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Cholesterol Alters the Binding of Ca^{2+} Channel Blockers to the Membrane Lipid Bilayer

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SUMMARY

X-ray diffraction and equilibrium binding techniques were used to study the effect of cholesterol on membrane binding of the charged 1,4-dihydropyridine (DHP) Ca^{2+} channel antagonist amlodipine and uncharged isradipine, nimodipine, and nitrendipine. Increases in membrane cholesterol content resulted in a marked decrease in DHP binding to cardiac phospholipid membranes, as expressed by the equilibrium partition coefficient ($K_{P[mem]}$). Between a 0:1 and 0.3:1 cholesterol to phospholipid mole ratio, the $K_{P[mem]}$ values for isradipine, nimodipine, and nitrendipine decreased by >50%, whereas that for amlodipine decreased by only 10%. Electron density profiles calculated from the X-ray diffraction data showed that the time-averaged locations for the DHPs and cholesterol in the membrane overlap, leading to the conclusion that the addition of cholesterol alters the lipid bilayer hydrocarbon core structure in a manner that makes drug partitioning into the membrane less energetically favorable. These data support the idea that drug interactions with the anisotropic membrane environment are complex and may be greatly influenced by cholesterol composition. This effect of cholesterol was also observed for phenylalkylamine (verapamil) and benzothiazepine (diltiazem) Ca^{2+} channel blockers. The DHP amlodipine had the highest membrane partition coefficient ($K_{P[mem]} > 10^4$) and the slowest rate of dissociation and was affected least by membrane cholesterol content. The combination of electrostatic and hydrophobic bonding between amlodipine and membrane phospholipid may explain the high affinity of this drug for the membrane bilayer with normal and elevated cholesterol. The results of this study show that cholesterol content differentially affects the membrane-binding properties of the charged DHP amlodipine, compared with other Ca^{2+} channel blockers. These data help explain the biological distribution of these drugs and the distinct pharmacokinetics of amlodipine versus other Ca^{2+} channel blockers.

The DHP Ca^{2+} channel blocker amlodipine has distinct pharmacological properties that appear to be mediated by its charged ethanolamine ($pK = 9.02$) side group (1, 2). Based on its observed membrane location and crystal structure, the charged side group of amlodipine is predicted to have electostatic binding with the phospholipid headgroup while the rest of the molecule has hydrophobic interaction with the fatty acyl chains (3).

The molecular basis for the distinct pharmacokinetics of amlodipine may be its strong interaction with the membrane lipid bilayer (2, 3). Several lines of evidence support a "membrane pathway" for the DHP binding mechanism, in which Ca^{2+} channel binding is preceded by drug partitioning into and lateral diffusion through the lipid bilayer phase (4–9). Although the DHP binding site on the Ca^{2+} channel α_1 subunit has not yet been directly determined, biochemical studies suggest that DHPs bind the protein near the cytoplasmic surface of the membrane (10). These studies indicate that DHP partitioning into the target membrane lipid bilayer represents an important component in its overall activity.

In this study, we examined the effect of cholesterol content on the membrane binding of the charged DHP amlodipine versus the uncharged DHPs nimodipine, nitrendipine, and isradipine, as well as phenylalkylamine (verapamil) and benzothiazepine (diltizaem) Ca^{2+} channel blockers. Cholesterol is an abundant plasma membrane component that significantly affects membrane structure and physiology (11). Cholesterol content has been shown to change substantially in arterial smooth muscle as a function of age (12) and under various pathological conditions, including experimental diabetes (13), chronic cigarette smoking (14), and dietary atherosclerosis (15). Specifically, smooth muscle plasma membranes isolated from rabbits with dietary atherosclerosis showed an 80% elevation

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ABBREVIATIONS: DHP, 1,4-dihydropyridine; C:Pl mole ratio, cholesterol to phospholipid mole ratio; HEPES, (*N*-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]; LSR, light sarcoplasmic reticulum; BPC, bovine cardiac phosphatidylcholine; PC, phosphatidylcholine; DOPC, dioleoyl phosphatidylcholine; MLV, multilammelar membrane vesicles.

in the C:PL mole ratio, from 0.38:1 to 0.68:1 (15). Studies in which smooth muscle membrane cholesterol was increased *in vitro* have also shown an elevation in transmembrane extracellular Ca^{2+} uptake, as well as changes in the activity of Ca^{2+} channel blockers (16, 17).

