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Differential Membrane Interactions of Calcium Channel Blockers

IMPLICATIONS FOR ANTIOXIDANT ACTIVITY

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ABSTRACT. Lipid peroxidation causes cellular damage during aging and various diseases, including atherosclerosis. Chronic administration of highly lipophilic calcium channel blockers (CCB) may reduce lipid peroxidation as a result of concentration in cell membranes and altering physico-chemical properties of the lipid bilayer. In this study, small angle X-ray scattering was used to examine reconstituted cardiac membrane lipid bilayers in the presence of CCB with various antioxidant activities, including nisoldipine, nifedipine, and diltiazem. Analysis of one-dimensional electron density profiles demonstrated that these compounds have different molecular distributions relative to the center of the membrane: diltiazem (± 14–22 Å), nifedipine (± 12–22 Å), and nisoldipine (± 7–22 Å). The overall hydrocarbon core width for control samples was 44 Å and was unaffected by the addition of drugs at these concentrations (< 1% by mass). High resolution differential scanning calorimetry indicated that CCB markedly perturbed the thermotropic properties of liposomes, including thermal phase transition temperature and enthalpy, relative to control samples. The effects of these compounds on membrane thermotropic properties correlate with their reported antioxidant activities. These data support the hypothesis that calcium channel blockers have potent physico-chemical interactions with the membrane lipid bilayer, which may underlie their antioxidant activity. BIOCHEM PHARMACOL 51;5:653–660, 1996.

KEY WORDS. antioxidants; calcium channel blockers; liposomes; X-ray diffraction

As the primary disease process underlying cerebral and myocardial infarction, atherosclerosis is the chief cause of death in the United States and Western Europe. Oxyradical damage of membranes and lipoproteins represents an early step in atheroma development [1] and may be retarded or blocked by lipophilic pharmacological agents, including organic voltage-sensitive CCB§ [2–6]. The primary pharmacological role for CCB is to decrease vascular contractility and arterial tone by modulating transmembrane influx of calcium into arterial smooth muscle cells (for review, see Ref. 7). However, the antioxidant activity of CCB appears to be unrelated to specific modulation of ion channels, as will be discussed.

Inhibition of lipid peroxidation by CCB has been characterized *in vitro* by independent laboratories using various membrane [8–14] and lipoprotein [15–17] preparations. Inhibition of lipid peroxidation by CCB appears to be independent of calcium transport. Both active and inactive isomers of DHP appear to have equipotent, concentration-dependent lipid antiperoxidative activity in isolated membrane, whole cell, and heart preparations [10, 18]; antioxidant activity of CCB may be related to trapping of free radicals as opposed to inhibition of the initiation step of oxidation [8–11]. By partitioning into the membrane hydrocarbon core, the chemistry of CCB may facilitate scavenging of free radicals by electron-donating and radical-resonating mechanisms, thereby breaking the lipid peroxidation chain reaction. The relative antioxidant potency of CCB parallels the measured equilibrium membrane-based partition coefficients measured for these compounds (nisoldipine > nifedipine ≫ diltiazem) [19, 20]. These observations support the hypothesis that the antioxidant activity of these DHPs is related to interactions with phospholipid and protein constituents of the membrane as opposed to specific pharmacological modulation of voltage-gated ion channels. A similar mechanism of antioxidant activity has been postulated for other lipophilic antioxidants, including vitamin E, which has been shown by fluorescence polarization techniques to interact strongly with liposomes [21].

To explore the physico-chemical basis for antioxidant activity of CCB, this study utilized experimental techniques that provide direct information concerning the equilibrium membrane location of these compounds and concentration-depen-

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§ *Abbreviations:* CCB, calcium channel blocker(s); DHP, 1,4-dihydropyridine; DSC, differential scanning calorimetry; DMPC, dimyristoyl phosphatidylcholine; BCPC, bovine cardiac phosphatidylcholine; CUS, cooperative unit size; T_m, thermal phase transition temperature, and r.h., relative humidity.

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dent membrane thermodynamic effects. The results of this study demonstrated that CCB have distinct sites of membrane interaction and alter membrane thermodynamic properties in a manner that may be correlated with relative antioxidant activity.

