Interaction of Calcium Channel Antagonists with Calcium: Spectroscopic and Modeling Studies on Diltiazem and Its Ca^{2+} Complex

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Using spectral techniques, the solution conformation of diltiazem was studied in acetonitrile with special reference to the effect of Ca^{2+} on the drug structure. Complete assignment of the proton resonances in the ^{1}H-NMR spectrum of the drug was made using one- and two-dimensional spectral analyses. A two-dimensional ^{1}H-NOESY spectrum (in the phase-sensitive mode) was obtained to identify the *inter*proton connectivities in the drug molecule. A molecular modeling program involving Monte Carlo simulation and energy minimization was employed to arrive at the structure of the drug. The program was run with and without the input of the *inter*proton distances derived from the NOESY cross peaks. Both the protocols led to a structure of the drug which was generally similar to that reported from X-ray diffraction data on crystalline diltiazem hydrochloride (Kojic-Prodic, et al. *Helv. Chim. Acta* 1984, *67*, 916–926). However, significant differences between the two structures were seen in the orientations of the substituent groups attached to the benzothiazepine ring. Substantial changes in the circular dichroic (CD) and ^{1}H-NMR spectra of diltiazem were observed on addition of Ca^{2+} up to a mole ratio of 0.5 Ca^{2+} per drug. Relatively large changes were seen in ^{1}H resonances of the *N*-methyl protons and the methylene protons attached to the heterocyclic nitrogen. Analysis of the binding isotherms from CD data at 22 ± 1 °C indicated a 2:1 drug:Ca^{2+} "sandwich" complex with an estimated dissociation constant of 140 μM. One-dimensional difference NOE and two-dimensional NOESY spectra revealed *inter*proton connectivities between two drug molecules that were compatible with the sandwich complex formation. The *inter*proton distances derived from the volume integrals of the NOESY cross peaks were used as geometrical constraints in modeling the Ca^{2+}-bound conformation of diltiazem. The minimum-energy conformation corresponded to the sandwich complex where Ca^{2+} was coordinated to three oxygens in each of the two drug molecules. Combined with our earlier data on the ability of diltiazem to translocate Ca^{2+} across the lipid bilayer in synthetic liposomes (Ananthanarayanan, V. S.; Taylor, L.; Pirritano, S. *Biochem. Cell Biol.* **1992**, *70*, 608–612), the structural data presented here point to a role for Ca^{2+} in the interaction of diltiazem with its membrane-bound receptor.

The calcium channel antagonists are a group of structurally diverse compounds that block the influx of extracellular Ca^{2+} through several types of calcium channels.[1] They are extensively used as drugs in the treatment of many cardiovascular disorders.[2] As in the case of other bioactive molecules, understanding the molecular basis of the action of these drugs rests on the characterization of the structures prevailing at the sites of their interaction with the respective membrane-bound receptors (or receptor regions of the calcium channels[1]). This knowledge will be of immense value in designing potent analogs of these drugs. In this regard, the available information on calcium channel antagonists has been obtained largely through studies of drug analogs using criteria such as chemical functionality and hydrophobicity.[1c,d] While such studies have had some success, especially in the case of the dihydropyridines,[1d,3] they tend to be empirical. A better strategy to accomplish structure–function correlation would require a knowledge of the bioactive conformation of the drug prevailing at the site of its interaction with the membrane-bound receptor. This, in turn, would require structure determination under conditions where the drug–receptor interactions are maximal. In the analogous case of peptide hormones, such conditions have been simulated by the use of membrane-mimetic solvents as well as lipid micelles.[4] Similar studies aimed at the bioactive conformations of calcium channel antagonists are, however, unavailable. While the crystal structures of some of these drugs have been known for quite some time,[5-7] it is possible that the bioactive conformation would be different from that found in the crystal structure.[8] Of particular significance to the bioactive structures of calcium channel antagonists are reports of the Ca^{2+} dependence of the drugs' binding to the calcium channels.[1d,9] The allosteric interactions between different classes of calcium channel antagonists at the dihydropyridine receptor also seem to be Ca^{2+}-dependent.[1c,9] On the basis of these observations, we have initiated a detailed structural study, using spectroscopic techniques, on calcium channel antagonists and their interaction with Ca^{2+} in low-dielectric media.[10] This paper deals with diltiazem [(+)-3-acetoxy-5-(2-(dimethylamino)ethyl)-2-(4-methoxyphenyl)-2,3-dihydro-1,5-benzothazepin-4(5*H*)-one] which is widely used as a protective agent in cases of ventricular ischemia and other forms of cardiac arrhythmia.[11]

