Partitioning and location of Bay K 8644, 1,4-dihydropyridine calcium channel agonist, in model and biological membranes

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ABSTRACT Several lines of evidence suggest that nonspecific drug interaction with the lipid bilayer plays an important role in subsequent recognition and binding to specific receptor sites in the membrane. The interaction of Bay K 8644, a 1,4-dihydropyridine (DHP) calcium channel agonist, with model and biological membranes was examined at the molecular level using small angle x-ray diffraction. Nonspecific drug partitioning into the membrane was examined by radiochemical assay. Nonspecific binding characteristics of [^{3}H] Bay K 8644 were determined in both dipalmitoyl phosphatidylcholine (DPPC) vesicles above and below their thermal phase transition (T_m) and rabbit skeletal muscle light sarcoplasmic reticulum (LSR). In DPPC, the partition coefficient, K_p, was 14,000 above the T_m (55°C) versus 160 in the gel phase (2°C). The K_p determined in LSR membranes was 10,700. These values for both DPPC and LSR membranes can be compared with K_p = 290 in the traditional octanol/buffer system.

Using small-angle x-ray diffraction, the equilibrium position of the electron-dense trifluoromethyl group of Bay K 8644 in DPPC (above T_m) and purified cardiac sarcolemmal (CSL) lipid bilayers was determined to be consistently located within the region of the first few methylene segments of the fatty acyl chains of these membranes. This position is similar to that observed for the DHP calcium channel antagonists nimodipine and Bay P 8857. We suggest this particular membrane location defines a region of local drug concentration and plane for lateral diffusion to a common receptor site. Below the DPPC membrane T_m, Bay K 8644 was shown to be excluded from this energetically favored position into the interbilayer water space. Heating the DPPC bilayer above the T_m (55°C) showed that this exclusion was reversible and indicates that drug-membrane interaction is dependent on the bilayer physical state. The absence of any specific protein binding sites in these systems allows us to ascertain the potentially important role that the bulk lipid phase may play in the molecular mechanism of DHP binding to the specific receptor site associated with the calcium channel.

INTRODUCTION

1,4-DHP calcium channel antagonists and agonists play an important role in the excitation-contraction mechanism of cardiac and smooth muscle by modulating the transmembrane influx of extracellular calcium (for review see Janis et al., 1987). This class of calcium channel blockers have been shown to bind with both specificity and high affinity to protein receptors in a variety of cell types. Bay K 8644, a potent DHP calcium channel agonist, has a positive inotropic effect on smooth and cardiac muscle contraction (Schramm et al., 1983). It binds to receptors in rabbit ventricular membranes with an apparent equilibrium dissociation constant (K_d) of 2.4 nM (Janis et al., 1984). Competitive binding studies demonstrate that Bay K 8644 is displaced from its high-affinity binding site by the calcium channel antagonist nitrendipine with a K_i of 0.1 nM and an apparent Hill slope of 0.98 (Janis et al., 1984), suggesting a common high-affinity binding site for the calcium channel agonist and antagonist (see also Hamilton et al., 1987). Other specific binding studies, however, indicate separate binding sites for the agonist and antagonist (Kokubun et al., 1986).

A mechanism for DHP binding to their specific protein receptor has been described by Rhodes et al. (1985). This pathway involves the DHP partitioning into the membrane bilayer where it assumes an energy favorable position and orientation before rapidly diffusing in a plane defined by that position to a specific receptor-binding site. Calculated, diffusion-limited rates for the membrane pathway are approximately three orders of magnitude greater than a direct aqueous approach of binding for the DHP. High partition coefficients for several DHPs with K_p ranging from 5,000 to 150,000 (Herbette et al., 1986) and low DHP receptor density in the cardiac sarcolemma of one receptor per square micron (Colvin et al., 1985) makes DHP nonspecific interaction with the membrane highly probable. Further, rapid lateral diffusion for an active DHP analogue (Chester et al., 1987), specific binding studies (Affolter and Coronado, 1985; Valdivia and Coronado, 1988) and patch-clamp investigations (Kokubun and Reuter, 1985) have provided experimental support for the membrane bilayer pathway for DHP binding to its receptor.

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Genetic studies have implicated a specific binding site in the transmembrane portion of the beta-adrenergic receptor (Kobilka et al., 1987*b*, 1988). Previous neutron diffraction studies have observed the location of the beta adrenergic antagonist propranolol (Herbette et al., 1983) to be near the hydrocarbon core/water interface of lipid bilayer membranes. This position may in turn define a region of localized drug concentration in equilibrium with the high-affinity receptor site buried in the membrane.

The primary structure of the DHP receptor from rabbit skeletal muscle has been deduced from its DNA sequences (Tanabe et al., 1987). The polypeptide is structurally very similar to the voltage-dependent sodium channel with four units of high homology that putatively comprise six transmembrane alpha helices and may serve as the channel for calcium (Tanabe et al., 1987). Similar to that suggested for the beta-adrenergic receptor (Kobilka et al., 1988), the transmembrane helices of the calcium channel may be critical for the binding of highly lipophilic DHPs.

In the present study we asked whether the calcium channel agonist Bay K 8644 occupies the same region in the CSL lipid bilayer as the antagonist Bay P 8857. Structural analysis of the interaction of Bay K 8644 with purified CSL and DPPC multilamellar vesicles, combined with the kinetics of nonspecific binding of the drug to rabbit LSR and DPPC vesicles have been used to examine the so called "nonspecific" membrane interactions of the drug. Bay K 8644 contains an ortho trifluoromethyl substituent to the phenyl ring, whereas Bay P 8857 has an iodoethylester group extending from the 5′ carboxylate substituent of the pyridine ring. The high electron density of these halogen substituents, in contrast to the lipid bilayer, allows the time-averaged position of the ligand in the membrane bilayer to be determined by small angle x-ray diffraction. The effect of the lipid phase on drug partitioning and membrane location was examined with the DPPC bilayers above and below its thermal phase transition (T_m). The equilibrium partition coefficient of [^{3}H] Bay K 8644 in LSR against buffer was also investigated. These results suggest that the nonspecific interactions of Bay K 8644 with the membrane bilayer may result in a well-defined location, orientation, and conformation necessary for intrabilayer receptor binding.

METHODS AND MATERIALS

Chemicals

Bay K 8644 and Bay P 8857 were gifts from Miles Laboratories Inc., New Haven, CT. [^{3}H]Bay K 8644 (66.9 Ci/mmol) was obtained from New England Nuclear Corp., Boston, MA. The labeled drug was stored in the dark at −12°C. Unlabeled drug was stored at 4°C, in the dark. Both drugs were dissolved in 100% ethanol due to their limited aqueous solubility. Dipalmitoylphosphatidylcholine was purchased from Avanti Biochemicals, Birmingham, AL, and stored in powder form at 4°C. Bay P 8857 was used after recrystallization, and drug and lipid purity was examined by thin-layer chromatography (TLC) before and after diffraction experiments to verify structural integrity. All other chemicals were reagent grade and any solutions were made using glass distilled deionized water.