MATERIALS AND METHODS

All chemicals used were reagent grade or better and made up in ultra-pure deionized water. DMPC, BCPC, and cholesterol were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL). Nisoldipine was provided by the Bayer Pharmaceutical Division (West Haven, CT). Nifedipine and diltiazem were purchased from the Sigma Chemical Co. (St. Louis, MO). The BCPC primary fatty acid composition was determined by gas-liquid chromatography to be as follows: 18:2 linoleic acid (30%), 16:0 palmitic acid (22%), 18:1 oleic acid (13%), 20:4 arachidonic acid (11%), and 20:3 homogamma linoleic acid (5%) (Avanti Polar Lipids, Inc.).

Differential Scanning Calorimetry

DSC was used to analyze drug-containing and control multilamellar DMPC vesicles prepared by the following procedure. The lipid DMPC has a well-defined T_m of 22.9°. To prepare vesicles, 100-μL aliquots of a 0.03 M solution of DMPC in chloroform was dried to a thin film by evaporation under N_2 gas, and residual solvent was removed by vacuum overnight. The dried lipid was then rehydrated for 10 min at 50° in 100 μL of sample buffer (0.5 mM HEPES, 2.0 mM NaCl, pH 7.3) containing various concentrations of diltiazem, nisoldipine, or nifedipine, and then vortexed for 1 min to form multilamellar vesicles. Aliquots (15 μL) of vesicles containing 0.300 mg of lipid were placed into DSC sample pans and sealed hermetically. DSC was carried out using a TA Instruments (New Castle, DE) DSC 2910 Differential Scanning Calorimeter, and the data were analyzed using a TA Instruments Thermal Analyst 2000 system. The calorimeter was calibrated for baseline, heat flow, and temperature using an indium standard of known mass. The scan rate was 2°/min starting at 6° and ending at 29.5°. Samples were scanned repeatedly over this range until there was no change in the observed calorimetric trace. Typically, there was no difference between the first and subsequent scans for a given sample. The reference was 15 μL of sample buffer. The change in CUS was determined by the following formula:

$$\Delta CUS = (\Delta Hvh/\Delta H \cdot F_w)_{drug} - (\Delta Hvh/\Delta H \cdot F_w)_{control}$$

where ΔCUS is the change in the cooperative unit size, ΔHvh is the van't Hoff enthalpy, ΔH is the calorimetric enthalpy, and F_w is the formula weight of the lipid undergoing transition.

Preparation of Membrane Vesicles for X-ray Diffraction

BCPC multilamellar liposomes were prepared for X-ray diffraction experiments [22]. Samples containing cholesterol and phospholipid at a 0.5:1 mole ratio dissolved in chloroform were dried down with a stream of N_2 gas to a thin film on the sides and bottom of a glass 13 × 100 mm test tube while vortexing. Residual solvent was then removed by vacuum. For X-ray diffraction experiments, a specified volume of buffer (0.5 mM HEPES, 2.0 mM NaCl, pH 7.3) was added to the dried lipid preparation, yielding a final phospholipid concentration of 5 mg/mL. Multilamellar vesicles were formed by vortexing the buffer and lipids for 3 min. Drug dissolved in ethanol was added to experimental samples at a final drug/lipid mole ratio of 1:50 and an ethanol concentration not exceeding 2%. This residual ethanol was removed during the dehydration process.

Preparation of Oriented Membrane Samples for X-ray Diffraction

Oriented multilamellar membrane samples in the presence and absence of drugs were prepared by centrifugation in Lucite sedimentation cells containing an aluminum foil substrate [23]. Samples were centrifuged in an SW-28 rotor (Beckman Instruments, Inc., Fullerton, CA) at 35,000 *g* for 1 hr at 5°. On completion of centrifugation, more than 90% of the supernatant was removed, and the "multibilayer" samples were mounted on curved glass supports. The samples were partially dehydrated overnight in glass vials containing a saturated salt solution ($ZnSO_4$) to define a specific relative humidity of 95% at 5°. Samples were then placed in sealed brass canisters in which temperature and relative humidity were again controlled.

Small Angle X-ray Diffraction Data Collection and Reduction

Small angle X-ray diffraction studies were carried out by aligning the membrane multibilayers at near-grazing incidence with respect to the X-ray beam. The radiation source was a collimated, monochromatic X-ray beam (Cu K_α X-ray, $\lambda = 1.54$ Å) from an Elliot GX-18 rotating anode X-ray generator (Enraf Nonius, Bohemia, NY) operated at 40 kV and 30 mA in the Biomolecular Structure Analysis Center at the University of Connecticut Health Center. A helium tunnel was positioned between the sample and detector to reduce scattering from the air. The experimental method utilized a single Franks mirror defining a focused, nickel-filtered line source where $K_{\alpha 1}$ and $K_{\alpha 2}$ are unresolved.