The results of this study demonstrate that cholesterol affects the membrane-binding properties of Ca^{2+} channel blockers. These data also show that amlodipine, in particular, binds strongly to the membrane bilayer and is least affected by elevated cholesterol. The high affinity of amlodipine for the membrane bilayer may mediate its long duration of activity as a Ca^{2+} channel blocker, as well as its potential to affect the atherosclerotic process at very low (picomolar) concentrations, relative to other Ca^{2+} channel blockers (18, 19).

Materials and Methods

All chemicals used were reagent-grade and made up in glass-distilled deionized water. Labeled [^{3}H]isradipine, [^{3}H]nimodipine, [^{14}C]DOPC, [^{3}H]verapamil, and [^{3}H]cholesterol were obtained from New England Nuclear Corporation (Boston, MA). [^{3}H]Amlodipine was provided by Pfizer via Amersham (Amersham, England). [^{3}H]Diltiazem was also obtained from Amersham. Unlabeled amlodipine was provided by Pfizer Central Research (Groton, CT). The drugs were stored at −12°, and the photosensitive drugs were protected from light. Due to the limited aqueous solubility of some of the drugs, labeled drugs were dissolved in 100% ethanol. The final concentration of ethanol in all binding reaction mixtures was <0.001%.

Unlabeled DOPC, cholesterol, and BPC lipids were purchased from Avanti Polar Lipids, Inc. (Pelham, AL). The purity of the BPC lipids was assessed using thin layer chromatography, as described previously (20). The primary fatty acid components of the BPC lipids were determined by the method of Christie (21), using fatty acid methyl esters. Gas-liquid chromatographic analysis showed the following constituents: 18:2 linoleic acid (29.9%), 16:0 palmitic acid (22.5%), 18:1 oleic acid (13.3%), 20:4 arachidonic acid (11.4%), and 20:3 homogamma linoleic acid (4.6%), with small amounts (<1% each) of palmitoleic acid (16:1), linolenic acid (18:3), 11,14-eicosadienic acid (20:2), and myristic acid (14:0) (Avanti Polar Lipids, Inc.).

Preparation of biological membranes. LSR vesicles were isolated from New Zealand white rabbit hind leg and back white muscle, by a modification (22) of the method of Harigaya and Schwartz (23). The protein content was determined by the method of Lowry *et al.* (24) and phosphate by the methods of Chester *et al.* (20). A colorimetric assay was used to measure total cholesterol in the LSR by a modification of the cholesterol oxidase method (25), as described by Chester *et al.* (26). Cholesterol was extracted from native membrane lipids using a chloroform/methanol/water system, as described by Folch *et al.* (27). The efficiency of cholesterol extraction was quantitated with [^{3}H] cholesterol and [^{14}C]DOPC analogs that were incubated with the membranes.

Preparation of MLVs for X-ray diffraction and binding assays. For binding experiments and structure studies, MLVs were used. The lipid used for these MLV was cardiac BPC. MLVs were also useful for filtration experiments, because they efficiently bind to glass fiber filters used for nonspecific membrane kinetic experiments. Using radiolabeled phospholipid and cholesterol analogs, it was shown that 80% of the MLV lipid was reproducibly retained on the filter after filtration experiments (three trials, 18 determinations). The kinetics of nonspecific DHP interactions with MLVs and unilamellar vesicles were similar, indicating that these drugs gain access to all available sites in the bilayers that comprise the MLVs (see also Ref. 6).

Phospholipid/cholesterol MLVs for X-ray diffraction and partition coefficient measurements were prepared as described in detail previously (20, 28). Phospholipid (either synthetic or native) and cholesterol dissolved in chloroform were dried down as a thin film on the bottom of 13- × 100-mm glass test tubes, by vortexing under vacuum. For X-ray diffraction experiments, a specified volume of 0.5 mM HEPES, pH 7.27, 2 mM NaCl, was added to the dried lipid preparation, yielding a final lipid phosphorus concentration of 6.36 mM. For partition coefficient experiments, a specified volume of 150 mM NaCl, pH 7.0, 10 mM Tris, was added to the dried lipid preparation, yielding a final lipid phosphorous concentration of 2.54 mM. The vesicles were then formed by vortexing of the buffer and lipids for 3 min.