Skeletal sarcoplasmic reticulum and cardiac sarcolemmal membrane isolation and lipid preparation

Purified LSR membranes were isolated from rabbit skeletal muscle as previously described (Watras et al., 1984). 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). CSL lipid extraction and analytical procedures have been described in full detail in a previous communication (Chester et al., 1987). Lipid phosphorus was determined by a modification of the method of Chen et al. (1956) as described previously (Chester et al., 1987). Polar and neutral lipid composition was analyzed by TLC on 250 μm silica gel 60 TLC plates (EM Reagents, E. 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. The various lipid classes were visualized under longwave UV light after staining with 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. Lipid class composition was assessed by scanning TNS fluorescence with a model SL-2DUV soft laser scanning densitometer (Zeineh Biomedical Instruments, Inc., Fullerton, CA) or scraping of bands for phosphate assay.

The phospholipid composition of the CSL membranes was primarily phosphatidylcholine (45%) and phosphatidylethanolamine (36%) with lesser amounts of phosphatidylserine and sphingomyelin (8%), phosphatidylinositol (7%), and phosphatidylglycerol (1%). Cholesterol accounted for ~13 mol% of the phospholipid content.

Multilamellar vesicle preparation

CSL multilamellar lipid vesicles were prepared in the presence or absence of known amounts of Bay K 8644 and Bay P 8857 essentially by the method of Bangham (1965). A specified volume of 0.5 mM Hepes, pH 7.27, 2 mM NaCl buffer containing the drug was added to the dried lipid preparation yielding a final drug-to-lipid ratio of 1:35 or 1:40. DPPC multilamellar vesicles were prepared as follows. The desired amount of lipid dissolved in $CHCl_3$:MeOH (19:1) was placed in a glass test tube and dried down to a thin film under N_2. Buffer (0.5 mM Hepes, 2.0 mM NaCl, pH 7.27), in the presence and absence of known Bay K 8644 and Bay P 8857 concentrations, was added to provide a final lipid concentration of 5 mg/ml. This solution was then sonicated above its T_m (48°C) to form a cloudy white suspension of multilamellar vesicles as assayed by electron microscopy. The membrane samples were stored at 4°C until use.

Preparation of multibilayer samples for x-ray diffraction

Multilayer samples were prepared as described in detail (Chester et al., 1987). Briefly, 50 μl of the multilamellar vesicle preparation described above was added to lucite sedimentation cells containing an aluminum foil substrate. 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", which have a single 100-μm-diameter hole in the top (Chester et al., 1987), and the pelleted vesicles spin dried at 65,000 g for 4 h. On completion of the spin dry process, the samples were mounted on curved glass supports and rehydrated in sealed brass canisters containing a saturated salt solution to define a specific relative humidity.

The salts used for controlling humidity were LiCl, 13%; MgCl, 32%; $NaNO_2$, 66%; $(NH_4)_2SO_4$, 81%; and $ZnSO_4$, 93%.

Small angle x-ray diffraction with multibilayer samples

Small-angle x-ray diffraction studies were carried out by aligning the samples at near grazing incidence with respect to the x-ray beam. The radiation source was a collimated, monochromatic x-ray beam (CuK_α x-ray, λ = 1.54 Å) from an Elliott GX-18 rotating anode x-ray generator (Marconi Avionics, Ltd., Borehamwood, UK). The experimental method utilized a single Franks' mirror defining a line source where $K_{\alpha 1}$ and $K_{\alpha 2}$ are unresolved. Sample temperature was controlled for the diffraction experiments. Upon completion of data collection, lipid and drug integrity were assessed by TLC.

Diffraction data collection and reduction

The data were recorded on both Kodak DEF-5 film (Eastman Kodak Co.) and a Braun position-sensitive 1-D detector (Innovative Technologies, Inc., South Hamilton, MA). Relative intensities for the diffraction orders were obtained either by scanning x-ray films with a Zeineh model SL-2DUV soft laser scanning densitometer (Biomed Instruments Inc., Fullerton, CA) or directly from digitized computer plots of the detector data using an integration routine. After repeated cycles of data reduction, the systematic error was determined to be <5% of the integrated intensities for each of the diffraction orders I (h = 1–6 for CSL and h = 1–7 for DPPC). To examine the effect of the systematic error, we recalculated electron density profiles for all the data with random 5% changes (increase or decrease) in all the diffraction orders. Whereas this deliberate 5% random error in the values of the integrated intensities modulated the electron density profiles, the differences observed between the sample with and without drug were preserved and did not affect the interpretation of the data. Thus, based on the signal to noise of our data, experimental uncertainty in the data and the potential for introducing errors based on how we integrate the various lamellar reflections, the changes that we have observed appear to be meaningful and are self-consistent when comparing different membrane lipid samples.

Data reduction (background and other geometrical corrections) for either method of data collection has been described previously (Herbette et al., 1985*a*). The lamellar intensity functions from the cardiac sarcolemmal samples collected with the electronic detector were corrected by a factor of $s^2 = (2 \sin \theta/\lambda)^2$. The Lorentz correction factor (one