Bragg's diffraction orders from the multilamellar samples were recorded on both Kodak DEF-5 film (Eastman Kodak Co., Rochester, NY) and a Braun position-sensitive 1-D detector (Innovative Technologies, Inc., Newburyport, MA). In addition to direct calibration of the detector system, mica, lead stearate, and cholesterol were used to verify the calibration and sample-to-detector distances. The sample-to-detector distance ranged from 100 to 150 mm.

Relative intensities for the diffraction orders were obtained directly from digitized computer plots of the detector data using an integration routine. After three cycles of data collection and reduction for control and test samples, the systematic error was determined to be less than 4% of the integrated

intensity of the diffraction orders (I), consistent with previous studies in our laboratory using this lipid bilayer preparation [24]. Each individual diffraction peak was background-corrected using a linear subtraction routine that averaged the noise. After accounting for experimental error associated with repeated sample data collection and integration of the lamellar reflections for both control and drug-containing samples, the changes observed appear to be both meaningful and general. All of the profiles were compared using an identical number of diffraction orders in the analysis. The lamellar intensity functions from the oriented membrane samples were corrected by a factor of $s = 2\sin\theta/\lambda$, the Lorentz correction, in which λ is the wavelength of the X-ray radiation (1.54 Å) and θ is the Bragg angle equal to one-half of the angle between the incident beam and the scattered beam. A swelling analysis was used to assign unambiguous phases to the experimental structure factors [25].

RESULTS

Differential Scanning Calorimetry

The effects of nisoldipine, nifedipine, and diltiazem on thermotropic properties of DMPC membrane bilayers are demonstrated in Figure 1 and Table 1. The gel-liquid crystal phase transition temperature (T_m) of control samples was 22.9° and the enthalpy of transition was 33.0 J/g. Relative to control samples, all of the drugs markedly altered the T_m of DMPC vesicles (−1.54° to −0.33°) and completely abolished the pretransition phase at all drug/lipid mole ratios. These molecules also perturbed the enthalpy (ΔH) associated with the T_m over a range of −9.09 to 1.86 J/g. Decreases in ΔH are due to the inability of a fraction of the molecules in the system to participate in the thermal phase transition. The average error associated with these DSC determinations was ± 0.05° (ΔT_m) and ± 1.2 J/g (ΔH).

Small Angle X-ray Diffraction

X-ray scattering from oriented BCPC/cholesterol lipid bilayers produced six strong, reproducible diffraction orders at 5° (Fig. 2). The unit cell periodicity or *d*-space for the control BCPC/cholesterol sample was 56.0 Å, consistent with reported dimensions of intact and reconstituted cardiac sarcolemmal preparations under similar experimental conditions [24, 26]. In the presence of drug (1:50 drug/phospholipid mole ratio), the membrane bilayer unit cell periodicity increased over a small range from 56.4 Å (nifedipine) to 57.0 Å (nisoldipine). Equatorial diffraction was examined to determine the average acyl-chain packing distance. At 25°, a single, very broad reflection was observed at 1/4.8 $Å^{-1}$, consistent with a liquid crystalline state membrane bilayer.

One-dimensional electron density profiles generated from the phased X-ray diffraction data demonstrated a centrosymmetric lipid bilayer structure (Fig. 3). The two peaks of electron density on either side of the figure correspond to phospholipid headgroups while the minimum of electron density at the center of the membrane is associated with terminal meth-