Preparation of oriented membrane multilayers for X-ray diffraction. Oriented membrane multilayer samples were prepared as described in detail in a previous communication (7). Briefly, 50 μl of the BPC membrane suspension were added to Lucite sedimentation cells containing an aluminum foil substrate. The normal SW-28 rotor (Beckman Instruments, Inc., Fullerton, CA) bucket caps were then replaced with "spin dry caps" (containing a 100-μm hole in the center), and the pelleted vesicles were spin dried at 65,000 × g for 3 hr, under centrifuge vacuum, at 15°. 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 relative humidity for X-ray samples were LiCl, 13%; $Mg(Cl)_2$, 32%; K_2CO_3, 45%; $Mg(NO_3)_2$, 55%; and $NaNO_2$, 66%.

Small-angle X-ray diffraction data collection and reduction. 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, $\lambda = 1.54$ Å) from an Elliot GX-18 rotating anode X-ray generator (Enraf Nonius, Bohemia, NY). The experimental method used a single Franks' mirror defining a line source where $K_{\alpha 1}$ and $K_{\alpha 2}$ are unresolved. Sample temperature was controlled during the diffraction experiments.

The diffraction data from the MLV samples were recorded on both Kodak DEF-5 film (Eastman Kodak Co.) and a Braun position-sensitive one-dimensional detector (Innovative Technologies, Inc., Newburyport, MA). Relative intensities for the diffraction orders were obtained directly from digitized computer plots of the detector data, using an integration routine. Data reduction (background subtraction and other geometrical corrections) for either method of data collection has been described previously (29). The lamellar intensity functions from the BPC samples collected with the electronic detector were corrected by a factor of $s = 2\sin\theta/\lambda$, the Lorentz correction.

To phase the lamellar reflections for each experiment, a hydration series, or swelling analysis, was carried out (30). We used at least 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.

Drug-membrane partition coefficients. Drug partition coefficients into MLVs composed of DOPC and BPC lipids were experimentally measured using vacuum filtration, as described in detail previously (6). To compare the binding data from these model membranes with those from an intact native membrane system, we used LSR (0.9 ± 0.1 nmol of phospholipid/μg of protein). Because LSR does not contain any specific DHP receptor sites, DHP binding to this membrane is entirely nonspecific. The filtration method was selected for this study because of its ability to separate membrane-bound radioligand from free radioligand rapidly and efficiently.

To obtain total nonspecific binding, the filters for these experiments were not washed. Reaction mixtures containing drug but no membrane were used to correct for the total nonspecific binding from binding of drug directly to filters. All filters were counted for radioactivity. The amount of drug in the filtrate was determined by subtracting the number of moles of drug bound to the membrane from the total number of moles of drug added to the reaction mixtures. This information, along with the total number of moles in the reaction mixture and the amount of lipid present, corrected for the recovery of membrane on the filters, allowing membrane partition coefficients to be calculated for each trial.

The incubation reaction consisted of a fixed phospholipid concentration, 20 μg/ml phospholipid, in pH 7.0 buffer (10 mM Tris, 150 mM NaCl, 21°). In experiments examining the effect of cholesterol on drug partitioning, the phospholipid concentration was kept constant. Cholesterol was added to the lipid suspension in choloroform, before vesicle formation, at C:PL mole ratios of 0:1 to 0.6:1. To multilammelar DOPC and BPC membranes, as well as unilammelar LSR vesicles, radiolabeled drug was added to produce a final concentration of drug in the reaction mixture of 5×10^{-10} M. These aqueous drug concentrations were similar to the K_d concentrations of these drugs. The aqueous concentration of drug was not significantly depleted during the partitioning reactions, because the membrane volume was relatively very low.

Membrane partition coefficients were calculated using the following equation:

$$K_{P[mem]} = \frac{\text{(g of drug bound to membrane/g of lipid)}}{\text{(g of drug in supernatant/g in buffer)}}$$

The amounts of drug on the filter (bound to membrane) and in the filtrate were determined as described above.