Experimental Section

Materials. Diltiazem hydrochloride was purchased from Sigma Chemical Co. (St. Louis, MO), deuterated acetonitrile (CD_3CN) from MSD Isotopes (Canada), acetonitrile (CH_3CN) and $Mg(ClO_4)_2$ from Fisher Scientific (Canada), $Ca(ClO_4)_2$ from VWR Scientific (Canada), $Zn(ClO_4)_2$ from GFS Chemicals (Columbus, OH). CH_3CN was dried by treatment with molecular sieves (4 Å) and was kept under argon. All perchlorate salts were

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lyophilized overnight to remove any trace of water as detected by ^1H NMR.

Methods. Circular Dichroism. CD spectra were recorded using a JASCO-J600A spectropolarimeter equipped with a microprocessor for spectral accumulation and data manipulation. All spectra were measured at room temperature (22 ± 1 °C) in dry CH_3CN using a quartz cell of 0.1-cm path length. The mean molar ellipticity $[\theta]$ (deg cm^2 dmol^{-1}) was measured in the absence and presence of $Ca(ClO_4)_2$. The latter was dissolved in CH_3CN and added in 5-μL aliquots to the drug solution inside the CD cell to yield the desired drug:Ca^{2+} ratios. Correction for solvent baseline and dilution effects were routinely made. Drug concentration varied between 1.1 and 2.2 mM.

NMR Spectroscopy. ^1H-NMR spectra were recorded using Bruker AC-200 and AM-500 instruments at ambient temperature (≈22 °C) in the former and at 30 °C in the latter instrument. The drug concentration used was 5.5 mM in CD_3CN. The resonance of the residual proton of CH_3CN in the deuterated solvent was used as an internal reference (a quintuplet set at 1.93 ppm). Analysis of the one-dimensional ^1H-NMR spectrum provided a straightforward way to assign the proton resonances of diltiazem. Two-dimensional homonuclear correlation spectroscopy (COSY) data were employed to confirm these assignments. The standard COSY sequence[12] was used. Titration of the drug with Ca^{2+} was done by adding, with mixing, aliquots of a stock solution of $Ca(ClO_4)_2$ in CD_3CN to the drug solution inside the 5-mm NMR tube to obtain the desired drug/Ca^{2+} mole ratios. Dilution of the sample by Ca^{2+} addition was less than 5%. Spin–lattice relaxation times (T_1) were determined for all protons of diltiazem with and without Ca^{2+} by the inversion recovery method. One-dimensional proton nuclear Overhauser effect (NOE) difference spectra on diltiazem and its Ca^{2+} complex were obtained using the 500-MHz instrument; an off-resonance control spectrum was subtracted from the individual spectra obtained by selective irradiation of each proton signal. The saturation duration of 5 s also served as the relaxation delay for both the control and on-resonance free induction decays (FIDs). The decoupler was gated off during acquisition. Eight scans were acquired for both the control and on-resonance FIDs. A cycle of alternate acquisition of these FIDs was repeated four times for a total of 32 scans for the final difference FID. A 90° ^1H pulse width of 18.6 μs was used. Two-dimensional NOESY spectra were run in the phase-sensitive mode. At least 256 t_1 increments were used with 128 scans per t_1 value. The initial value of the evolution time was 3 μs and subsequent increments were 143 μs. A random variation of 20 ms was added or subtracted from the mixing time in each pulse sequence to minimize contributions from coherent magnetization transfer between scalar-coupled nuclei. The NOE enhancements were linear for mixing times ranging from 0.25 to 1.5 s, indicating negligible contribution from spin–spin diffusion in this range. An optimal mixing time of 1 s was chosen to obtain NOE cross peaks with good signal-to-noise ratios. The two-dimensional NOESY plots were based on a 2K × 2K matrix and are shown in the positive value mode after zero-filling twice in the $f1$ dimension and multiplying with a squared sine-bell function shifted by $\pi/2$ in both dimensions. There were two dummy scans prior to the experiments with a relaxation delay of 3 s between each scan. *Inter*proton distances were evaluated from the volume integrals of the NOESY cross peaks using the 1/(distance)^6 relationship to measure the extent of transfer of magnetization between spin systems during the NOE mixing period.[13] The distance of 2.4 Å between protons H-7 and H-8 in ring C (see Figure 1) was used as the internal standard for this purpose.