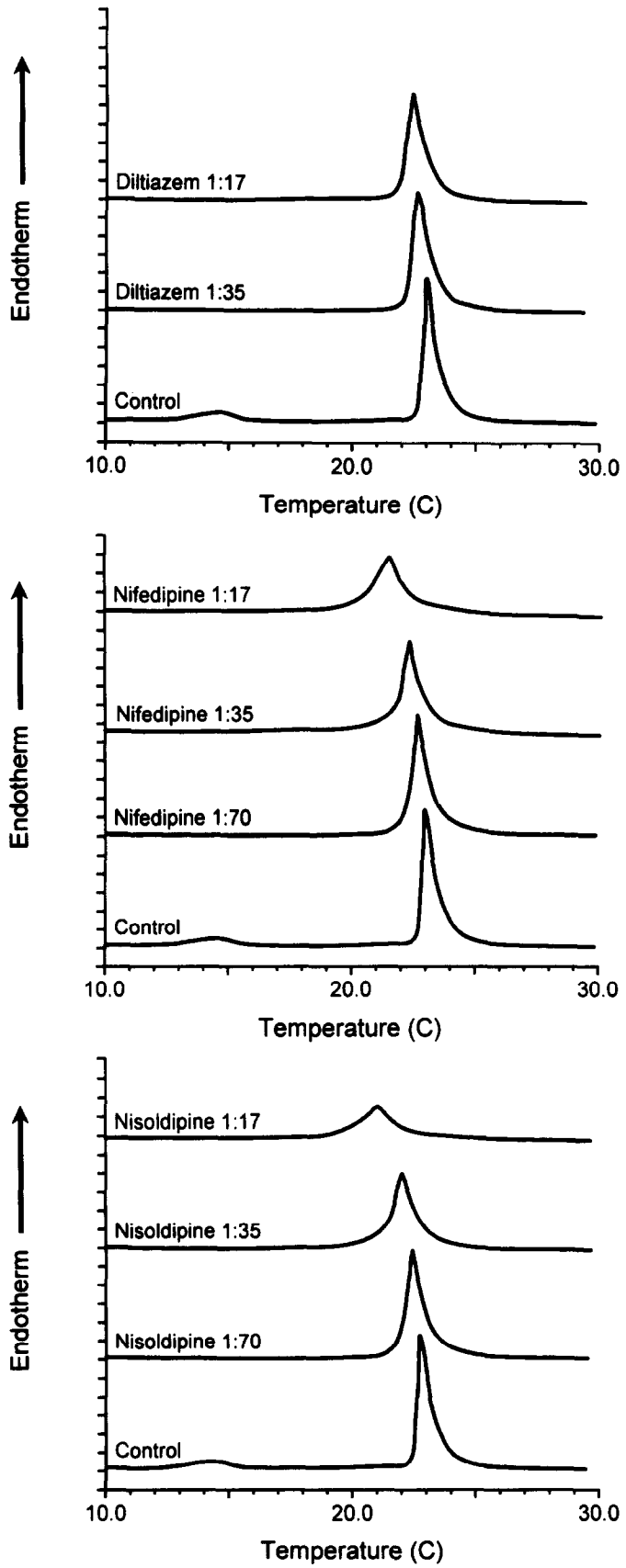

FIG. 1. Concentration-dependent effects of the calcium channel blockers diltiazem (top panel), nifedipine (center panel), and nisoldipine (bottom panel) on high resolution differential scanning calorimetry heating thermograms of DMPC vesicles. The drug:lipid mole ratios were successively increased from 1:70 to 1:17. The scans represent the average of two trials consisting of three samples for each drug concentration.

TABLE 1. Change in transition temperature and enthalpy for the drugs nisoldipine, nifedipine, and diltiazem as a function of drug/lipid mole ratio

	ΔT_m*			ΔH†		
	1:17	1:35	1:70	1:17	1:35	1:70
Nisoldipine	−1.54	−0.71	−0.34	−9.09	−0.37	0.80
Nifedipine	−1.42	−0.63	−0.33	−3.89	−1.27	1.86
Diltiazem	−0.66	−0.37	—‡	−0.76	−0.21	—

* ΔT_m = change in the temperature of the gel-liquid crystal phase transition (°); ΔT_m control, 22.9°; average error ± 0.05.

† ΔH = change in the enthalpy of the gel-liquid crystal phase transition (J/g); ΔH control, 33.0 J/g; average error ± 1.2.

‡ — = No effect observed.

ylene segments. The intrabilayer headgroup separation for the control sample was 44 Å (the measured distance between the phospholipid headgroup peaks). Electron density profiles for membrane bilayers in the presence and absence of nifedipine, nisoldipine, or diltiazem are superimposed in Fig. 3. Subtraction of the control profile from the drug-containing profile at identical resolution yielded positive differences in electron density that are indicated by the filled areas in the center of each figure.