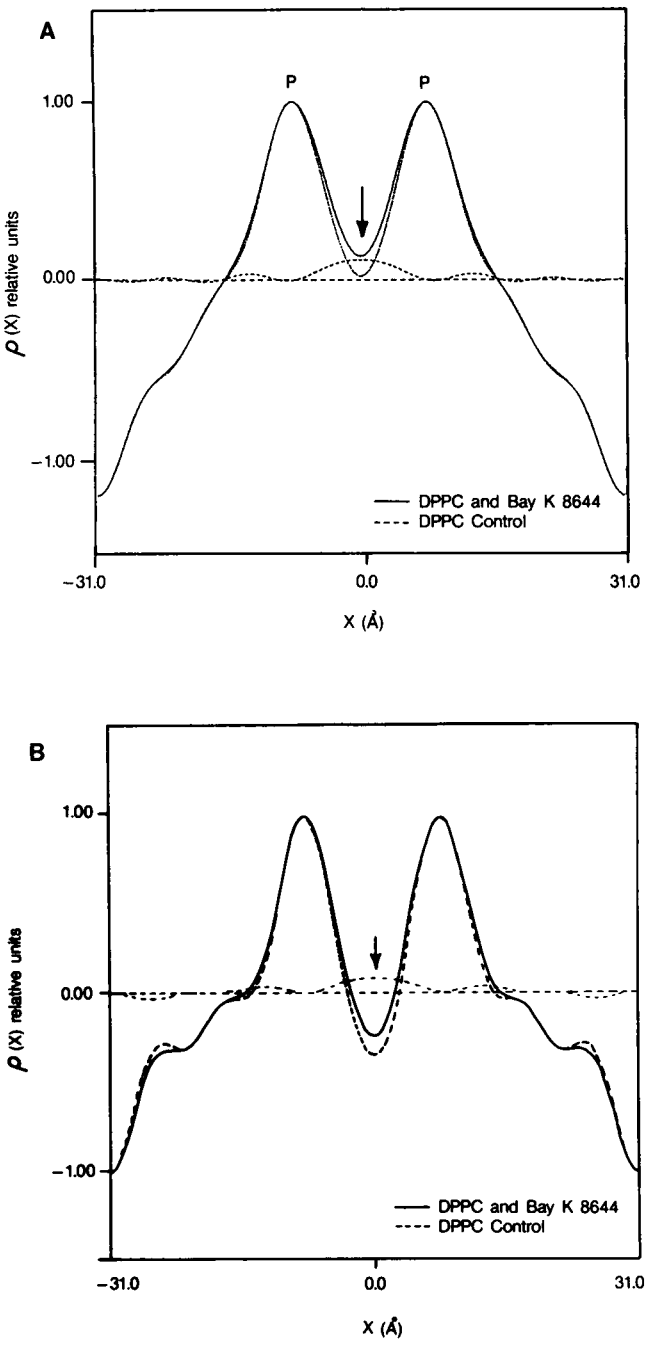

FIGURE 1 (*A*) $D/2$ shift in the experimental electron density profiles of DPPC membranes below their thermal phase transition (15°C) in the absence (····) and presence (—) of Bay K 8644 in a 1:37.5 drug:lipid ratio at 81% relative humidity (9 Å resolution). The phosphate atom of the headgroup is labeled with a "*P*." Superimposition of these profiles demonstrate that they are identical within experimental error except for an increase in electron density in the interbilayer water space (---) when the bilayer is in the gel state. The unit cell repeat distance is 61 Å for the bilayers. This observation was independent of hydration (13–93%); 8° correction factor applied to data. (*B*) Same as *A* but with a correction factor of s^1 applied to the data for samples with and without drug.

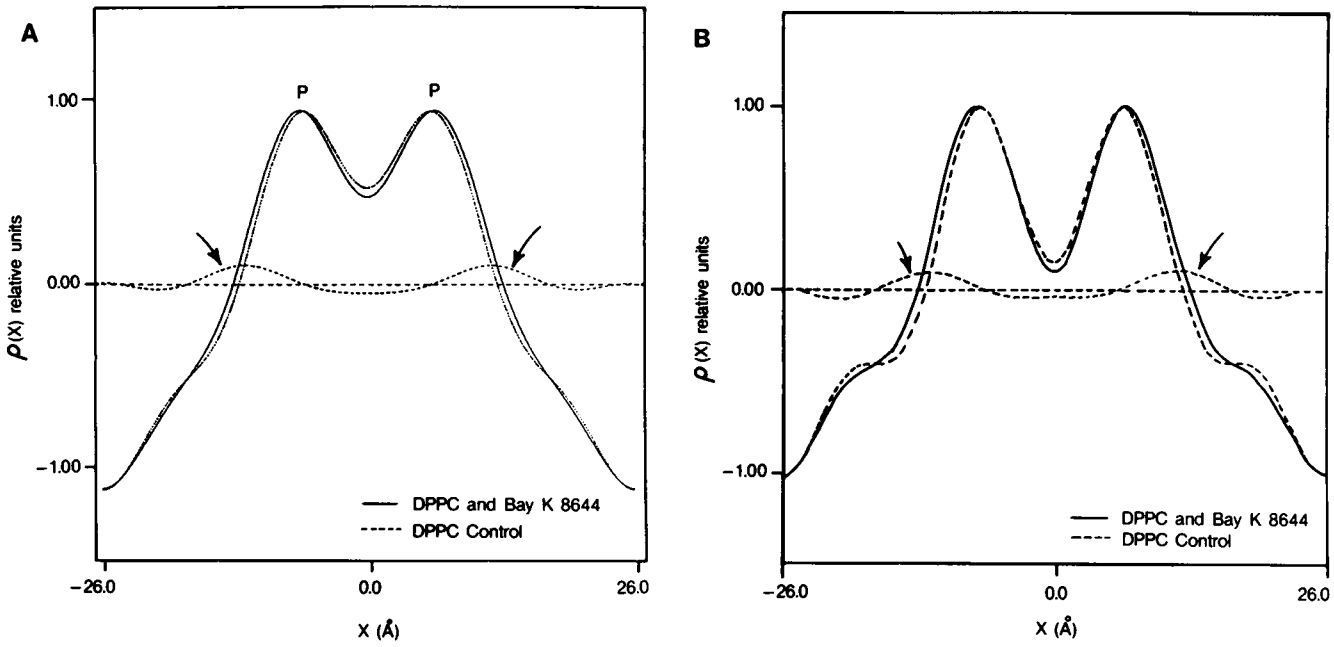

FIGURE 2 $D/2$ shift in the experimental electron density profiles of DPPC membranes above their thermal phase transition (55°C) in the absence (·····) and presence (—) of Bay K 8644 in a 1:37.5 drug:lipid ratio at 81% relative humidity (four diffraction orders for 14 Å resolution). There was a 0.9-Å decrease in the unit cell repeat distance for the DPPC in the presence of Bay K 8644 relative to the control. When superimposing these profiles with a unit cell repeat of 52 Å, there is no significant change beyond experimental error of electron density in the interbilayer water space as seen in Fig. 1. The only statistically significant change in electron density is an increase within the bilayer as marked by the arrows and consistent with CSL as well as DOPC in the liquid crystalline state (data not shown). This drug location in DPPC above the thermal phase transition was observed over a variety of relative humidities. This change in electron density from the interbilayer water space to within the bilayer as a function of bilayer state is reversible; 8° correction factor applied to data. (*B*) Same as *A* but with a correction factor of s^1 applied to the data for samples with and without drug.

factor of s) arises from the cylindrical curvature of the multilayers and hence is a weighting function for the intersection of the reciprocal lattice of the multilayer with the Ewald sphere. The second factor of s was necessary because the CSL lamellar diffraction arcs were significantly larger than the height of the detector window due to the inherent mosaic spread of the membrane multilayer samples. The second factor of s was further confirmed by comparing the electron density profiles calculated from data collected on the electronic detector where the lamellar scattering function was corrected by s^2 with electron density profiles calculated from data obtained on film where the entire reflection arc was integrated and corrected by s^1.