Molecular Modeling. This was performed using a Biograf version 2.2 software (Molecular Simulations, Inc., Waltham, MA) on a Personal Iris computer. Using the Monte-Carlo method,[14] two sets of random conformers of the drug (without and with Ca^{2+}) were generated, one with and the other without the incorporation of the distance constraints derived from the NOESY data. In each case, the conformational energies of the generated structures were minimized using the generic force field DREIDING[15] and applying a distance cut off of 9 Å for nonbonded interactions. In the constrained Monte Carlo method, a range of NOESY-based distances was input in the harmonic force field using a force constant of 25 kcal/mol. A dielectric constant of 37.5 was used in these calculations to represent the polarity of the solvent, CH_3CN. A conjugate gradient method was used in the energy minimization so as to achieve a convergence to 0.1 kcal/mol per Å. In modeling the Ca^{2+} complex of diltiazem, the Ca^{2+} ion specific parameters described by Hori et al.[16] were integrated into the Biograf software.

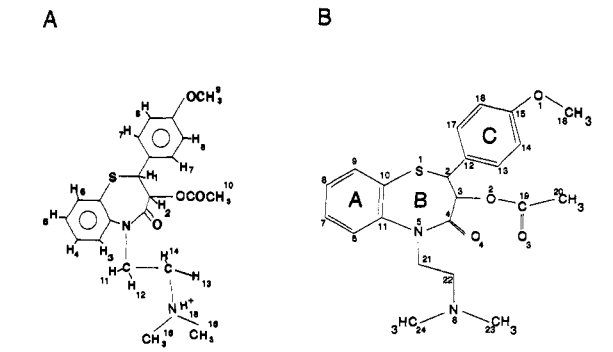

Figure 1. Chemical structure of diltiazem showing the numbering of A (left panel), protons in the molecule as used in labeling the NMR chemical shifts; B (right panel), carbons in the molecule as represented in the X-ray crystal structure.[5]

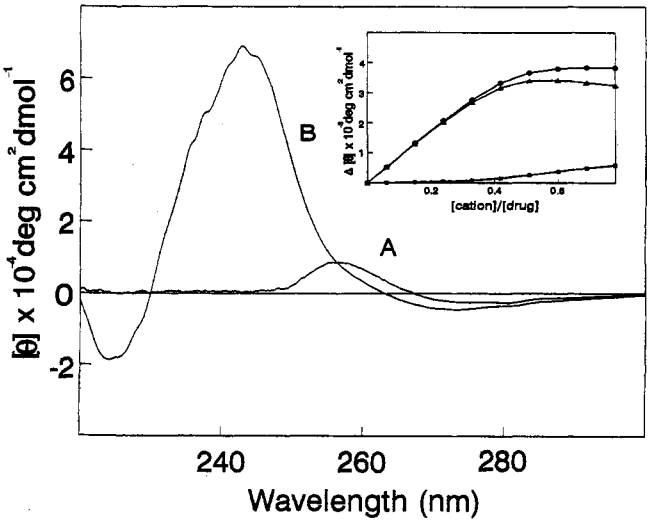

Figure 2. CD spectra of (A) diltiazem and (B) diltiazem + 1.0 molar equiv of $Ca(ClO_4)_2$ in CH_3CN at 22 ± 1 °C. Inset: Ca^{2+} binding curves obtained from changes in $[\theta]$ at 270 nm ($[\Delta\theta]$): (●) experimental data, (▲) computed curve corresponding to the 2:1 drug–Ca^{2+} complex, (■) computed curve for the 1:1 drug–Ca^{2+} complex. The method of Reuben[17] was used to obtain the computed curves (see text).

Results

For use in the interpretation of the CD and NMR data presented below, the chemical structure of diltiazem is shown in Figure 1, indicating the two labeling schemes for the ring structures and the protons, one used when dealing with NMR data and the other when relating to the X-ray structure.[5]

Studies on the Free Drug. CD Data. In the wavelength range 220–300 nm, the CD spectrum of diltiazem in CH_3CN displayed a positive band at 255 nm and two negative bands centered, respectively, at 275 and 225 nm (Figure 2); the latter is relatively much smaller in magnitude and is not observable in the figure. The negative bands in CH_3CN are shifted by about 10 nm compared to those reported in methanol by Kojic-Prodic et al.[5] Using the interpretation of these authors, the CD spectrum of diltiazem in CH_3CN shows that the molecule assumes the *S* configuration at the two asymmetric centers (see Figure 1).