The marked increases in electron density were attributed to the equilibrium location of CCB in membrane bilayers (see also Refs. 24 and 27). In particular, addition of CCB produced an increase in electron density relative to the center of the membrane bilayer of ± 14–22 Å (diltiazem), ± 12–22 Å (nifedipine), or ± 7–22 Å (nisoldipine). In samples containing nisoldipine, a bimodal distribution was observed with peaks at ± 9 Å and ± 19 Å from the center of the membrane bilayer while the other molecules had single peaks of electron density distribution centered at ± 18 Å (nifedipine) and ± 19 Å (diltiazem). These data demonstrated that electron density distributions for these CCB molecules in the membrane bilayer differ under identical experimental conditions.

In addition to positive increases in electron density following addition of CCB analogs, a broadening in the terminal methylene trough was observed (Fig. 3). This change is attributed to intercalation of CCB molecules near the hydrocarbon core/water interface, resulting in an alteration in the lateral packing of the phospholipid molecules and thus an increase in

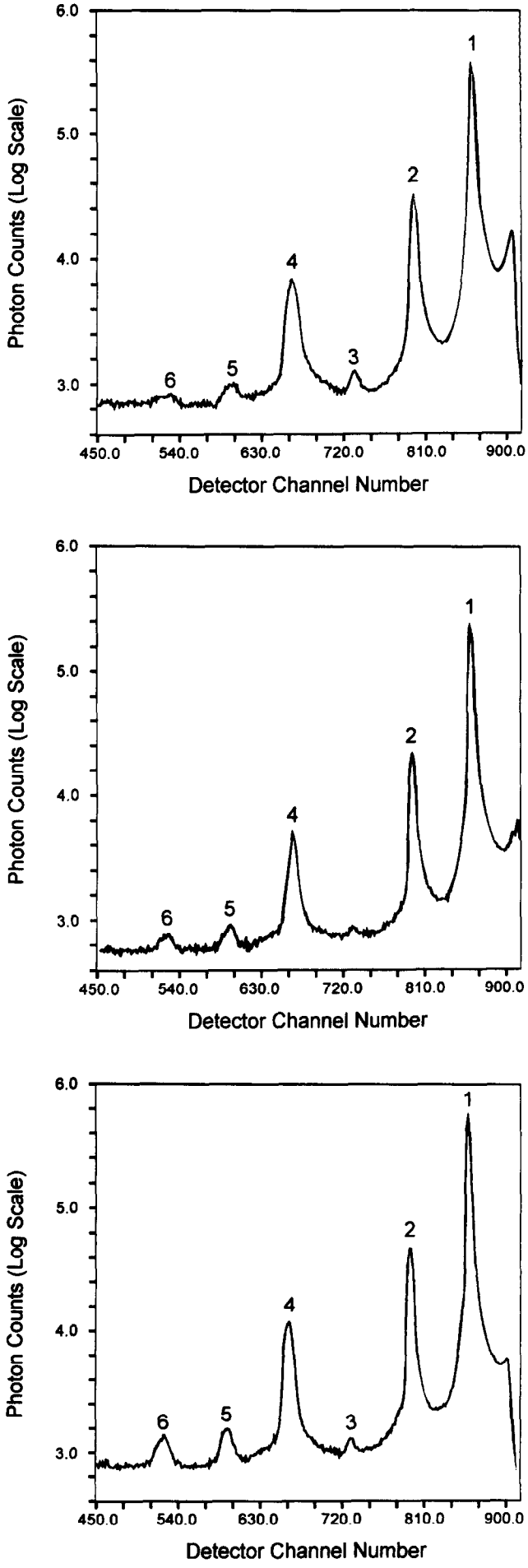

FIG. 2. Representative X-ray diffraction intensities from oriented cardiac phospholipid/cholesterol membrane samples incubated with either diltiazem (top panel), nifedipine (center panel), or nisoldipine (bottom panel) at a 1:50 drug/phospholipid mole ratio (samples contained less than 1% drug), by mass). Six diffraction orders were observed from oriented cardiac phospholipid/cholesterol samples at 5° and 95% relative humidity. The diffraction pattern is used to calculate, by Fourier analysis, the time-averaged electron density distribution of the membrane lipid bilayer in the absence or presence of drug (see Fig. 3). This figure illustrates the differences observed in the relative intensities of the X-ray diffraction data between the various drug-containing sample, for example diffraction peaks 3, 5 and 6.

volume occupied by the acyl-chain segments near the center of the membrane bilayer.

