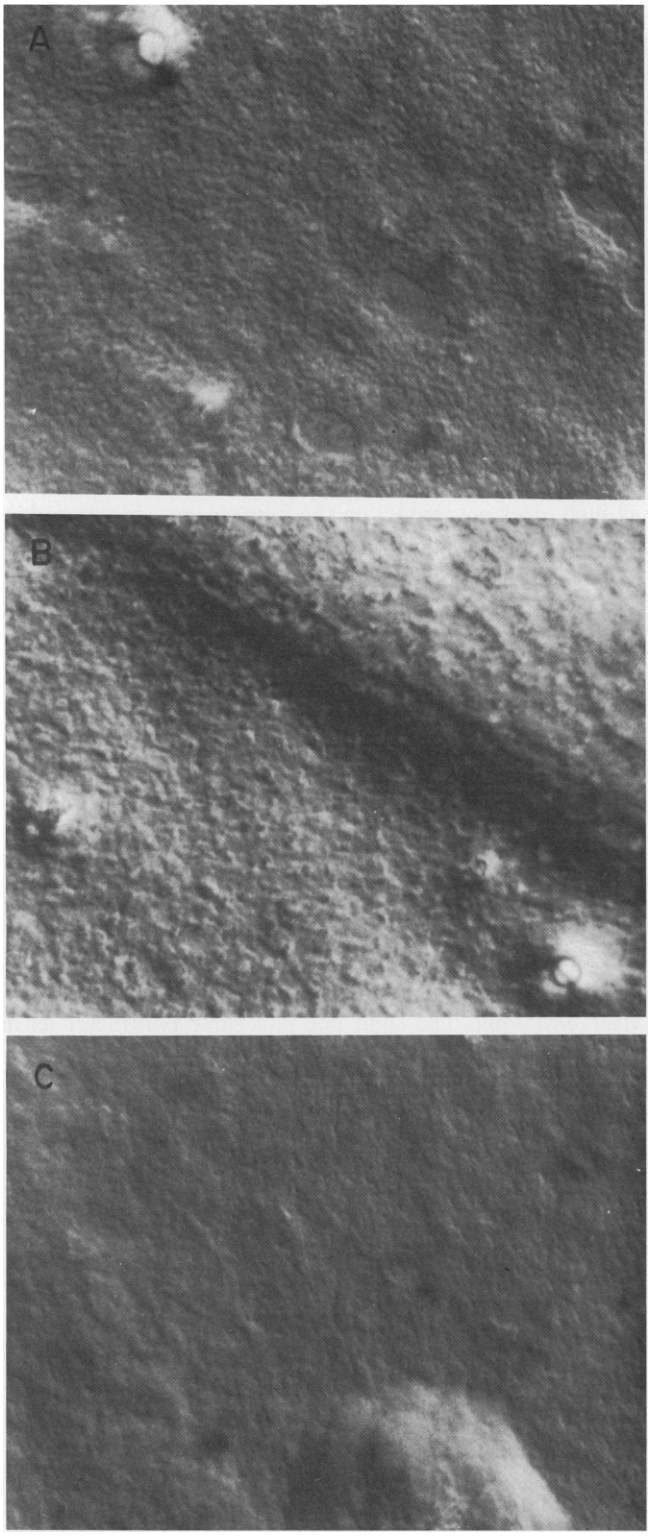

FIGURE 3 Nomarski photomicrographs of multilayers hydrated to (*A*) 66%, (*B*) 81%, and (*C*) 88% relative humidity to illustrate the relative uniformity of the multibilayer pellets. 364×.