Table I. ^1H Chemical Shifts and Spin–Lattice Relaxation Times (T_1) for Diltiazem and Its Ca^{2+} Complex in CD_3CN at 22 °C

	free drug		drug + Ca^{2+} (2:1 mole ratio)	
proton	chemical shift (ppm)	T_1[a] (s)	chemical shift (ppm)	T_1 (s)
H-1	5.03[b]	2.10[b]	5.05	1.16
H-2	5.03[b]	2.10[b]	5.26	0.90
H-3	7.59	2.04	7.61	1.80
H-4	7.62	2.33	7.53	1.28
H-5	7.37	2.29	7.35	1.23
H-6	7.74	3.19	7.76	1.56
H-7	7.39	1.76	7.40	1.00
H-8	7.03	2.52	6.93	1.44
H-11	4.16	0.59	4.13	0.49
H-12	4.64	0.67	4.47	0.35
H-13	3.11	0.77	3.29	2.07
H-14	3.39	0.74	3.48	0.40
H-15[c]	2.15	3.48	2.19	3.12
CH_3-9	3.81	1.74	3.80	1.01
CH_3-10	1.81	1.56	1.82	1.01
CH_3-16	2.73	1.09	2.86	0.58

[a] T_1 values were determined to any accuracy of three significant numbers above 1s and four significant numbers below 1s. [b] These two protons could not be discriminated in the free drug but are resolved in the Ca^{2+} complex. [c] The assignment of this NH^+ proton was made on the basis of the COSY connectivities as well as the observation that addition of a trace of D_2O diminished its resonance significantly while it increased the resonance of the residual water peak shown in Figure 4F.

NMR Data. The proton resonances were fully assigned from the one-dimensional spectrum, using their respective chemical shifts, multiplicities, and coupling constants. Two-dimensional COSY spectra obtained under the same conditions (data not shown) reaffirmed these assignments. Both H-1 and H-2 (refer to Figure 1A) appeared at 5.03 ppm and could not be differentiated because of overlap; they were, however, resolved in the drug–Ca^{2+} complex (see below). The ^1H chemical shift data for diltiazem in CD_3CN are presented in Table I. Two-dimensional NOESY spectral data on diltiazem were collected so as to obtain further information of its molecular geometry. The NOESY spectrum of the drug is shown in Figure 3A. In order to verify that the *inter*proton connectivities observed as cross peaks in the NOESY spectra are not due to artifacts (e.g., for t_1 noise and t_2 ridge which could cause problems in assignments of some cross peaks such as those involving H-16 in Figure 3A), we also carried out a complete one-dimensional NOE difference spectral analysis of the drug molecule. All the NOESY connectivities (as well as additional ones not observable under the NOESY set up conditions) were also seen in the one-dimensional NOE data. Figure 4A shows some representative difference NOE spectral data. The *inter*proton connectivities obtained from the NOESY spectra as well as a few of the important additional connectivities obtained from the one-dimensional NOE data are shown in Table II. These connectivities (except those involving H-1 and H-2 which were not distinguishable) were used in deriving distance constraints (see Methods) for the molecular modeling of the uncomplexed form of the diltiazem molecule (see below).

Studies on the Drug–Ca^{2+} Complex. CD Spectral Changes. Progressive addition of Ca^{2+} to diltiazem in CH_3CN produced dramatic changes in the drug's CD spectrum as shown in Figure 2. The positive and negative CD bands of the drug increased in magnitude sizably on Ca^{2+} addition until saturation was obtained at 0.5 mole ratio of Ca^{2+} (Figure 2, inset). A large shift in the positive band position from 255 to 243 nm was also seen. The binding isotherm was analyzed using the method of Reuben[17] which provides for the following multiple equilibria:

$$2(\text{drug}) + Ca^{2+} \rightleftharpoons [(\text{drug})_2 \cdot Ca^{2+}]$$

$$[(\text{drug})_2 \cdot Ca^{2+}] + Ca^{2+} \rightleftharpoons 2[\text{drug} \cdot Ca^{2+}]$$

The results shown in the inset in Figure 2 demonstrate that the observed CD changes during Ca^{2+} binding arise almost solely from the formation of the 2:1 drug–Ca^{2+} complex (known as the "ion sandwich" complex[18]). Addition of Mg^{2+} caused changes in diltiazem CD in CH_3CN similar to those caused by Ca^{2+} (data not shown). A similar but much reduced (by more than six times) CD change was observed with Zn^{2+} whereas Na^+ addition elicited no CD changes even at a 4 M excess of the cation (data not shown).