DISCUSSION

A number of biochemical studies indicate that organic calcium channel blockers have lipid antiperoxidation activity that is independent of specific modulation of calcium channels. The mechanism for CCB antiperoxidative activity is not well understood, but may be related to physico-chemical interactions with the membrane lipid bilayer. The results of this study indicate that CCB occupy time-averaged locations in the membrane and alter basic thermodynamic properties of the lipid bilayer. These membrane interactions contribute to a *non-receptor*-mediated mechanism for CCB lipid antiperoxidation activity, as suggested by previous studies utilizing isolated membrane [8–12] and lipoprotein [15–17] preparations. The compounds examined in this study have high membrane-based partition coefficients that vary from 10^2 (diltiazem) to 10^3 (nifedipine) and 10^4 (nisoldipine) [19, 20]. Thus, chronic administration of these compounds would result in their accumulation in lipoproteins and cellular membranes in concentrations exceeding plasma drug levels by as much as four orders of magnitude, as in the case of nisoldipine.

High resolution differential scanning calorimetry demonstrated that these compounds altered the basic thermodynamic properties of the membrane lipid bilayer in a concentration-dependent manner. The effects of the DHP analogs nisoldipine and nifedipine on both the ΔH and T_m of DMPC samples were much more pronounced than the benzothiazepine, diltiazem (Table 1 and Fig. 1). Moreover, marked differences in thermotropic effects were observed between the two DHP compounds. Specifically, nisoldipine had the most pronounced effect on the enthalpy of the thermal phase transition (−9.09 J/g at a 1:17 drug/lipid mole ratio). This decrease in enthalpy is due to the inability of a fraction of the lipids to participate in the phase transition, as evidenced by a broadening in the endotherm (Fig. 1). With respect to changes in T_m, both nisoldipine (−1.54°) and nifedipine (−1.42°) had similar effects on T_m at a 1:17 drug/lipid mole ratio. At an identical concentration, the effects of diltiazem on ΔH (−0.76 J/g) and T_m (−0.66) were much less pronounced. Differences in the membrane effects of these compounds may be attributed to an alteration in physico-chemical interactions with neigh-