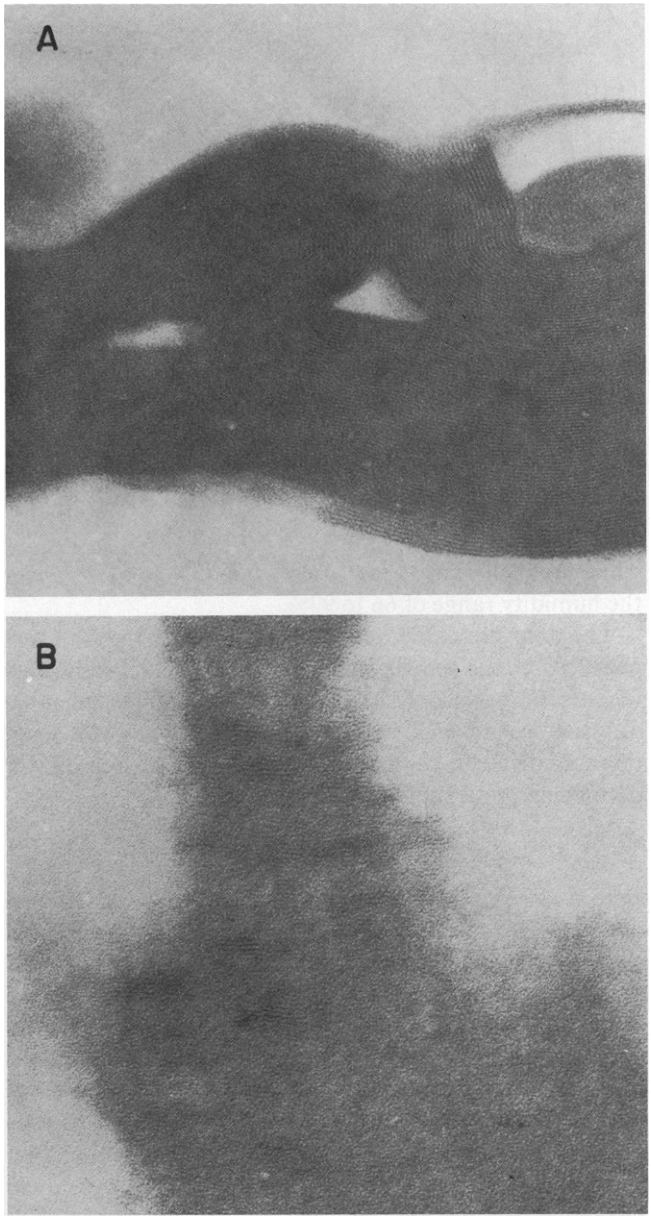

FIGURE 4 Transmission electron micrographs of thin sections cut perpendicular to the multibilayer plane. Samples were fixed with 2% OsO_4 vapor in an atmosphere of (*A*) 81% and (*B*) 98% relative humidity. 312,000×.

X-ray diffraction studies were performed in parallel with the FRAP experiments to verify the changes in multilayer unit cell repeat as a function of relative bilayer hydration. These studies demonstrated that the multilayers were well ordered, as indicated by low mosaic spread, and swelled (increase in the average multilayer unit cell repeat) as the bilayers were hydrated. These findings are consistent with both light and electron microscope observations, demonstrating the reasonable uniformity of these samples. Low resolution profile structures generated from these diffraction data demonstrated that the phosphate-to-phosphate distance across the bilayer remains constant (39 Å)

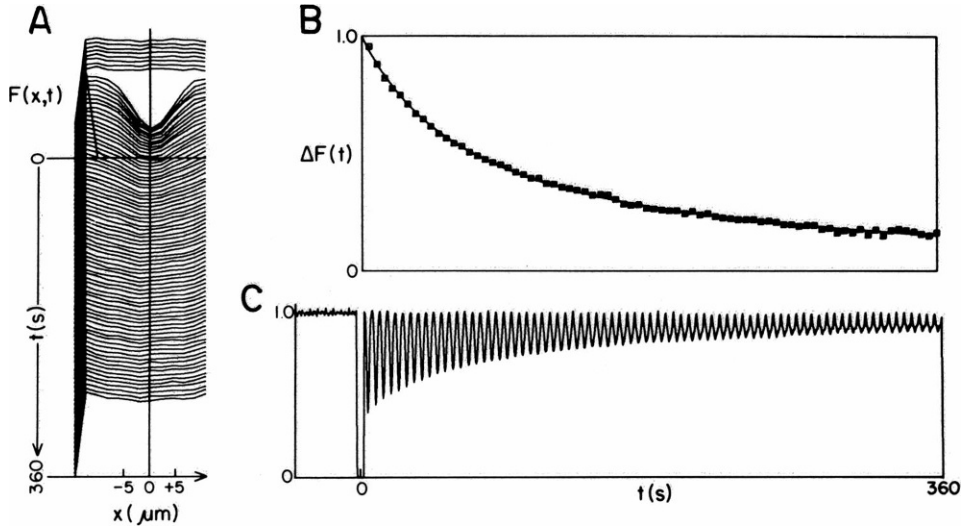

FIGURE 5 Typical FRAP data obtained for $DiIC_{16}$ at 55% relative humidity. (*A*) Stack of 84 individual twelve point scans through the bleached area, highlighting the flat pre-bleach intensity and return to this profile after a 40-ms bleach pulse (dip in scan profile). (*B*) Linear sequence of all the data during the series of fluorescence scans. (*C*) Fluorescence depletion coincident with the bleach point. Solid line is a three-parameter least squares fit of the data presented in *B*.