Drug dissociation rates from membranes. The time dependence of drug release from model membranes was measured by filtration with 5×10^{-10} M concentrations of [^{3}H]isradipine, [^{3}H]nimodipine, and [^{3}H]amlodipine incubated with 20 μg/ml BPC MLVs, at a 0:1 and 0.3:1 C:Pl mole ratio, for 30 min at 25° in pH 7.0 buffer (10 mM Tris, 150 mM NaCl, 21°). All solutions were filtered through Whatman GF/C glass fiber filters, on a Brandel cell harvester, but not washed. Control reaction mixtures contained drug but no membranes. Three filters with membranes and control filters were immediately counted for radioactivity (maximal binding of drug to the membranes). The remaining filters were attached to support pins and submerged in 400 ml of buffer at 25°, with stirring. At appropriate time intervals, filters with and without membranes were removed from the buffer and assayed for radioactivity, and the percentage of drug remaining bound to membranes was calculated for each drug. As in the other filtration experiments, ^{14}C-labeled phospholipid was used to verify that membrane was not lost during incubation on the filter.

Results

Effect of increasing cholesterol on the electron density profiles of BPC oriented membranes. To examine the effect of cholesterol on the structure of phospholipid membrane bilayers, X-ray diffraction techniques were utilized. Oriented membrane multilayers prepared in the presence and absence of cholesterol gave clearly defined, reproducible, diffraction patterns (Table 1). In Fig. 1, the correctly phased electron density profiles for BPC membranes in the presence of 0:1, 0.3:1, and 0.6:1 C:PL mole ratios were superimposed. Six-order diffraction was obtained for these samples over a humidity range from 13 to 84% relative humidity at 15°.

At the resolution of the electron density profiles (8 Å), the effect of cholesterol on the structure of the BPC membrane could be clearly seen and compared (Fig. 1). There was a substantial successive increase in the electron density within the hydrocarbon core of BPC membranes from approximately 5 to 16 Å from the bilayer center. This increase over an extent of approximately 11 Å is consistent with the cholesterol molecule being oriented in the membrane with its long axis parallel to the phospholipid acyl chains and the hydroxyl group located in the vicinity of the phospholipid carbonyl group (31, 32). The increase in electron density through this large region of the hydrocarbon core would be attributed to the contribution of