NMR Changes and Spectral Analysis. Significant changes in the ^1H-NMR spectrum of diltiazem were observed[19] on addition of $Ca(ClO_4)_2$ in CD_3CN up to a mole ratio of 0.5 Ca^{2+}. Substantial changes in the positions of the methylene protons H-12, H-13, and H-14 and in the *N*-methyl protons H-16 were observed (compare spectra A and F in Figure 4). Reuben's analysis[17] of the binding isotherms obtained from the H-12 and H-13 proton data showed a predominantly 2:1 drug–Ca^{2+} complex as in the case of the CD data. The consensus value for the dissociation constant of this complex obtained from the CD and NMR data was 140 μM.

Details of the conformational changes associated with Ca^{2+} binding were sought from one- and two-dimensional NOE spectral analyses. Calcium addition caused some of the *inter*proton connectivities in diltiazem to disappear and some new ones to arise. This is seen from the NOESY spectrum shown in Figure 3 and NOE difference spectra shown in Figure 4 (spectra G–K). Knowledge of the 2:1 drug–Ca^{2+} stoichiometry suggested that some of the above connectivities might arise from *inter*molecular interactions in the sandwich complex. The absence of any nonspecific *inter*molecular interactions, such as those arising from aggregation, was confirmed by the fact that the observed connectivities were unaffected by a 10-fold change in the drug concentration.

Molecular Modeling. The structures of free and Ca^{2+}-bound forms of diltiazem were obtained by using the modeling protocol described in the Experimental Section. In both cases, structures were computed with and without using the distance constraints derived from the NOESY data. In the case of the free drug, the X-ray crystal structure of diltiazem hydrochloride reported by Kojic-Prodic et al.[5] was used as the starting conformation. The torsional angles of this structure were then allowed to change freely using the Monte Carlo technique. The energies of 200 random structures of the drug thus generated were then minimized incorporating the distance constraints derived from the NOE data (Table II). This yielded one lowest energy conformer whose structure is shown in Figure 5A. In the unconstrained modeling protocol, this minimum energy structure was used as the starting conformation and, using the Monte Carlo protocol, its torsional angles were allowed to change randomly as before. The energies of the resulting 200 conformers were then minimized without the restrictions imposed by the NOE data. The structure of the lowest energy conformer