while unit cell repeats increased from 53.4 to 61.5 Å over the humidity range of 66 to 93%.

The data presented above suggest that the multilayers produced by the spin dry technique are very well suited for the determination of fluorescent drug and phospholipid diffusion coefficients by the FRAP method. Moreover, these studies can be performed at very low, non-bilayer perturbing probe/lipid ratios.

Fluorescence

Before FRAP analysis of drug (probe) diffusional dynamics, the multibilayers were examined for sample and substrate (aclar) autofluorescence in the absence of added fluorophore. The fluorescence intensity associated with the aclar was very low in comparison with other substrates examined (plastic or mylar). This autofluorescence was bleached with the laser bleach pulse but failed to recover during the time frame used in these studies. The sample autofluorescence intensity was somewhat greater than that associated with the aclar substrate and did recover upon bleaching. A diffusion coefficient of 1.37×10^{-8} cm^2/s, at 81% relative humidity, was calculated which is similar to phospholipid values obtained with $DiIC_{16}$.

The origin of the sample autofluorescence was examined and determined to be associated with the isolated phospholipids. Fig. 2 *b* represents fluorescence scans of TLC-separated phospholipids before and after TNS staining. The association of this fluorescence with the phospholipid was maintained in two-dimensional TLC, using two different solvent system schemes. Moreover, when examined at high concentration in chloroform, these lipids elicited broad excitation and emission spectra with maxima at ~4,000 and 5,250 Å, respectively. The fluorescence intensity at 5,500 Å, the cut-off of the dichroic mirror and barrier filters, is ~44% that observed at the emissions maximum. In any experiment, this autofluorescence intensity contributed <5% of the total intensity obtained from either Ns-R or the $DiIC_{16}$ at $1:10^4$ probe/lipid ratios and, as such, does not impact on the fluorescence signal used in the determination of diffusion coefficients.

The FRAP data obtained for Ns-R and $DiIC_{16}$ at probe-to-lipid ratios of $1:10^4$ are presented in Figs. 5–7. To obtain an accurate assessment of both the diffusion coefficient, *D*, and percent recovery, the overall time of data collection was varied such that half maximal recovery was observed after 10–20 of the total of 75 post-bleach scans. Fig. 5 is a display of one set of data obtained from the $DiIC_{16}$ probe at 55% relative humidity. Fig. 5 *B* is a linear sequence of all the data during the series of fluorescence scans with a focused laser beam. In Fig. 5 *A*, the same data are presented as a stack of 84 individual 12 point scans recorded at 4.8-s intervals before and after bleaching. Fig. 5 *C* shows the fluorescence depletion coincident with the position of the bleaching pulse as a function of the time after bleaching. The solid line is a three-parameter least squares fit to the data corresponding to an extrapolated fluorescence recovery (i.e., mobile fraction) of 98%.

Distinct changes in the diffusion coefficients for both the drug and $DiIC_{16}$ were observed as a result of alterations in relative bilayer hydration (Figs. 6 and 7). Fig. 6 illustrates the least squares fit of the recovery data for Ns-R and $DiIC_{16}$ at 55 (Fig. 6 *A*) and 98% (Fig. 6 *B*) relative humidity. Note the time scales and the fact that the diffusion rates increase 50- and 200-fold for $DiIC_{16}$ and Ns-R, respectively, as bilayer hydration was increased from 55 to 98%. Moreover, there was a fourfold difference in diffusion coefficient between Ns-R and $DiIC_{16}$ at 55% while no difference was apparent at 98% relative humidity. The relationship of drug and lipid diffusion coefficients as a function of relative bilayer hydration is shown in Fig. 7. From the FRAP data obtained in this study, the Ns-R DHP drug analogue, which is active as a calcium channel antagonist (Table I), has a diffusion coefficient of 3.8×10^{-8} cm^2/s at maximal bilayer hydration. This value is identical with that obtained for phospholipids under identical conditions. Moreover, diffusion coefficients were simi-