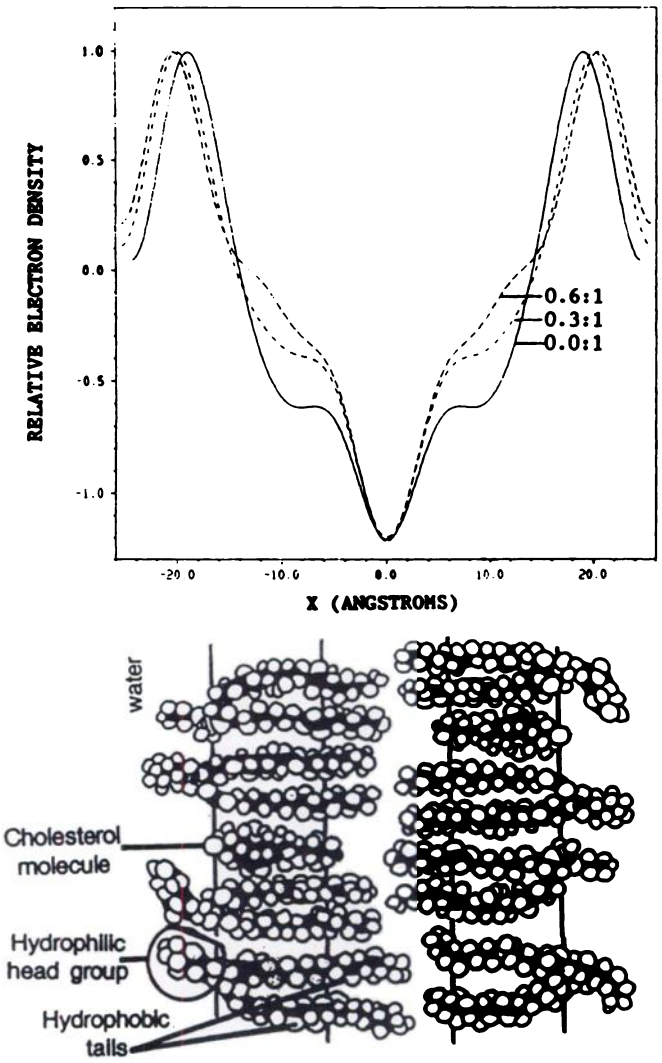


Fig. 1. Membrane bilayer electron density profiles. In the upper panel, comparison of electron density profiles for BPC oriented membrane multilayers in the absence (——) and presence of a 0.3:1 (– – –) and 0.6:1 (— —) C:Pl mole ratio. The two maxima of electron density at the sides of the figure correspond to the electron-dense phosphate headgroups, whereas the minimum of electron density in the center of the figure corresponds to the membrane bilayer center. The profiles were placed on the same scale, to match the peaks of electron density with the electron density minima. The profiles were correlated with a phospholipid/cholesterol membrane bilayer in the lower panel. The long axis of the cholesterol molecule is aligned parallel to the phospholipid acyl chains. The successive addition of cholesterol to the lipid bilayer produced a broad increase in electron density within the hydrocarbon core in a region approximately 11 Å in width, approximately the length of the heterocyclic ring structure of cholesterol. Further, there was an outward displacement in the phosphate headgroups after the addition of cholesterol.

TABLE 1

Lorentz-corrected structure factors and membrane bilayer width (*D*) for BPC with and without cholesterol

Structure factors		
0:1 C:PL (*D*=49 Å)	0.3:1 C:PL (*D*=51 Å)	0.6:1 C:PL (*D*=51 Å)
1 = −0.1333	1 = −0.1313	1 = −0.1322
2 = −0.0091	2 = −0.0158	2 = −0.0213
3 = +0.0295	3 = +0.0134	3 = 0.0000
4 = −0.0486	4 = −0.0504	4 = −0.0418
5 = +0.0086	5 = +0.0117	5 = +0.0148
6 = −0.0089	6 = −0.0136	6 = −0.0190

the electron-rich conjugated ring structure of cholesterol, as well as the effect of cholesterol on ordering the acyl chain region of the bilayer (i.e., decreasing *trans-gauche* isomerizations) in the portion of the hydrocarbon core region adjacent to the glycerol backbone (11). These data confirm previous findings that indicate that the presence of cholesterol substantially affects the structure of the phospholipid bilayer by altering the phospholipid acyl chain packing characteristics (11, 33).

Effect of membrane cholesterol content on drug partitioning. Previous X-ray and neutron diffraction studies show that DHPs, including amlodipine and nimodipine, have a similar time-averaged location near the hydrocarbon core/water interface, a region that overlaps the location of free cholesterol in the membrane (3, 7, 34). It was not surprising, therefore, based on the structure data as well as fluorescence polarization studies done under similar conditions (33), that membrane cholesterol content would significantly affect the binding of these drugs to the membrane.

Table 2 summarizes the effect of cholesterol content on the $K_{P[mem]}$ of the DHPs (amlodipine, isradipine, and nimodipine), as well as the phenylalkylamine verapamil and benzothiazepine diltiazem, at 21°. These results are expressed as the mean ± standard error, with a sample size of at least $n = 12$. These data show a strong negative correlation between membrane cholesterol content and drug partition coefficients. Interestingly, the effect of membrane cholesterol content on amlodipine partitioning was substantially less than for the other Ca^{2+} channel blockers, whereas the overall $K_{P[mem]}$ values for amlodipine were much higher. Although diltiazem is also a charged molecule and is readily soluble in water, it had the lowest $K_{P[mem]}$ values and was highly sensitive to cholesterol content. These results demonstrated that a positive charge alone does not predict either high $K_{P[mem]}$ values or a diminshed effect of cholesterol on drug partitioning. The same can be said for the charged verapamil compound, which has a membrane location similar to that of the DHPs in the membrane in the presence of cholesterol (data not shown).

To correlate the results from a synthetic PC lipid system (DOPC) with results in native PC lipids composed of heterogeneous acyl chains, we examined the partitioning of the DHPs in cardiac BPC (Table 3). Although the absolute partitioning values are lower overall, the effect of cholesterol is reproduced. The decrease in partitioning is attributed to the high percentage of diunsaturated linoleic acid (~30%) and saturated palmitic acid (~20%) in the BPC lipids, which would serve to increase the density of the hydrocarbon acyl chains, compared with the monounsaturated DOPC acyl chains (33).