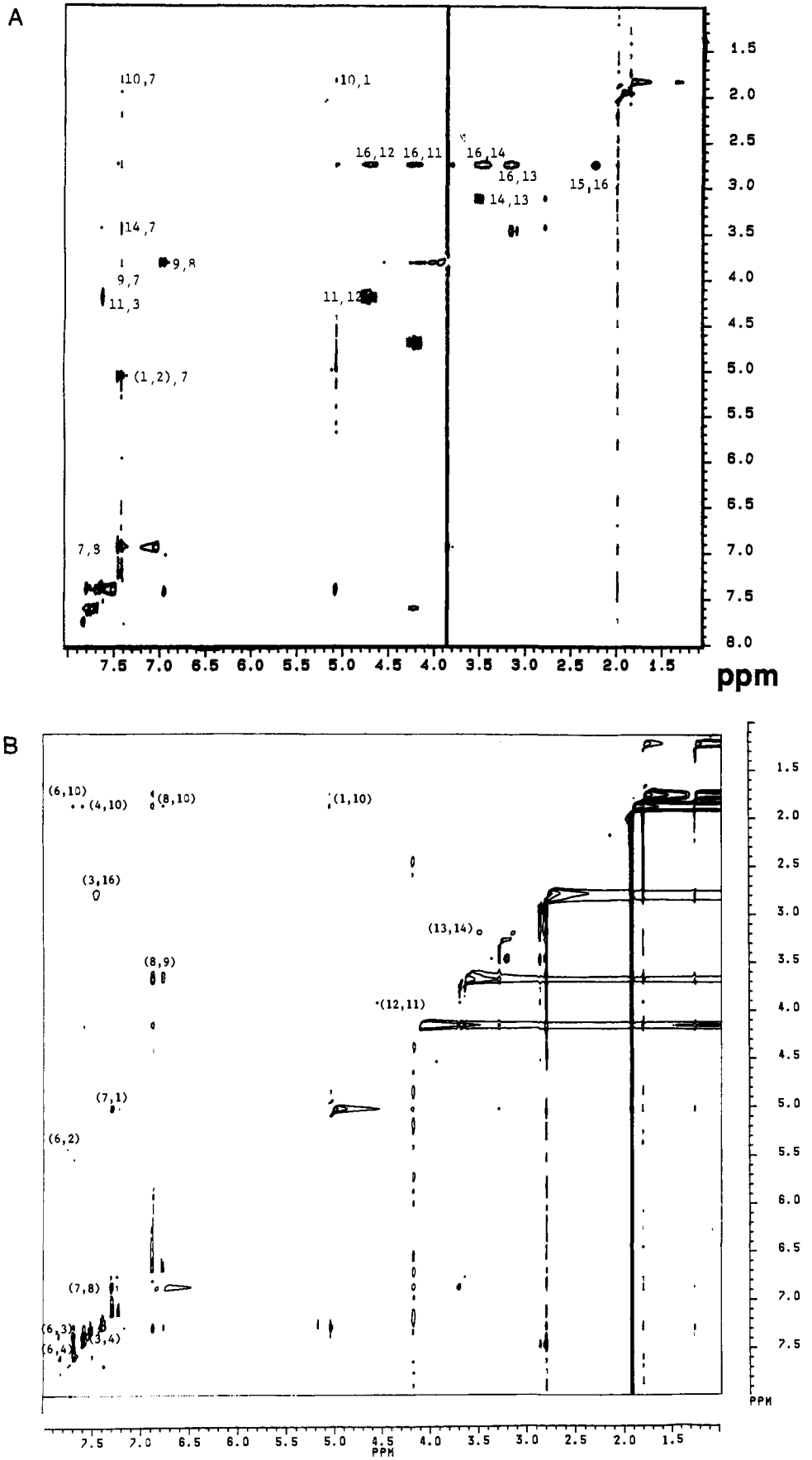

Figure 3. Two-dimensional phase-sensitive ^{1}H-NOESY spectra of diltiazem in CD_3CN at 22 ± 1 °C: (A) drug alone; (B) drug + 0.75 molar equiv of Ca^{2+}. The spectrum is shown in the positive mode. The diagonal peaks and cross peaks due to J correlation were observed in the negative phase (data not shown).

thus derived, as well as the structures of several other low energy conformers,[20] lay within an rms deviation of 1.5 Å from the minimum-energy structure (Figure 5A) derived with NOE distance constraints.[21] Shown in Figure 5B is