The inherent mosaic spread of the lamellar intensity for DPPC lipid samples was significantly smaller and thus a correction factor of s^2 resulted in electron density profiles with excessive rippling, indicative of overcorrection of the data. In addition, using an approximate correction of s^1 (the Lorentz correction factor) resulted in a somewhat overcorrection of the data (Figs. 1 *B* and 2 *B*). With no correction factor applied (Fig 1 *A* and 2 *A*) the electron density profile is considerably damped, indicating the data is somewhat undercorrected. Therefore, the true correction factor for the DPPC data appears to be some value of s raised to a fractional power >0 but <1. We present in Figs. 1 and 2 DPPC data with a correction factor of s^0 and s^1. It should be noted that whereas the correction factors modulated the shape of the various electron density profiles, the differences attributed to the presence of the drug were preserved as long as the correction factors were applied identically to the control and drug sample data.

Phasing the data

To phase the lamellar reflections for each experiment, a hydration series or swelling analysis was carried out (Moody, 1963). We used three sets of intensity data at different relative humidities each with unique unit cell repeat distances to assign an unambiguous phase combination to the experimentally obtained structure factors. An algorithm devised by Stamatoff and Krimm (1976) was used to compute the delta values for all possible phase combinations with the most probable profile structure possessing the least deviation (Herbette et al., 1985*a*).

Modeling of electron density profiles

Step-function equivalent profiles with step widths constrained by the resolution of the experimental data were fitted to the experimental profile to examine perturbations in the electron density of the membrane due to the addition of the halogenated drugs. The calculated step-function equivalents were Fourier-transformed once to generate the continuous structure factor function, which was truncated at a resolution equivalent to the experimental data (six diffraction orders). When the calculated profile structure and its intensity function correlated, within experimental error, with the experimental profile structure, the calculations were terminated (Herbette et al., 1985*b*).

Partition coefficient measurements

The nonspecific binding of Bay K 8644 to isolated LSR membranes was determined by centrifugation of various concentrations of [^{3}H] Bay K 8644 as explained in detail by Herbette et al. (1988). Briefly, the membrane partition coefficients were determined by suspending the SR membrane vesicles (12.5 μg/ml) in buffer (10 mM Tris, 150 mM NaCl), pH 7.3, containing a specific drug concentration. The samples were centrifuged in polyethylene microcentrifuge tubes (400 μl) in a model SW28 swinging bucket rotor (Beckman Instruments Inc., Fullerton, CA) for 1 h at g_{av} = 81,000 at 5°C. Control experiments contained the same reaction mixture, but were not centrifuged. The tip of the centrifuge tube containing the membrane pellet was cut off; the excess water above the pellet was blotted dry and placed in scintillation fluid to be counted for ^{3}H radioactivity. The question of how much of the drug is in the membrane bilayer versus water in the interbilayer or intravesicular spaces of the pellet was addressed in a previous paper with the more hydrophilic drugs propranolol and timolol (Herbette et al., 1983). When comparing the amount of drug or number of counts associated with phospholipid in the pellet (determined by phosphate assay) with the amount of drug associated with the aqueous supernatant, it is clear that for partition coefficients $>10^3$, most of the drug is associated with lipid and not buffer. In fact, after blotting the membrane pellet, only 5–10% of the pellet's total mass represents water (Herbette et al., 1983).

The tip of the control tubes were also cut off at the same location, blotted to remove excess buffer, and counted. These counts represented the amount of drug nonspecifically bound to the surface of that portion of the tube. Control counts were subtracted from the total pellet counts to give the actual counts reflecting drug associated only with the membrane pellet.

The amount of labeled and nonlabeled drug added to each centrifuge tube was corrected to account for any loss of drug during transfer from the reaction mixtures to the microcentrifuge tubes. The free drug concentration to which the membrane vesicles were exposed during centrifugation was determined by correcting for the total amount of drug added to the tubes, i.e., the amount of drug remaining in the supernatant after binding to the walls of the centrifuge tube, calculated using the labeled drug specific activity (adjusted for counter efficiency).

Membrane partition coefficients were calculated using the following equation: (grams of drug bound to membrane/grams lipid)/(grams of drug in supernatant/grams buffer). The amount of drug in the pellet (bound to membrane) and in the supernatant were determined as described above. The amount of lipid was adjusted for the recovery of membrane in the pellet after centrifugation. The Bay K 8644 partition coefficient was obtained using drug concentrations over the range of 10^{-7} to 10^{-10}M.

RESULTS

Partition coefficients

The partition coefficient, K_p, for [^{3}H] Bay K 8644 in LSR was determined to be 10,700 by a graph of log K_p vs. $-$log [drug concentration (M)]. This K_p was independent of drug concentration over the range of 1×10^{-7} m to 1×10^{-10} M. This is in agreement with K_p values reported for other DHPs (Herbette et al., 1986). The octanol/buffer partition coefficient, on the other hand, was determined to be 290 ± 10 (SD, n = 3). The partition coefficient of Bay K 8644 in DPPC was highly dependent on the state of the membrane such that below its thermal phase transition (2°C), the K_p was 160 ± 520 while in the liquid crystalline state, at 55°C, the K_p was 14,000 ± 1,600 (SD, n = 14).

Small angle x-ray diffraction studies

CSL electron density profiles

Membrane multilayers prepared in the presence and absence of Bay K 8644 at different lipid/drug ratios gave clearly defined, reproducible diffraction patterns. To optimize the diffraction quality of our CSL samples, we examined the multibilayers under a variety of temperature and hydration conditions. We found that the highest resolution was obtained at bilayer hydrations of 55 and 66% and low temperatures 5–10°C. At 66% relative humidity and 6°C, for example, we observed six sharp lamellar diffraction orders with a unit cell repeat distance of ~56 A for a resolution of ~9 A (see Table 1). Previous studies demonstrated strong similiarity in the profile structures for the intact sarcolemma versus extracted lipids due to the high lipid-to-protein ratios in the CSL (Herbette et al., 1985*a*).