These data suggest that amlodipine has distinct chemical and physical properties that affect its interactions with the membrane bilayer, relative to those of the other Ca^{2+} channel blockers. Amlodipine is an amphipathic molecule with a charged 2-aminoethoxymethyl substituent at the 2-position of the dihydropyridine ring ($pK_a = 9.02$), which, in its crystal structure, extends away from the hydrophobic portion of the molecule (3). Although amlodipine is soluble in water and has a low partition coefficient in a hydrophobic octanol/buffer system ($K_{P[oct]} = 30$), its $K_{P[mem]}$ values were substantially higher than those of the neutral DHPs, which are nearly insoluble in water. Molecular modeling of amlodipine, based on its location in the membrane bilayer and crystal structure, predicts that the charged portion of the drug molecule interacts with the anionic oxygen in the phospholipid headgroup while the hydrophobic DHP and chlorophenyl ring structures penetrate into the hydrocarbon core, as illustrated in Fig. 2. The combination of ionic and hydrophobic bonding between amlodipine and the phospholipid molecules may help to explain the high $K_{P[mem]}$ values observed for this drug, as well as the weaker dependence of its membrane partitioning on membrane cholesterol content (Fig. 3).

The $K_{P[mem]}$ values measured for amlodipine and nimodipine into BPC/cholesterol MLVs were very similar to $K_{P[mem]}$ measurements in intact skeletal LSR, despite the fact that the LSR contains protein (0.9 ± 0.1 nmol of lipid/μg of protein) and a heterogeneous phospholipid composition (Table 4). These results argue that DHP partitioning depends primarily on the C:PL mole ratio.

Dissociation of drugs from membranes as a function of cholesterol content. In addition to affecting the $K_{P[mem]}$ values for the DHPs, membrane cholesterol content substantially modulated the dissociation of these drugs from the membrane. Table 5 compares the rate of dissociation for these drugs from BPC membranes in the presence and absence of cholesterol. The $t_{1/2}$ of dissociation for nimodipine was 2-fold longer in the absence of cholesterol (8 min), compared with a 0.3:1 C:PL mole ratio (4 min). In contrast, the dissociation of amlodipine was essentially unaffected by cholesterol content, and its rate of dissociation was >1 order of magnitude slower than that of the other drugs in the presence of cholesterol.

TABLE 2

Drug partition coefficients into DOPC membranes as a function of the C:PI mole ratio

Drug concentrations were maintained at 5×10^{-10} M. Phospholipid concentration was 20 μg/ml (pH 7.0, 21°).

Drug	Partition coefficient		
	0:1 C:PL	0.3:1 C:PL	0.6:1 C:PL
Amlodipine	34,400 ± 1,300	21,800 ± 700	12,000 ± 400
Isradipine	11,400 ± 100	6,000 ± 400	2,200 ± 200
Nimodipine	6,300 ± 300	2,700 ± 200	1,200 ± 100
Verapamil	5,600 ± 300	2,700 ± 400	800 ± 300
Diltiazem	900 ± 30	600 ± 30	200 ± 10

TABLE 3

DHP partition coefficients into cardiac PC membranes as a function of the C:PI mole ratio

Drug concentrations were maintained at 5×10^{-10} M. Phospholipid concentration was 20 μg/ml (pH 7.0, 21°).

Drug	Partition coefficient		
	0:1 C:PL	0.3:1 C:PL	0.6:1 C:PL
Amlodipine	21,300 ± 1,200	19,300 ± 1,300	7,700 ± 500
Nitrendipine	10,100 ± 500	4,700 ± 400	800 ± 200
Isradipine	5,800 ± 400	2,900 ± 200	900 ± 100
Nimodipine	5,300 ± 400	2,100 ± 200	470 ± 30

Discussion

The results of this study demonstrate the complexity of drug interaction with the membrane. The chemical and crystal structure of the drug alone does not provide sufficient information with which to predict drug/membrane interactions. Traditional methods to predict the "lipophilicity" of drugs by measuring partition coefficients into nonpolar solvents such as octanol appear to be inadequate for DHPs and other drugs (Table 4). The equilibrium and kinetic parameters of DHP binding to the