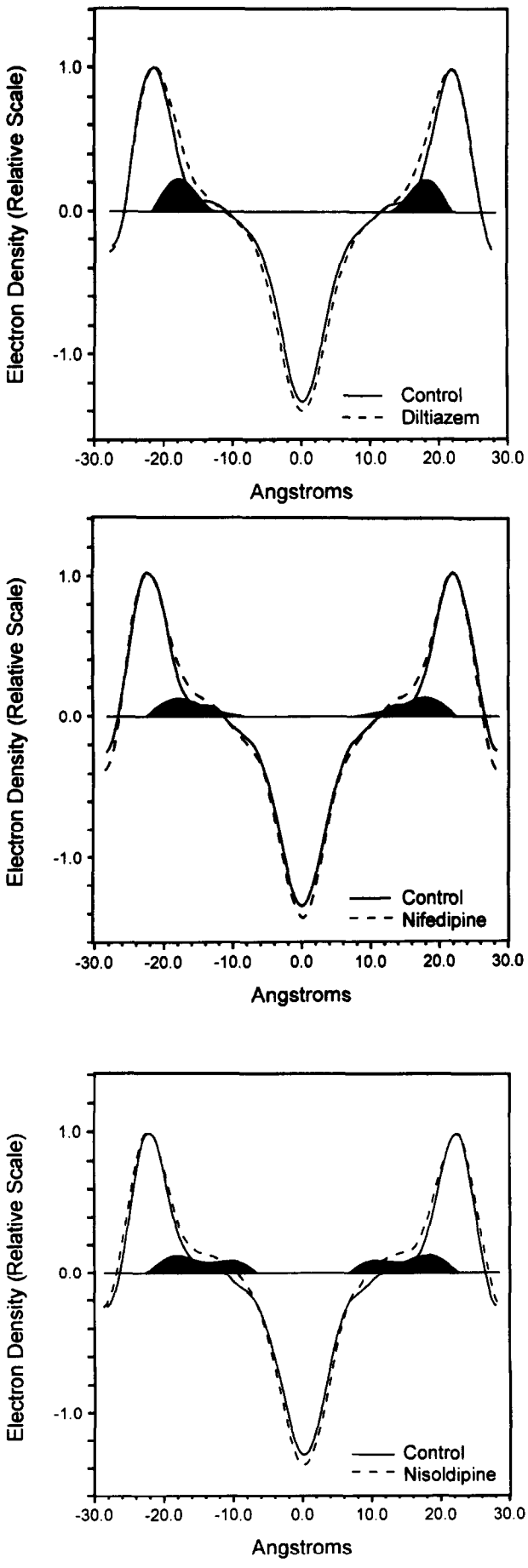

FIG. 3. One-dimensional electron density profile (Å versus electrons/Å^3) of a centrosymmetric cardiac membrane lipid bilayer in the absence and presence of either diltiazem (top panel), nifedipine (center panel), or nisoldipine (bottom panel) at 5° and 95% relative humidity. The peaks of electron density correspond to the phospholipid headgroups, while the minimum of electron density at the center of the bilayer correlates with the terminal methylene segments that have a relatively high hydrogen to carbon ratio. Positive differences in electron density corresponding to time-averaged drug distribution are represented by the shaded regions in the center of the membrane bilayer.