As we further dehydrated the CSL multibilayers below 55% relative humidity, a second lattice of diffraction peaks along the lamellar meridional axis appears with peak ratios of $1:\sqrt{3}:2:\sqrt{7}$ suggestive of H_{II} phase lipid (Gruner, 1985). The H_{II} phase was also observed when the temperature was gradually raised above 10°C at these lower relative humidities (55 and 66%). The hexagonal phase reversibly disappeared as the temperature was again reduced below 10°C. The phase transition may compensate for the increase in free energy of this heterogenous lipid system as a result of significant increases in repulsive electrostatic, hydration, and steric forces as the apposing bilayers come closer together as previously described in detail (Gruner, 1985; Kirk et al., 1984). For example, the interbilayer pure water space at 55% rela-

TABLE 1 Structure factors for CSL in the absence and presence of Bay K 8644/Bay P 8857, d = 56 A

Absence of Bay K 8644/Bay P 8857		Presence of Bay K 8644		Presence of Bay P 8857	
(h)	*F(h)*	*(h)*	*F(h)*	*(h)*	*F(h)*
1	−0.7979	1	−0.7810	1	−0.7854
2	−0.0979	2	−0.0943	2	−0.0807
3	+0.0458	3	0.0	3	0.0
4	−0.5473	4	−0.5340	4	−0.5148
5	+0.1438	5	+0.1533	5	+0.1757
6	−0.1772	6	−0.2691	6	−0.2842

tive humidity is only 4.6 Å and the pressure exerted between the bilayers under these conditions is *ln* 17.5 dyns/cm^2 by the equations of McIntosh et al. (1987).

$$P = RT/V_w[ln\,(P/P_o)],$$

in which R is the molar gas constant, T is temperature, V_w is the molar volume of water (18 cm^3/mol), and P/P_o is the relative vapor pressure (McIntosh et al., 1987; Mason, R. P., and D. W. Chester, submitted for publication).

Examination of the membrane profile structures in the presence and absence of drug demonstrates that control and drug-containing profiles are identical in unit cell repeat distance (see Figs. 3–6). The bilayer does not appear to be significantly perturbed by the DHPs at 1:40 and 1:35 drug-lipid concentrations (Bay K 8644 and Bay P 8857 representing ~1% of the membrane mass). This observation is supported by the consistency in unit cell repeat distances over a range of drug:lipid ratios and very similar electron density profiles with the exception of the following features. Calculated step-function equivalent profiles indicate the phosphate headgroups are displaced outward by 1 Å by the presence of the agonist in the bilayer without affecting the calculated 7.5 Å width of the headgroup (Figs. 5 and 6). There is a significant increase in electron density approximately located at $x = \pm 16.5$ Å

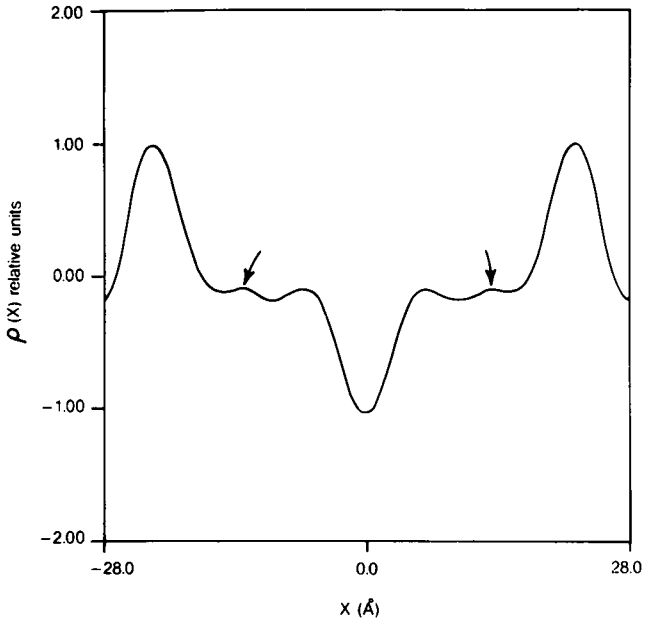

FIGURE 3 One-dimensional electron density profile of a CSL lipid bilayer in the presence of the DHP antagonist Bay P 8857 with a 1:35 drug:lipid molar ratio. A unit cell repeat distance of 56 Å was measured at 66% relative humidity and 6°C. Arrow indicates the putative position of the iodinated antagonist which is similar to that observed for Bay K 8644 in Fig. 4 within the limits of our resolution.

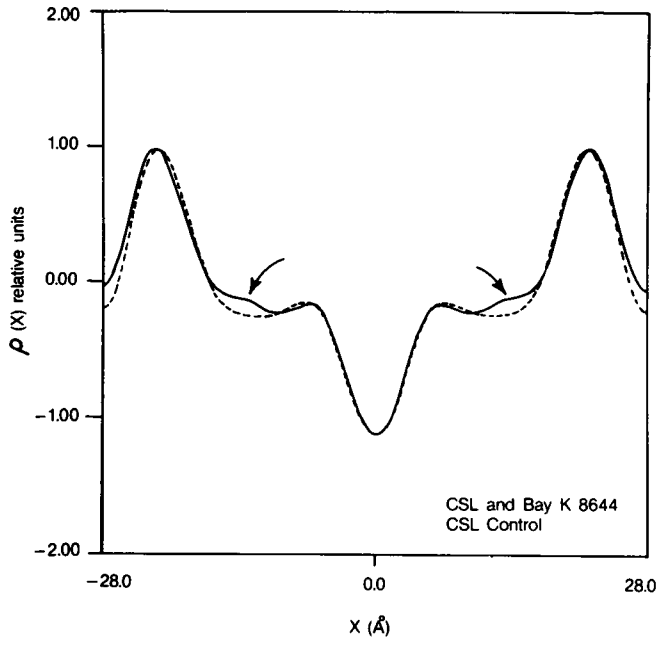

FIGURE 4 Distance across a single bilayer unit cell (A) vs. electron density for purified cardiac sarcolemmal lipid membrane in the presence and absence of Bay K 8644 at 66% relative humidity and 6°C. Superimposition of the one-dimensional electron density profiles shows a significant increase in the hydrocarbon core of the bilayer adjacent to the headgroup (within the first few methylene segments) which we attribute to the equilibrium location of the Bay K 8644.

from the center of the bilayer corresponding to the first few methylene segments of the acyl chains (Fig. 7). This discrete increase in electron density is attributed to the triflouromethyl substituent of the Bay K 8644 molecule. This equilibrium position is present over a variety of drug concentrations (DHP:lipid molar ratios of 1:10 to 1:40, which correlate well with the relative changes in electron density) and bilayer hydrations (55–81%). Fig. 3 demonstrates that Bay P 8857 has a similar location, within the resolution of our system, as the Bay K 8644.