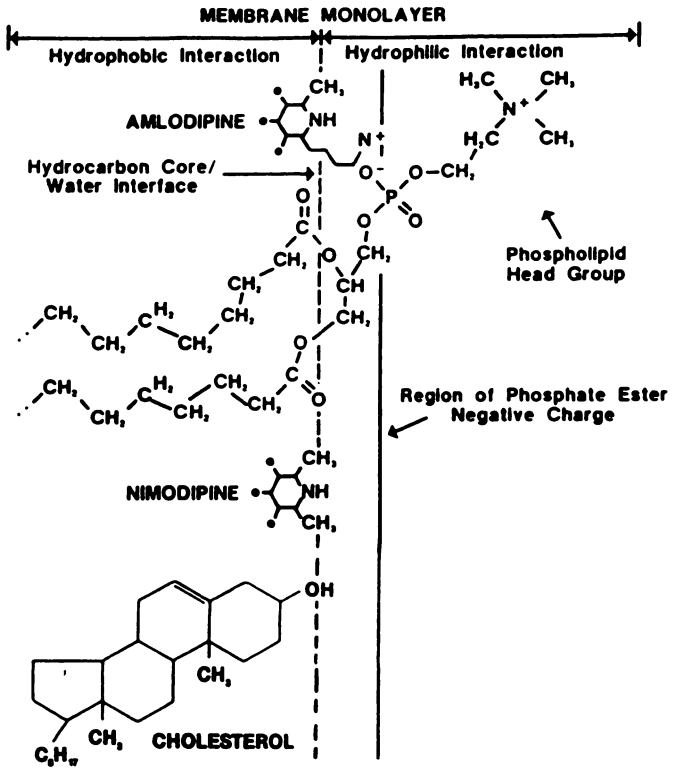
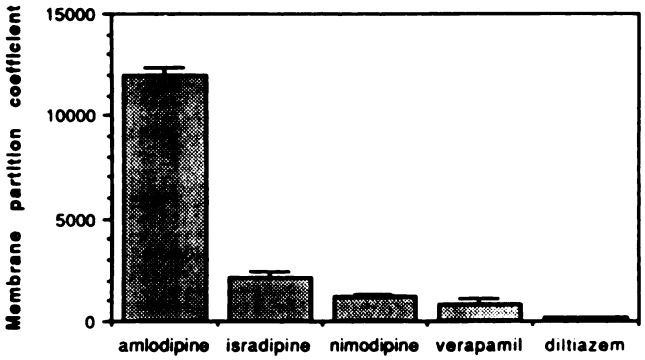


Fig. 2. Molecular model of drug/membrane interactions. This figure illustrates the possible interactions of amlodipine and nimodipine with a cholesterol/phospholipid monolayer. This model is based on the observed location of these drugs in the membrane and their crystal structures (3, 34). The location of amlodipine near the hydrocarbon core/water interface allows both hydrophobic interactions with phospholipid fatty acyl chains and electrostatic bonding between the charged ethanolamine side group and anionic oxygen of the phosphate moiety. These interactions may account for amlodipine's high $K_{P[mem]}$ values and slow dissociation from the membrane. The nimodipine chemical structure would predict primarily hydrophobic interactions with the membrane. The position and orientation of cholesterol in the membrane overlap the location of the DHPs (Fig. 1). By ordering the hydrocarbon acyl chains adjacent to the glycerol backbone, cholesterol affects the partitioning of DHPs to this region of the membrane. The ionic interactions of amlodipine with the phospholipid headgroup may account for the weaker dependence of its partitioning on membrane cholesterol content, compared with nimodipine.



a. Drug concentrations were maintained at 5x10-10 M.
DOPC membrane concentration was 20 ug/ml (pH 7.0, 21 C).
b. The Cholesterol:DOPC mole ratio was 0.6.

Fig. 3. Effect of membrane cholesterol content on drug partitioning. This figure compares the membrane partition coefficients for several Ca^{2+} channel blockers under atherosclerotic-like conditions of elevated membrane cholesterol (0.6 C:PI mole ratio). These data were obtained for DOPC membranes. Similar results were obtained with a cardiac BPC membrane system (Table 3). The data represents the mean ± standard error (12 experiments).

TABLE 4

Comparison of drug partition coefficients in native versus model systems

Drug	Partition coefficient		
	LSR (0.1:1 C:PL)	BPC/cholesterol (0.1:1 C:PL)	Octanol/buffer
Amlodipine	19,000 ± 700[a]	19,900 ± 1,200	30 ± 4[a]
Nimodipine	5,200 ± 400[b]	4,800 ± 400	260 ± 50[b]

[a] From Ref. 3.
[b] From Ref. 6.