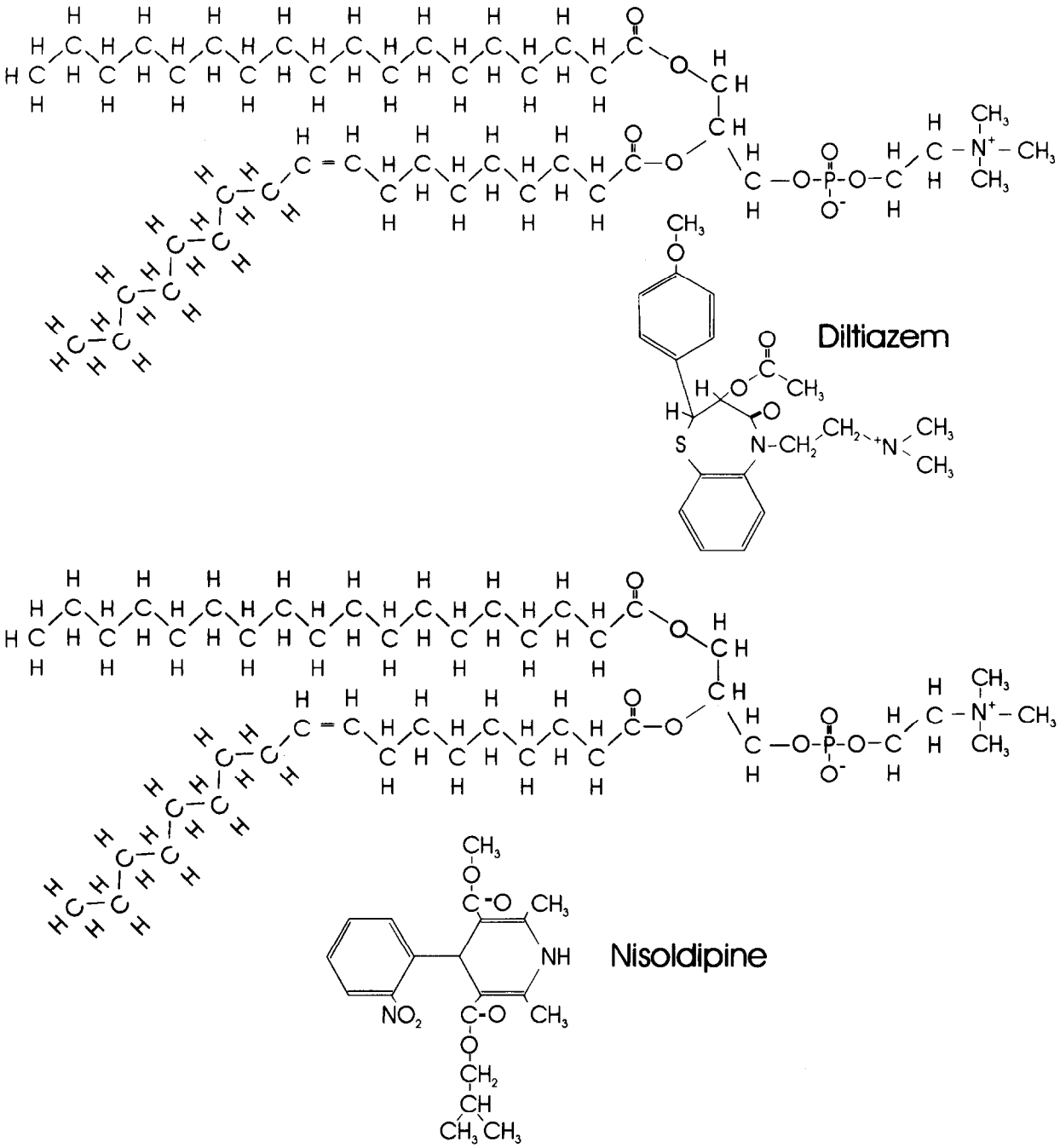

FIG. 4. Highly schematic illustration of molecular interactions of diltiazem (top panel) and nisoldipine (bottom panel) with membrane phospholipid, based on results of X-ray diffraction analysis. The orientation of the molecule is postulated from its chemical properties for the purposes of this figure. The drug location is not static but distributed over a limited region of the lipid bilayer. The time-averaged distribution of nisoldipine is skewed towards the center of the membrane hydrocarbon core when compared with diltiazem. The time-averaged location of nisoldipine molecules near the acyl chain unsaturated bonds may underlie differences in its effects on membrane thermodynamic properties (Fig. 1) and lipid antiperoxidation activity (see Discussion).

boring phospholipid molecules (i.e. ionic and hydrogen bonding, steric effects). These changes in membrane thermotropic properties are similar to those observed for other organic compounds that putatively concentrate in the upper half of the hydrocarbon core of the lipid bilayer, adjacent to the phospholipid headgroups [28]. This conclusion regarding CCB membrane location is supported directly by small angle X-ray scattering data.

Small angle X-ray diffraction was used to ascertain the time-averaged location of the three CCB in oriented cardiac phospholipid/cholesterol membrane bilayers under controlled experimental conditions. One-dimensional electron density profiles generated from the diffraction data are superimposed in Fig. 3. Positive differences in electron density following addition of the CCB analogs are indicated by the shaded areas at the center of the profiles. Molecular distributions relative to

the center of the membrane hydrocarbon core were as follows: diltiazem (± 14–22 Å), nifedipine (± 12–22 Å), and nisoldipine (± 7–22 Å). (The intrabilayer headgroup separation width of both control and drug-containing samples was 44 Å). These findings are consistent with DSC results, which indicate that these CCB analogs are located in the upper half of the hydrocarbon core, adjacent to the phospholipid headgroups.

Membrane structure data demonstrate that diltiazem, nifedipine, and nisoldipine have different time-averaged distributions in the membrane that contribute to their thermotropic effects. Distinct chemical and steric interactions of these compounds with neighboring phospholipid molecules underlie their differences in molecular distribution. In particular, the covalent structure of the uncharged nisoldipine molecule includes a bulky *s*-butyl group at the C-3 position of the DHP ring which may only be accommodated deeper in the hydrocarbon core, where there is relatively greater molecular volume. Thus, the time-averaged distribution of nisoldipine is skewed towards the center of the membrane bilayer. By contrast, the *charged* benzothiazepine diltiazem favors a membrane location with a more limited time-averaged distribution near the phospholipid headgroups. The insertion of diltiazem into the membrane hydrocarbon core may be sterically constrained due to the large heterocyclic ring structure of the molecule.

By partitioning to specific regions in the membrane hydrocarbon core, CCB may inhibit the propagation of lipid peroxidation by directly scavenging free radicals and/or by altering intermolecular packing of phospholipid molecules. Nisoldipine, in particular, has a time-averaged location near the phospholipid acyl chain unsaturated double bonds, an important target for lipid peroxidation, as illustrated in Fig. 4. In addition to directly scavenging free radicals, CCB molecules intercalate into the membrane hydrocarbon core and disrupt phospholipid intermolecular packing, as evidenced by changes in thermotropic properties including ΔH and T_m. As a consequence, CCB may interfere with rapid propagation of unstable free electrons between adjacent phospholipid acyl chains. Indeed, the effects of these compounds on membrane thermotropic properties (Table 1) correspond well with their antiperoxidation activities in microsomal and lipoprotein systems (nisoldipine > nifedipine ≫ diltiazem), as previously reported [8–10, 17]. This study provides a direct physico-chemical explanation for the differential lipid antiperoxidative activities of these representative CCB analogs. This mechanism of antiperoxidation activity may be analogous to vitamin E, which has been shown to modulate the microviscosity of reconstituted liposomes, as monitored by fluorescence polarization [21].

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