DPPC electron density profiles

Drug location was examined in DPPC vesicles above and below its thermal phase transition (see Table 2 and Figs. 1 and 2). The state of the lipids was shown to change from gel to liquid crystalline state by calculating the unit cell repeat distance, D, as previously described (Franks and Lieb, 1979). The D spaces for DPPC bilayers above and below the phase transition temperature were 52 and 61 Å, respectively. This significant decrease in intrabilayer thickness is attributed to chain melting in the hydrocarbon core of the bilayer with *trans* to *gauche* conformation changes as the system is heated above its T_m. The change

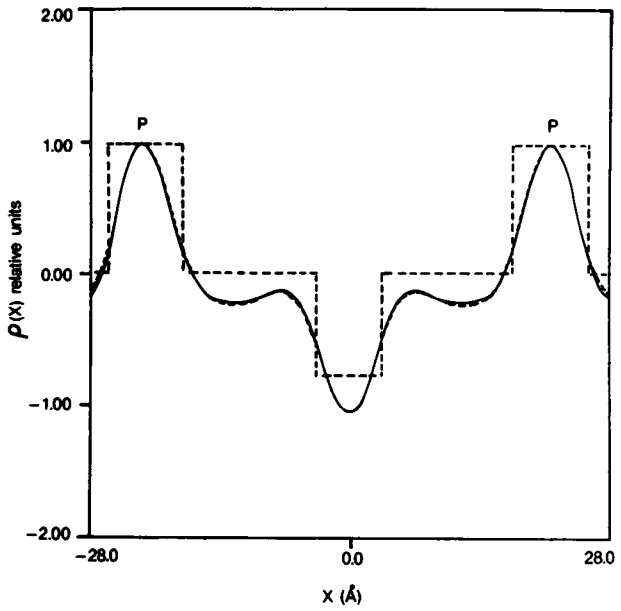

FIGURE 5 Distance across a single bilayer (A) vs. electron density for purified cardiac sarcolemmal lipid membrane in the absence of Bay K 8644 at 66% relative humidity and 6°C. The peaks in the one-dimensional electron density profile (—) refer to the phosphate headgroups (labeled "P"), whereas the central trough of minimum electron density corresponds to the terminal methyl groups in the bilayer center. The measured unit cell repeat distance was 56 Å. The experimental profile was modeled (---) with a series of four unique steps (headgroup region, acyl chains, terminal trough, and water space) with widths determined by the resolution of the system as described in the Methods and previously explained in detail (White and King, 1985). The electron density profile calculated from the modeled step function is superimposed on the experimental profile (· · · ·).

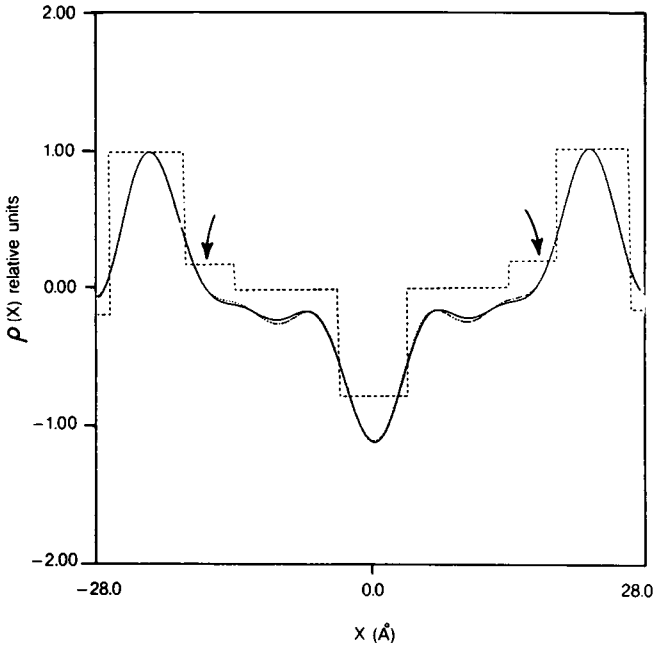

FIGURE 6 One-dimensional electron density profile of a CSL lipid bilayer in the presence of Bay K 8644 with a 1:40 drug:lipid molar ratio at 66% relative humidity and 6°C. Relative to the control, the only significant increase in electron density was modeled by a discrete step (*arrow*) with a center of mass ±16.5 Å from the bilayer center attributed to the halogenated Bay K 8644 molecule. Further, the step specifically modeling the phospholipid headgroup was displaced outward by ~1 Å while maintaining its width of 7.5 Å. The unit cell repeat distance is identical to the control (56 Å) and the profile is on the same scale as in Fig. 5. The electron density calculated from the modeled step function is superimposed on the experimental profile (· · · · ·).

in D space as a function of heating the sample was very sharp and reproducible. The correlation between change in D space and change in the physical state from gel to liquid crystalline has been well described in the literature.

In the liquid crystalline state, a statistically significant change in electron density is attributed to the trifluorinated "tag" in a location consistent with that observed for CSL below the phosphate head group (Fig. 2). Below the thermal phase transition, the lipids are highly ordered in the gel state resulting in higher resolution diffraction (9 Å) at 81% relative humidity. The electron density profiles for the drug sample and control are similar throughout the bilayer in the gel state with a significant increase in electron density located in the interbilayer water space only (Fig. 1). These results suggest the drug is excluded or "frozen out" of the bilayer during a change from liquid crystalline to gel physical state. This extrusion is reversible when the bilayer is returned to the liquid crystalline state and was independent of bilayer relative humidity (66–93%).

DISCUSSION

To examine the nonspecific membrane interactions of Bay K 8644, the partition coefficient and the molecular location have been studied in both biological and model membrane systems. We suggest for this study (see also Marqusee and Dill, 1986) that the anisotropic bilayer structure, in contrast to a bulk phase solvent such as octanol with invariant properties throughout, has very different physical and chemical characteristics as a function of distance across the bilayer normal axis which will affect drug-lipid interaction. The bilayer also differs from isotropic solvents because of its high surface-to-volume ratio. Drug partitioning and location in the bilayer would be expected to exploit these differences in achieving an energetically favorable location, orientation, and conformation (Simon et al., 1979; Herbette et al., 1986). For example, the partitioning of Bay K 8644 into LSR membranes was nearly two orders of magnitude greater than in the octanol/buffer system, similar to that

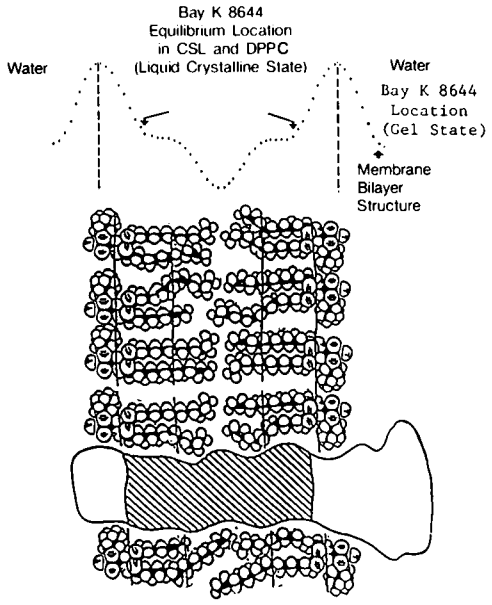

FIGURE 7 Summary of location for Bay K 8644 in CSL and DPPC in the liquid crystalline state and for Bay K 8644 in DPPC in the gel state. The electron density profile is correlated with a hypothetical membrane bilayer with a transmembrane protein. The Bay P 8857 has a similar membrane location as the Bay K 8644 in CSL (Figs. 3 and 4).

observed for Bay P 8857 (Herbette et al., 1986). Bay K 8644 partitioning is further affected by the thermal phase transition of the membrane bilayer. In DPPC membranes, the K_p value was also nearly two orders of magnitude greater above the thermal phase transition than below. We would expect this because the membrane bilayer is highly ordered in the gel phase with acyl chains in the all-*trans* conformation resulting in an increase in lipid density with exclusion of most of the drug from the bilayer (Fig. 1). As the membrane is melted, the bilayer becomes less ordered, as indicated by the change in D space from 61 to 52 Å, with a subsequent reduction in lipid packing density permitting higher drug partitioning into the bilayer. Similar effects on solute partitioning was recently described for benzene (DeYoung and Dill, 1988).