TABLE 5

$T_{1/2}$ of drug dissociation from cardiac PC membranes

Concentrations of drugs were 5 × 10^{-10} M. Membrane concentration was 20 μg/ml (pH 7.0, 21°).

Drug	$t_{1/2}$	
	0:1 C:PL mole ratio	0.3:1 C:PL mole ratio
	min	
Amlodipine	120 ± 3	120 ± 3
Nimodipine	8 ± 0.5	4 ± 0.5
Isradipine	5 ± 0.5	3 ± 0.5

membrane bilayer are highly dependent on lipid composition, especially cholesterol content, because cholesterol substantially modulates the structure of the membrane hydrocarbon core (Fig. 1). Small-angle X-ray diffraction studies show that the time-averaged location of DHP analogs and cholesterol overlap in the membrane (Fig. 1). These structural results suggest that an increase in free cholesterol reduces the available volume for drug partitioning in the membrane hydrocarbon core. This conclusion is also supported by fluorescence polarization studies (33).

Cholesterol may affect drug distribution in biological membranes. These data have important implications for understanding biological drug distribution and pharmacokinetics under both normal and pathological conditions in which plasma membrane cholesterol content is substantially elevated.

A membrane bilayer pathway for drug binding would predict that the membrane concentration of drug, as opposed to the aqueous concentration, is in equilibrium with an intrabilayer receptor site (for review of this model, see Ref. 35). The results of this study indicate that the membrane concentration of certain Ca^{2+} channel blockers would be highly affected by dietary atherosclerosis, in which the cholesterol content has been shown to increase by 80% in isolated arterial smooth muscle plasma membranes (15) and by 50% in cardiac membranes (36). For example, an 80% increase in the C:PL mole ratio, from 0.38:1 to 0.68:1 (15), would be expected to reduce the membrane concentration of nimodipine by at least 4-fold. Based on the measured concentration of DHP receptors in cardiac sarcolemma (similar to the concentration in smooth muscle sarcolemma) of approximately one Ca^{2+} channel receptor/10^6 phospholipid molecules (37), a $K_{P[mem]}$ of 500 for nimodipine at a 0.6:1 C:PI mole ratio would result in a drug to receptor ratio of approximately 0.5:1 at the nimodipine K_d (4 × 10^{-10} M aqueous concentration) (6). This was calculated directly from the amount of drug in the membrane at these C:PL mole ratios, with the concentration of nimodipine in the buffer being 5 × 10^{-10} M (the aqueous drug concentration is not

significantly depleted during these experiments). In contrast, the high membrane partition coefficients of amlodipine ($K_{P[mem]} > 7.7 \times 10^3$) (Table 3) at a 0.6:1 C:Pl mole ratio would result in a drug to receptor ratio of 8:1 when the aqueous concentration is 5×10^{-10} M. Thus, after its depletion from plasma and interstitial compartments, amlodipine can remain sequestered in the membrane bilayer of the normal or atherosclerotic target tissue at sufficient concentrations for continued activity over time.

The strong interactions of amlodipine with membrane phospholipid may explain its long duration of activity as a Ca^{2+} channel blocker (1) and its described effect on atherogenesis (18, 19). It is not clear, however, whether the potential antiatherosclerotic activity of Ca^{2+} channel blockers is related to their ability to modulate the Ca^{2+} channel specifically or is due to interaction with other protein and/or lipid sites in the membrane. For example, we have recently observed that amlodipine is able to reverse the effect of cholesterol enrichment on alterations of membrane lipid structure (38).

Conclusion. This study demonstrated that cholesterol has a significant effect on the interactions of Ca^{2+} channel blockers with the membrane bilayer. The charged DHP amlodipine had the highest membrane affinity and was least affected by membrane cholesterol content. The combination of strong electrostatic and hydrophobic bonding between amlodipine and the phospholipid molecules may help to explain the high $K_{P[mem]}$ values observed for this drug, as well as the weaker dependence of its membrane partitioning on membrane cholesterol content and acyl chain composition, compared with the neutral DHPs. Finally, the interactions of amlodipine with the membrane may be part of the molecular basis for its pharmacological properties, including its long duration of activity as a Ca^{2+} blocker and its ability to exhibit antiatherogenic activity, as evidenced by potent antiproliferative and antimigrational effects on arterial smooth muscle cells.

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