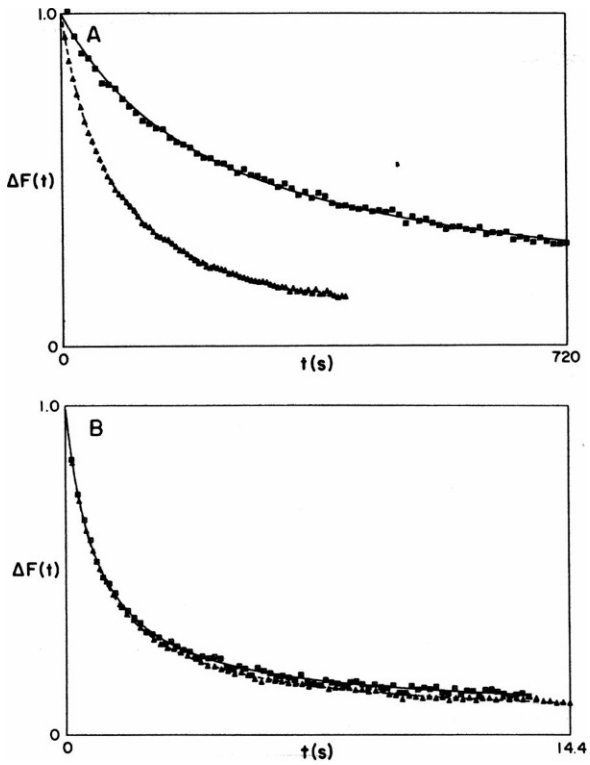

FIGURE 6 Decay of fluorescence depletion coincident with the bleach point for Ns-R (□) and $DiIC_{16}$ (△) at (*A*) 55% and (*B*) 98% relative humidity. The solid and dashed lines represent three-parameter least squares fits of the data.

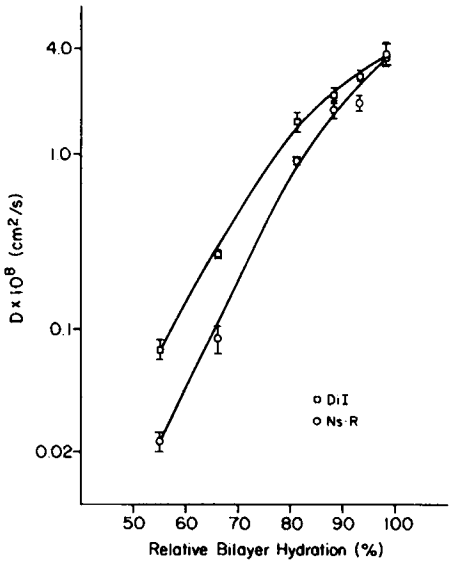

FIGURE 7 Diffusion coefficient, *D*, as a function of relative bilayer hydration. These average values and error bars (standard deviation) were determined from a minimum of five sets of data. The relative bilayer hydration of the samples was maintained by equilibration over saturated salt solutions which define a specific water vapor pressure (range 55–98%).

TABLE I
PERCENT INHIBITION OF SPECIFIC [^{3}H]NITRENDIPINE (5×10^{-11} M) BINDING IN GUINEA PIG ILLIUM SMOOTH MUSCLE MEMBRANES BY THE NISOLDIPINE-RHODAMINE ANALOGUES AS A FUNCTION OF METHYLENE SEGMENT LENGTH

Conc.	Percent inhibition		
	I	II	III
M			
10^{-10}	—	0.00 (5)	—
10^{-9}	1.6 ± 0.6 (4)*	—	24.8 ± 11.7 (3)
10^{-8}	18.2 ± 7.3 (4)	27.5 ± 17.6 (5)	61.9 ± 16.9 (4)
10^{-7}	54.8 ± 8.6 (4)	71.0 ± 10.4 (5)	90.0 ± 3.6 (4)
10^{-6}	92.0 ± (1)	—	87.4 ± 4.5 (4)

Probe I, $n = 2$; probe II, $n = 6$; probe III, $n = 11$.
*Refers to the number of experimental values collected.

lar for Ns-R concentrations ranging from 1:10^3 to 1:10^4, indicating that there are no concentration effects at these probe-to-lipid ratios on drug diffusional dynamics in these membranes. Despite the similarities in probe diffusion at complete bilayer hydration, it is interesting to note the substantial differences in diffusional dynamics as bilayer hydration is decreased. As discussed below, this may reflect some differences in the relative locations of these two probes within the bilayer.