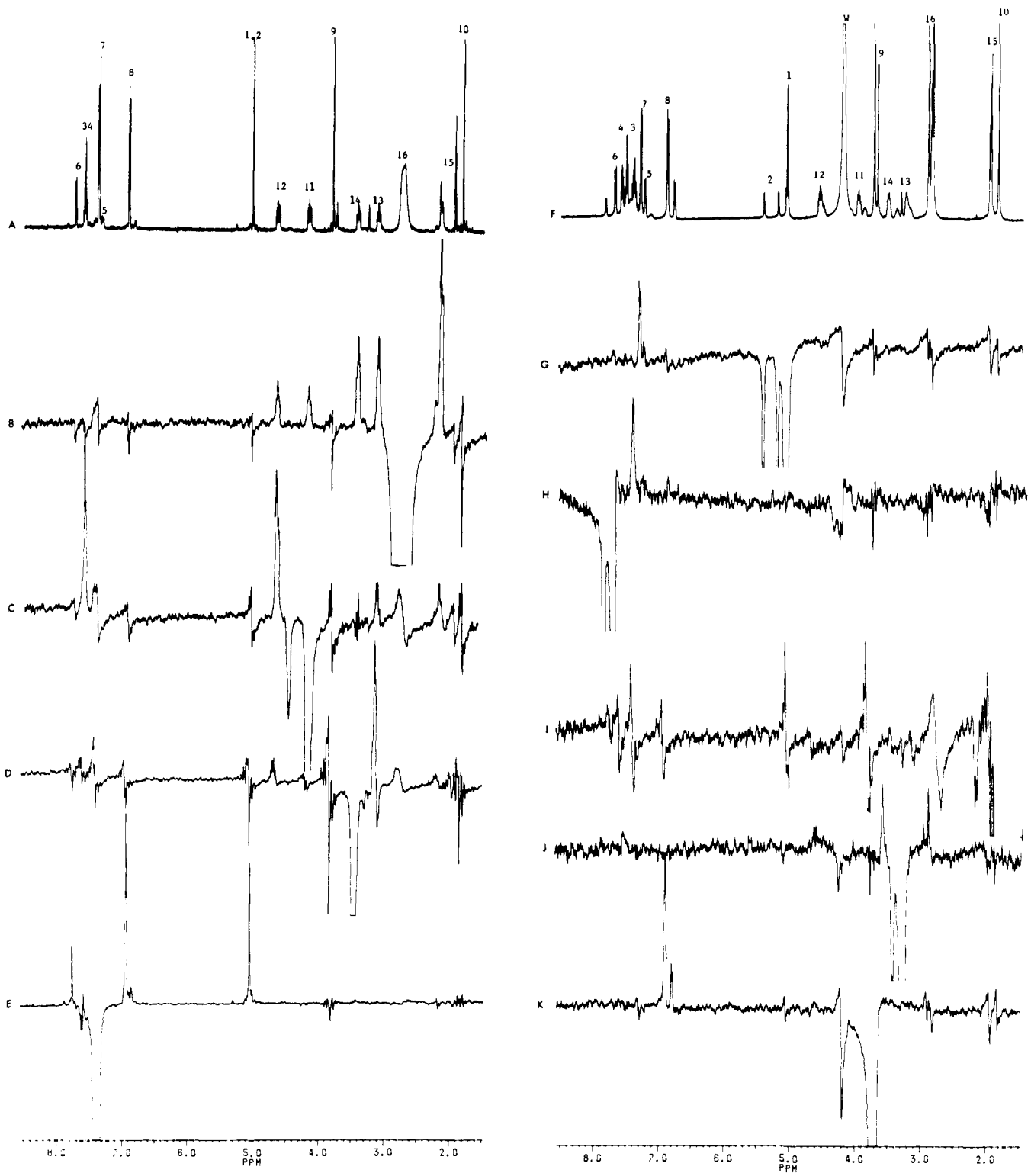

Figure 4. One-dimensional ^{1}H-NMR spectra and representative NOE difference spectra of diltiazem and its Ca^{2+} complex in CD_3CN at 22 ± 1 °C: (A) ^{1}H-NMR spectrum of the free drug; (B–E) NOE difference spectra in the free drug obtained by setting the decoupler frequency to irradiate: H-16 (2.13 ppm), H-11 (4.16 ppm), H-13 (3.15 ppm), and H-7 (7.39 ppm); (F) ^{1}H-NMR spectrum of diltiazem + 0.75 molar equiv of Ca^{2+}; (G–K) NOE difference spectra in the drug–Ca^{2+} complex caused, respectively, by irradiation of H-6 (5.05 ppm), H-6 (7.65 ppm), H-10 (1.80 ppm), H-13 (3.20 ppm), and H-9 (3.75 ppm). Irradiation duration was 5 s. In spectrum F, W indicates the residual water protons in the sample.

a superimposition of several such low-energy conformers on the minimum energy structure. This reveals a considerable degree of flexibility in the drug molecule in solution, particularly in the substituents attached to the benzothiazepine ring. The torsional angles describing the minimum-energy structure in Figure 5A are given in Table III. Comparison of this solution structure of diltiazem hydrochloride (in CH_3CN) with the X-ray structure[5] revealed relatively minor differences in the puckering of the benzothiazepine ring (Table III). However, significant differences were seen (Table III) in the relative orientations of the acetoxy group [reflected in the torsional angle C(3)–O(2)–C(19)–C(20)] and of the methylene groups (reflected in the torsional angle C(21)–C(22)–N(6)–C(24); labeling

Table II. Proton Connectivities in Diltiazem and Its Ca^{2+} Complex[a] Derived from NOESY Data in CD_3CN at 22 °C

	NOE[b]	
connected proton	free	with Ca^{2+}
1, 7	++	++
1(2), 10[c]	+	+
3, 6		+
3, 11	++	
3, 15		+
3, 16		+
4, 6		++
4, 10		+
4, 16		++
6, 10		+
7, 8	+++	+++
7, 9	+	+[d]
7, 10	+	++
8, 9	++	+++
8, 10		++
8, 12		+
9, 10		++
11, 12	+++	+++
11, 16	++	
12, 16	+	++[d]
13, 14	+++	+
13, 16	++	++[d]
14, 16	+++	+++[d]

[a] Drug:Ca^{2+} ratio is 2:1. [b] Based on the magnitude of volume integrals of cross peaks: +++, 1.5–3.0 Å; ++, 2.5–4.0 Å; +, 3.5–5.1 Å. [c] Protons 1 and 2 could not be discriminated in the free drug (see text). [d] These connectivities were discernible in the one-dimension difference NOE spectra.

is as in Figure 1B). In the drug structure shown in Figure 5A, the OCH_3 substituent of the phenyl ring C is positioned right over the ester and ketone groups attached to the 7-membered ring. This results in the formation of a pocket where the three of the four oxygens belonging to the above groups are in a geometrical arrangement predisposed to interact with a divalent cation; such a pocket is not seen in the X-ray structure.[5] The ester oxygen (O(1)) adds to the overall negative electrostatic field in this part of the drug molecule.