The results of low-angle x-ray scattering of Bay K 8644 with DPPC in the gel and liquid crystalline states correlate well with these drug partition studies. At ~9 Å resolution (81% relative humidity), there is no observable difference in the electron density profiles for the control vs. drug samples except for an increase in electron density indicating the presence of drug in the interbilayer water space (Fig. 1). Above the thermal phase transition, however, the interbilayer increase in electron density disappears, whereas there is an increase within the bilayer (Fig. 2) consistent with the idea that the drug has moved into the bilayer. This location within the liquid crystalline DPPC bilayer is consistent with the drug location for the CSL. Preliminary data has also demonstrated that Bay K 8644 is near the hydrocarbon core/water interface of dioleoylphosphatidylcholine (DOPC) membranes in the liquid crystalline state with 8 Å resolution.

We have shown that bilayer physical state can play an important role in drug-membrane interactions. It has been shown that cholesterol (Genz et al., 1986), alcohols (O'Leary et al., 1986; Pringle and Miller, 1979), and fatty acids (Eliasz et al., 1976) effect the ordering and hence, the thermal phase transition of biological membranes. As such, the presence of these molecules in the membrane bilayer may significantly affect nonspecific drug and, further, drug-receptor interactions. It would also be interesting to consider how the membrane bilayer structure and drug interactions are influenced by hypercholesterolemia in the disease atherosclerosis, for example.

The amphiphilic DHP appears to interact with both the charged headgroup region and the hydrophobic acyl chains of the bilayer core. Other drugs and small molecules with greater hydrophobicity can be located either near or at the central region of the bilayer core such as alkanes (White et al., 1981; McIntosh et al., 1980) and amiodarone (Trumbore et al., 1988). The distribution of solute in membrane bilayers at either of these two regions

TABLE 2 Structure factors for DPPC in the absence and presence of Bay K 8644

At 15°C, d = 61 A				At 55°C, d = 52 A			
Absence of Bay K 8644		Presence of Bay K 8644		Absence of Bay K 8644		Presence of Bay K 8644	
(h)	*F(h)*	*(h)*	*F(h)*	*(h)*	*F(h)*	*(h)*	*F(h)*
1	−0.8819	1	−0.9065	1	−0.9662	1	−0.9562
2	−0.2939	2	−0.2676	2	−0.1277	2	−0.1881
3	+0.2030	3	+0.1808	3	+0.1473	3	+0.1706
4	−0.2912	4	−0.2588	4	−0.1685	4	−0.1453
5	+0.0608	5	+0.0529				
6	−0.0500	6	−0.0400				
7	+0.0608	7	+0.0490				

appear to be thermodynamically favored over alternative locations because of enthalpic and entropic constraints (Marqusee and Dill, 1986).

Bay K 8644's location at the hydrocarbon core/water interface defines a region of localized drug concentration that may be in equilibrium with a specific receptor site at this intrabilayer position. For example, the DHP calcium channel antagonist, nimodipine, was identified at this position in sarcoplasmic reticulum using neutron diffraction (Herbette et al., 1986), whereas Bay P 8857 occupies a similiar location in CSL lipid bilayers (Fig. 3). This similar time-averaged location for the agonist and antagonist in the membrane may be related to a similiar high-affinity binding site at this particular depth in the bilayer (Janis et al., 1984; Hamilton et al., 1987) or separate binding sites on a structurally related portion of the receptor involved in the potential sensitive gating process as described by Kokubun et al. (1986). In either case these DHPs can laterally diffuse from this position (Chester et al., 1987) to the hydrophobic receptor site recently sequenced (Tanabe et al., 1987).

CONCLUSION

An examination of Bay K 8644 partitioning properties and location in the native and model bilayers reveals that drug interaction with the membrane bilayer is specific and is not adequately described by bulk solvent systems, such as octanol. The disparity between Bay K 8644 partitioning into native bilayers vs. octanol and the effect of the lipid phase on this partitioning highlights this point. Further, the consistent location of Bay K 8644 near the hydrocarbon core/water interface of model and heterogenous lipid bilayer systems in the liquid crystalline state suggest that the drug-membrane interaction is strongly influenced by their mutual chemical and physical characteristics. For example, the described time-averaged location of Bay K 8644 was dependent on the physical state of the lipid bilayer. Finally, this partitioning and location is consistent with the DHP antagonist Bay P 8857 and suggests a common membrane pathway mechanism for these drugs en route to a common sarcolemmal receptor.

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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.

Bangham, A. D., M. M. Standish, and J. C. Watkins. 1965. Diffusion of univalent ions across the lamellae of swollen phospholipids. *J. Mol. Biol.* 13:238-252.

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

Chester, D. W., L. G. Herbette, R. P. Mason, A. F. Joslyn, D. J. Triggle, and D. E. Koppel. 1987. Diffusion of dihydropyridine calcium channel antagonists in cardiac sarcolemmal lipid multibilayers. *Biophys. J.* 52:1021–1030.

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.

DeYoung, L. R., and K. A. Dill. 1988. Solute partitioning into lipid bilayer membranes. *Biophys. J.* 53:497a.

Eliasz, A. W., D. Chapman, and D. F. Ewing. 1976. Phospholipid phase transitions: effects of n-alcohols, n-monocarboxylic acids, phenyl alkyl alcohols and Squaternary ammonium compounds. *Biochim. Biophys. Acta.* 448:220–230.

Folch, J., M. Les, 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.

Franks, N. P., and W. R. Lieb. 1981. X-Ray and neutron diffraction studies of lipid bilayers. *In* Liposomes: From Physical Structure to Therapeutic Applications. Elsevier/North-Holland Biomedical Press, New York. 243–271.