DISCUSSION

Depending upon the pathway, specific binding of DHP to receptors in sarcolemmal membranes, in general, will be governed by such parameters as the rate of ligand diffusion, drug orientation/conformation required for active site binding, ligand concentration at the binding site, binding constraints imposed by receptor state, etc. Additionally, the rate of bilayer partition and subsequent effects on drug orientation/conformation must be considered when dealing with ligands that bind at receptor sites within the membrane. Rhodes and co-workers (1985) and Herbette et al. (1985) have proposed the membrane bilayer pathway for drug–receptor interaction (see Fig. 1), which provides an appropriate mechanism for optimizing drug orientation/conformation, thereby maximizing the overall rate for the receptor binding process. The theoretical diffusion-limited rate for this pathway was calculated to be some three orders of magnitude faster than that attainable directly via an aqueous route. A two-step binding mechanism is supported by the time-averaged location of DHP at the hydrocarbon core/water interface.[1,2] Moreover, the theoretical rate analyses presented by Rhodes et al. (1985) suggest that despite strong ligand anisotropy, the receptor–ligand interaction is not diffusion limited. As such, discrimination between the two pathways, aqueous and membrane, requires a thorough investigation of the microscopic rate constants associated with each step in the receptor binding process. Receptor binding kinetic analyses will also

provide some important information relative to the rate-limiting step in the overall mechanism.

The structure/activity relationships cited by Bolger et al. (1983) and Janis and Scriabine (1983) are indicative of the conformational and orientational requirements for receptor binding. This is further demonstrated with enantiomeric DHP pairs (e.g., 202,791) where the isomers possess either agonist or antagonistic activity with the agonist activity strongly dependent upon membrane potential (Kongsamut et al., 1985; Hof et al., 1985). This delicate balance of structure and activity is also demonstrated by an interesting feature of the fluorescent DHP analogue used in this study where the methylene segment length between the rhodamine and DHP strongly affects the ability of this ligand to displace nitrendipine from the DHP receptor (see Table I). It would appear that the observed activity differences are not related to minor differences in diffusion coefficient since, as concluded by Rhodes et al. (1985), receptor binding does not appear to be diffusion limited. Moreover, preliminary diffusion data with probe *I* ($n = 2$) yield a similar diffusion coefficient to that of Ns-R (probe III) at low bilayer hydration (66%), suggesting that the activity differences are related to alterations in ligand orientation or depth of penetration into the membrane.

Structure studies reveal the location of the DHP at the hydrocarbon core/water interface.[1,2] In the context of bilayer location, this region is the most constrained due to phospholipid packing and related surface tension. This location, on a time-averaged basis, would potentially define a concentration gradient centered about this interface within which DHP could diffuse. It is not unreasonable to anticipate that this locale could also correspond with that portion of the receptor protein containing the binding domain. The hydrocarbon core/water interface provides an interesting physical chemical environment for ligands such as DHP. As stated earlier, the properties of this interface would impose, for the sake of minimum energy, conformational and orientational constraints on the ligand as it diffuses along the interface to interact with the receptor. A close correlation between the time-averaged DHP location and receptor binding domain within the bilayer could begin to suggest a mechanism for voltage potential-dependent activity of these ligands involving configurational shifts in the binding domain affecting the interaction with ligand in a specific equilibrium minimum energy conformation.

In this study, we have begun to examine the diffusion limits for DHP in pure cardiac sarcolemmal lipid bilayers as an initial approach to understanding the diffusional dynamics of these drugs in native cardiac membranes. We have determined that the diffusion coefficient of an active DHP analogue (see Table I), Ns-R, is 3.8×10^{-8} cm^2/s, the same as that obtained for phospholipids under identical conditions. The diffusion coefficients observed here are similar to those obtained for NBD-labeled phospholipid by McCowen et al. (1981) and those determined by an independent method using the rate of pyrene eximer formation (Eisinger and Halperin, 1986). Rapid bilayer partition rates, indicated by the rate of nonspecific binding,[1] and rapid diffusion within the bilayer are processes that are significantly faster than the overall binding to the receptor, suggesting that these events "set up" the appropriate equilibrium conditions for drug binding to the receptor.