Genz, A., J. F. Holzworth, and T. Y. Tsong. 1986. The influence of cholesterol on the main phase transition of unilamellar dipalmitoylphosphatidylcholine vesicles. *Biophys. J.* 50:1043–1051.

Gruner, S. M. 1985. Intrinsic curvature hypothesis for biomembrane lipid composition: a role for nonbilayer lipids. *Proc. Natl. Acad. Sci. USA.* 82:3665–3669.

Hamilton, S. L., A. Yatini, K. Brush, A. Schwartz, and A. M. Brown. 1987. A comparison between the binding and electrophysiological effects of dihydropyridines on cardiac membranes. *Mol. Pharmacol.* 31:221–231.

Herbette, L. G., A. M. Katz, J. M. Sturtevant. 1983. Comparisons of the interactions of propranolol and timolol with model and biological membrane systems. *Mol. Pharmacol.* 24:259–269.

Herbette, L. G., T. MacAlister, T. F. Ashavaid, and R. A. Colvin. 1985*a*. 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. *Biochem. Biophys. Acta.* 812:609–623.

Herbette, L., P. DeFoor, S. Fleischer, D. Pascolini, and J. K. Blasie. 1985*b*. The separate profile structures of the functional calcium and the phospholipid bilayer within isolated 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.* 49:91–94.

Herbette, L. G., Y. M. Vant Erve, and D. G. Rhodes. 1989. Interaction of 1,4-dihydropyridine calcium channel antagonists with biological membranes: lipid bilayer partitioning could occur before drug binding to receptors. *J. Mol. Cell. Cardiol.* In press.

Israelachvili, J. N., S. Marcelja, and R. G. Horn. 1980. Physical principles of membrane organization. *Q. Rev. Biophys.* 13:121–200.

Janis, R. A., D. Rampe, J. G. Sarmiento, and D. J. Triggle. 1984. Specific binding of a calcium channel activator, [^{3}H]Bay K 8644, to membranes from cardiac muscle and brain. *Biochem. Biophys. Res. Commun.* 121:317–323.

Janis, R. A., P. J. Silver, and D. J. Triggle. 1987. Drug action and cellular calcium regulation. *Adv. Drug Res.* 16:309–591.

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.

King, G. I., and S. H. White. 1986. Determining bilayer hydrocarbon thickness from neutron diffraction measurements using strip-function models. *Biophys. J.* 49:1047–1054.

Kirk, G. L., S. M. Gruner, and D. L. Stein. 1984. A thermodynamic model of the lamellar to inverse hexagonal phase transition of lipid membrane-water systems. *Biochemistry.* 23:1093–1102.

Kobilka, B. K., R. A. F. Dixon, T. Frielle, H. G. Dohlman, M. A. Bolanowski, I. S. Sigal, T. L. Yang-Feng, U. Francke, M. G. Caron, and R. J. Lefkowitz. 1987*a*. cDNA for the human beta2-adrenergic receptor: a protein with multiple membrane-spanning domains and encoded by a gene whose chromosomal location is shared with that of the receptor for platelet-derived growth factor. *Proc. Natl. Acad. Sci. USA.* 84:46–50.

Kobilka, B. K., C. MacGregor, K. Daniel, T. S. Kobilka, M. G. Caron, and R. J. Lefkowitz. 1987*b*. Functional activity and regulation of human beta2-adrenergic receptors expressed in *xenopus* oocytes. *J. Biol. Chem.* 262:1–7.

Kobilka, B. K., T. S. Kobilka, K. Daniel, J. W. Regan, M. G. Caron, and R. J. Lefkowitz. 1988. Chimeric alpha-2, beta-2 adrenergic receptors: delineation of domains involved in effector coupling and ligand binding specificity. *Science (Wash. DC).* 240:1310–1316.

Kokubun, S., and H. Reuter. 1984. Dihydropyridine derivatives prolong the open state of Ca^{++} channels in cultured cardiac cells. *Proc. Natl. Acad. Sci. USA.* 81:4824–4827.

Kokubun, S., C. Prod'hom, C. Becker, H. Porzig, and H. Reuter. 1986. Studies on Ca channels in intact cardiac cells: voltage-dependent effects and cooperative interaction of dihydropyridine enantiomers. *Mol. Pharmacol.* 30:571–584.

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.

McIntosh, T. J., S. A. Simon, and R. C. MacDonald. 1980. The organization of n-alkanes in lipid bilayers. *Biochim. Biophys. Acta.* 597:445–463.

McIntosh, T. J., A. D. Magid, and S. A. Simon. 1987. Steric repulsion between phosphatidylcholine bilayers. *Biochemistry.* 26:7325–7332.

Moody, M. F. 1963. X-Ray diffraction pattern of nerve myelin: a method for determining the phases. *Science (Wash. DC).* 142:1173–1174.

O'Leary, T. J., P. D. Ross, and I. W. Levin. 1986. Effects of anesthetic tetradecanols on phosphatidylcholine phase transitions. *Biophys. J.* 50:1053–1059.

Pringle, M. J., and K. W. Miller. 1979. Differential effects of phospholipid phase transitions produced by structurally related long-chain alcohols. *Biochemistry.* 18:3314–3320.

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 antagonist to their active site. *Mol. Pharmacol.* 27:612–623.

Schramm, M. G., R. T. Thomas, and Franckowiak. 1983. Novel dihydropyridines with positive inotropic action through activation of Ca^{+2} channels. *Nature (Lond.).* 303:535–537.

Simon, S. A., W. L. Stone, and P. B. Bennett. 1979. Can regular solution theory be applied to lipid bilayer membranes? *Biochim. Biophys. Acta.* 550:38–47.

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.

Tanabe, T., H. Takeshima, A. Mikami, V. Flockerzi, H. Takahashi, K. Kangawa, M. Kojima, H. Matsuo, T. Hirose, and S. Numa. 1987. Primary structure of the receptor for calcium channel blockers from skeletal muscle. *Nature (Lond.).* 328:313–318.

Trumbore, M. W., D. W. Chester, J. Moring, D. Rhodes, and L. G. Herbette. 1988. Structure and location of amiodarone in a membrane bilayer as determined by molecular mechanics and quantitative x-ray diffraction. *Biophys. J.* 54:535–543.

Valdivia, H., and R. Coronado. 1988. Pharmacological profile of skeletal muscle calcium channel in planar lipid bilayers. *Biophys. J.* 53:555a.

Watras, J., F. C. Messineo, and L. G. Herbette. 1984. Mechanisms of fatty acid effects on sarcoplasmic recticulum. I. Calcium-fatty acid interaction. *J. Biol. Chem.* 259:1319–1324.

White, S. H., G. I. King, and J. E. Cain. 1981. Location of hexane in lipid bilayers determined by neutron diffaction. *Nature (Lond.).* 290:161–163.