Reducing bilayer hydration has profound effects on the diffusion of both Ns-R and $DiIC_{16}$, as illustrated in Figs. 6 and 7. The changes in diffusion coefficient that we have observed are qualitatively similar to that reported by McCowen et al. (1981) in a different lipid system, though considerably greater in magnitude over the same range of relative humidity. The differential decrease of Ns-R diffusion relative to $DiIC_{16}$ diffusion is of considerable interest to us and, as such, we are continuing to explore this phenomenon with other membrane probes. This differential effect is likely to be a reflection of different bilayer positions for these two probes, as illustrated in Fig. 8. The charged rhodamine moiety of the DHP analogue would be expected to extend further from the bilayer hydrophobic domain, interacting more strongly with the phospholipid headgroup region. This rhodamine position is supported by studies conducted by Loew and co-workers (1986), demonstrating that certain charged rhodamines (e.g., rhodamine 123) do not penetrate membrane bilayers and hence, remain outside the bilayer hydrophobic domain. This contention is also supported by some recent data (to be published) demonstrating that free rhodamine diffusion between bilayers under identical conditions is by far less constrained relative to the DHP–chromophore complex or phospholipids. It is anticipated that $DiIC_{16}$, on the other hand, would be embedded deeper into the bilayer. The general decrease of diffusivity with decreased hydration may reflect either surface condensation, interbilayer headgroup interactions, or some combination of the two, as discussed by McCowen et al. (1981). Differential diffusion changes can also be explained in the context of the free volume model of diffusion (Vaz et al., 1985). Surface condensation, decreasing the availability of free volume spaces for diffusional jumps, would preferentially slow down those probe molecules with the larger cross-sectional dimension at some critical plane within the bilayer. From the vantage point of the data presented herein, the relative locations, bilayer interactions at that locale, chromophore squatness, etc. may also play a role in defining the diffusional dynamics of small molecules in membranes.

SUMMARY

We have recently determined that two DHP analogues, nimodipine and Bay P 8857, are located near to the hydrocarbon core/water interface (Herbette et al., 1985).[2] This location is similar to that obtained for the amphipha-

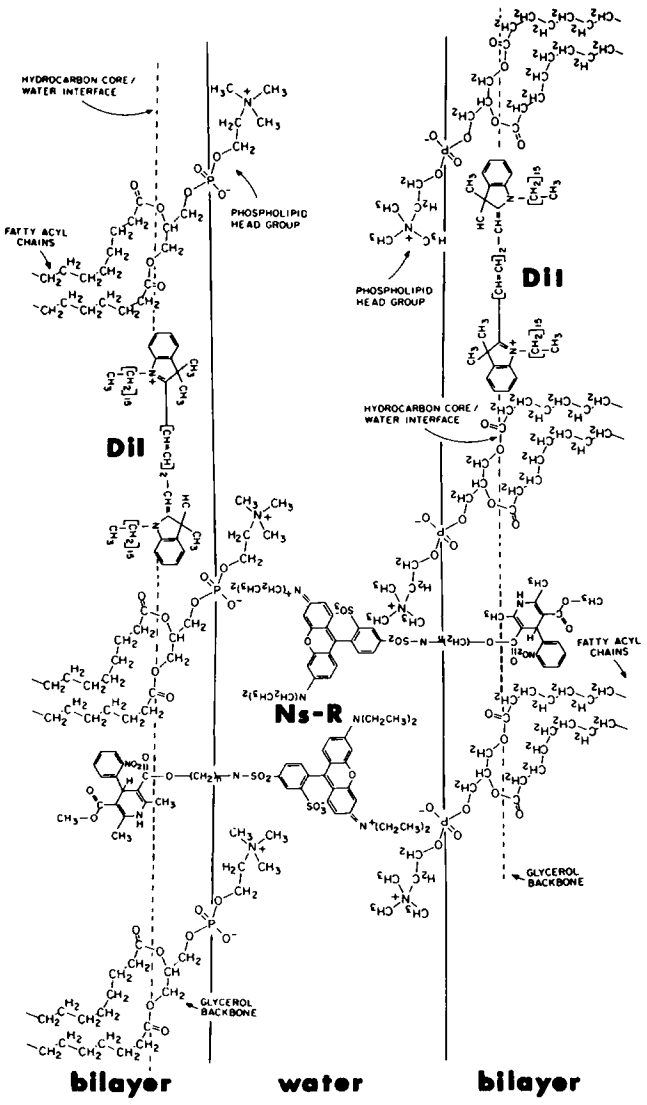

FIGURE 8 Illustration of the anticipated locations of Ns-R and $DiIC_{16}$ in cardiac sarcolemmal lipid bilayers. This figure shows the rhodamine moiety of the Ns-R complex associated with the phospholipid headgroup and aqueous milieu and the $DiIC_{16}$ probe buried deeper in the membrane near the hydrocarbon core/water interface.

thic beta-adrenergic receptor antagonist, propranolol (Herbette et al., 1985). The observation that these drugs partition to a specific region of the membrane bilayer (Herbette et al., 1985),[2] have specific orientation/conformation requirements, and diffuse rapidly in the plane of the bilayer ($3.8 \times 10^{-8} cm^2/s$) suggest that the physical characteristics of this interface provide a microenvironment that facilitates receptor–ligand interaction and binding. Moreover, it is anticipated that the receptor binding domain may also be located at the level of this interface. Since receptor state seems to play an important role in the receptor binding mechanism (Bean, 1984; Lee and Tsien, 1983), subtle changes in receptor site conformation, mediated by receptor state and/or membrane potential, could have substantial ramifications on the highly stereospecific binding and activity of these ligands, once they have diffused to and reside at the protein receptor site.

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REFERENCES

Affolter, H., and R. Coronado. 1985. Agonists of Bay K 8644 and CGP 28392 open channels from skeletal muscle transverse tubules. *Biophys. J.* 48:341–347.

Affolter, H., and R. Coronado. 1986. The sidedness of reconstituted calcium channels from muscle transverse tubules as determined by D-600 and D-890 blockade. *Biophys. J.* 49(2, Pt. 2):197a. (Abstr.)

Bangham, A. D., M. M. Standish, and J. C. Watkins. 1965. Diffusion of univalent ions across the lamellae of swollen phospholipids. *J. Mol. Biol.* 13:238.

Bean, B. P. 1984. Nitrendipine block of cardiac calcium channels: high affinity binding to the inactivated state. *Proc. Natl. Acad. Sci. USA.* 81:6388–6392.

Belleman, P., A. Schade, and R. Towart. 1983. Dihydropyridine receptor in rate brain labeled with [^{3}H]-nimodipine. *Proc. Natl. Acad. Sci. USA.* 80:2356–2360.

Bolger, G. T., P. Gengo, R. Klockowski, E. Luchowski, H. Siegel, R. A. Janis, A. M. Triggle, and D. J. Triggle. 1983. *J. Pharm. Exp. Ther.* 225:291–309.

Chen, P. S., Jr., T. Y. Toribara, and H. Warner. 1956. Microdetermination of phosphorus. *Anal. Chem.* 28:1756–1758.

Clark, N. A., K. J. Rothschild, D. A. Luipold, and B. 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.

Colvin, R. A., T. F. Ashavaid, and L. G. Herbette. 1985. Structure-function studies of canine cardiac sarcolemmal membranes. I. Estimation of receptor site densities. *Biochim. Biophys. Acta.* 812:601–608.

Eisinger, J., and B. I. Halperin. 1986. Effects of spatial variation in membrane diffusibility and solubility on the lateral transport of membrane components. *Biophys. J.* 50:513–521.

Folch, J., M. Lees, and G. A. Sloane-Stanley. 1957. A simple method for the isolation and purification of total lipids from animal tissues. *J. Biol. Chem.* 226:447–509.

Glossman, H., and D. R. Ferry. 1983. New Calcium Antagonists. Recent Developments and Prospects. Gustav Fischer Verlag, Stuttgart. 63–98.

Herbette, L. G., J. Marquardt, A. Scarpa, and J. K. Blasie. 1977. A direct analysis of lamellar x-ray diffraction from hydrated oriented multibilayers of fully functional sarcoplasmic reticulum. *Biophys. J.* 20:245–272.

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

Herbette, L. G., D. G. Rhodes, D. W. Chester, R. A. Colvin, J. G. Sarmiento, Y. Vant Erve, and A. M. Katz. 1985. Possible mechanism for cardiovascular drug-membrane interaction. *In* Pathobiology of

Cardiovascular Injury. Martinus Nijhoff Publishing Co., The Hague. 245–257.

Herbette, L. G. 1986. Techniques for determining membrane structure: the necessity for understanding cardiovascular drug-membrane interactions at a molecular level. *In* The Heart and Cardiovascular System. H. A. Fozzard et al., editors. Raven Press, New York. 263–287.

Hess, P., J. P. Lansman, and R. W. Tsien. 1984. Different modes of Ca^{2+} channel gating behavior favored by dihydropyridine Ca^{2+} agonists and antagonists. *Nature (Lond.)*. 311:538.

Hof, R. P., U. T. Ruegg, A. Hof, and A. Vogel. 1985. Stereoselectivity at the calcium channel: opposite action of the enantiomers of a 1,4-dihydropyridine. *J. Cardiovasc. Pharmacol.* 7:689–693.

Janis, R. A., and A. Scriabine. 1983. Sites of action of Ca^{+2} channel inhibitors. *Biochem. Pharmacol.* 32:3499–3507.

Janis, R. A., and D. J. Triggle. 1984. 1,4-Dihydropyridine Ca^{+2} channel antagonists and activators: a comparison of binding characteristics with pharmacology. *Drug Dev. Res.* 4:257–274.

Jones, L. R., S. W. Maddock, and H. R. Besch. 1980. Unmasking effects of alamethacin on the (Na/K+)-ATPase, B-adrenergic receptor-coupled adenylate cyclase and cAMP-dependent protein kinase activities in cardiac sarcolemmal vesicles. *J. Biol. Chem.* 255:9971–9980.

Kongsamut, S., T. J. Kamp, R. J. Miller, and M. C. Sanguinetti. 1985. Calcium channel agonist and antagonist effects of the stereoisomers of the dihydropyridine 202–791. *Biochem. Biophys. Res. Commun.* 130:141–148.

Koppel, D. E. 1979. Fluorescence redistribution after photobleaching. A new multipoint analysis of membrane translational dynamics. *Biophys. J.* 28:281–291.

Koppel, D. E., and M. P. Sheetz. 1983. A localized pattern photobleaching method for the concurrent analysis of rapid and slow diffusion processes. *Biophys. J.* 43:175–181.

Langs, D. A., and D. J. Triggle. 1985. Conformational features of calcium channel agonist and antagonist analogs of nifedipine. *Mol. Pharmacol.* 27:544–548.

Lee, K. S., and R. W. Tsien. 1983. Mechanism of calcium channel blockade by verapamil, D-600, diltiazem and nitrendipine in single dialysed heart cells. *Nature (Lond.)*. 302:790–794.

Loew, L. M., B. Ehrenberg, E. Fluhler, M.-D. Wei, and V. Burnham. 1986. A search for nerstian dyes to measure membrane potential in individual cells. *Biophys. J.* 49(2, Pt. 2):308a. (Abstr.)

McCowen, J. T., E. Evans, S. Diehl, and H. C. Wiles. 1981. Degree of hydration and lateral diffusion in phospholipid multibilayers. *Biochemistry*. 20:3134–3138.

McCloskey, M., and M.-M. Poo. 1986. Rates of membrane associated reactions: reduction in dimensionality revisited. *J. Cell Biol.* 102:88–96.

Rhodes, D. G., J. G. Sarmiento, and L. G. Herbette. 1985. Kinetics of binding of membrane-active drugs to receptor sites. Diffusion limited rates for a membrane bilayer approach of 1,4-dihydropyridine calcium channel antagonists to their active site. *Mol. Pharmacol.* 27:612–623.

Sarmiento, J. G., R. A. Janis, A. M. Katz, and D. J. Triggle. 1984. Comparison of high affinity binding of calcium channel blocking drugs to vascular smooth muscle and cardiac sarcolemmal membranes. *Biochem. Pharmacol.* 33:3119–3123.

Vaz, W. L. C., R. M. Clegg, and D. Hallman. 1985. Translational diffusion of lipids in liquid crystalline phase phosphatidylcholine multibilayers. A comparison of experiment with theory. *Biochemistry*. 24:781–786.