Modeling of the Ca^{2+}-bound form of diltiazem was performed mostly as described above for the free drug. In doing this, we sought self-consistency among all the data: the conformational change involving the environment of the asymmetric protons indicated by the CD spectral changes; the stoichiometry obtained from CD and NMR data and the relative changes in the chemical shifts of different protons. In the modeling protocol, two diltiazem hydrochloride molecules having structures corresponding to that shown in Figure 5A were placed 10 Å apart, and a Ca^{2+} ion was placed in their middle. Using the Monte Carlo method, the torsional angles of the drug molecules were then varied at random to obtain a total of 200 structures. The energies of these structures were then minimized using, from the data in Table II, only those NOE distance constraints for the drug–Ca^{2+} complex that would correspond to *intra*molecular connectivities. This yielded a minimum energy conformer whose structure is shown in Figure 5C. Subsequently, the NOESY distance constraints were removed, and using the Monte Carlo method, both the molecules in the dimeric cation complex were allowed freedom of torsional angles. The minimum-energy conformer of the resulting ensemble of 200 random conformers displayed a structure that was nearly identical to that shown in Figure 5C. As in the case of the free drug, the structures of many other low-energy conformers lay within an rms deviation of 2 Å from this structure.[21] Figure 5D shows the structures of several low-energy structures[22] superimposed on the minimum-energy conformation and illustrates the flexibility of the ring substituent groups. From the model of the drug–Ca^{2+} complex shown in Figure 5C, the proton pairs: *3,16*; 4,11; *4,16*; 7,8; 7,10; 8,9; *8,10*; *9,10*; 13,14; and 13,16 were measured to be within an *inter*molecular distance of 5 Å. All of these connectivities were observed in the one- and two-dimensional NOE spectra, suggesting dipolar coupling (Table II). In the case of the italicized proton pairs, the main contribution to the observed NOE arose from *inter*molecular connectivities.

The energy of the Ca^{2+}–drug complex was about 80 kcal/mol lower than that of the free diltiazem molecule shown in Figure 5A. In the complex, Ca^{2+} was situated in a cavity formed of the liganding oxygen atoms, O(2), O(3), and O(4), which were, respectively, 2.46, 2.48, and 2.43 Å away from the cation. These distances are comparable to those found in peptides and proteins.[16,23] The nonliganding O(1) was at a distance of about 5.3 Å from the cation. The benzene rings of the benzothiazepine moieties in the two participating drug molecules sustained an angle of 102° between them, while the substituent benzene rings were nearly parallel and bore an angle of about 10° between them.[24] The distance between the latter rings was 7.8 Å; this would be too high to cause significant ring current effects that are often observed in the NMR spectra of stacked aromatic rings. The methylene and dimethylamino moieties of the participating drug molecules appear to "seal" the cation-containing cavity and render the entire complex very compact in shape.

Discussion

Much effort has gone into obtaining the structure–activity relationship in diltiazem by way of altering the nature of the substituent groups of the benzothiazepine ring or the nature of the heterocyclic ring.[5] However, in the absence of structural details of the drug and its analogues, it is rather difficult to arrive at any underlying factor(s) governing the activity of the drug which, in turn, is related to its interaction with the membrane-bound receptor. In this study, we have addressed this problem by using NMR and molecular modeling techniques to examine the structure of diltiazem in the membrane-mimetic solvent CH_3CN[25] and by extending this approach to study the interaction of the drug with the physiologically relevant cation Ca^{2+}. As described in the preceding section, while the most of the structural features seen in the crystal including the twisted-boat conformation of the benzothiazepine ring is maintained in solution, an important difference between these two phases is found in the orientation of the acetoxy substituent of the heterocyclic ring. In solution, this group is extended away from the ring unlike in the crystal[5] where it is bent downward. The proximity and relative dispositions of the oxygen atoms in the ring substituent groups in diltiazem appear to create a template for the formation of the 2:1 drug–Ca^{2+} complex observed by CD and NMR analyses. The CD and NMR (^1H and ^{13}C) spectral changes saturate at a drug:Ca^{2+} mole ratio of 0.5 and indicate the sandwich Ca^{2+} complex to be predominant species at the saturating conditions. The substantially different CD spectrum as well as one- and two-dimensional ^1H-NMR spectra of this complex point to a relatively stable cation complex with a sufficiently life time to display distinct *inter*molecular NOEs (Figures 3 and 4). (In light of this, it is likely that the dissociation

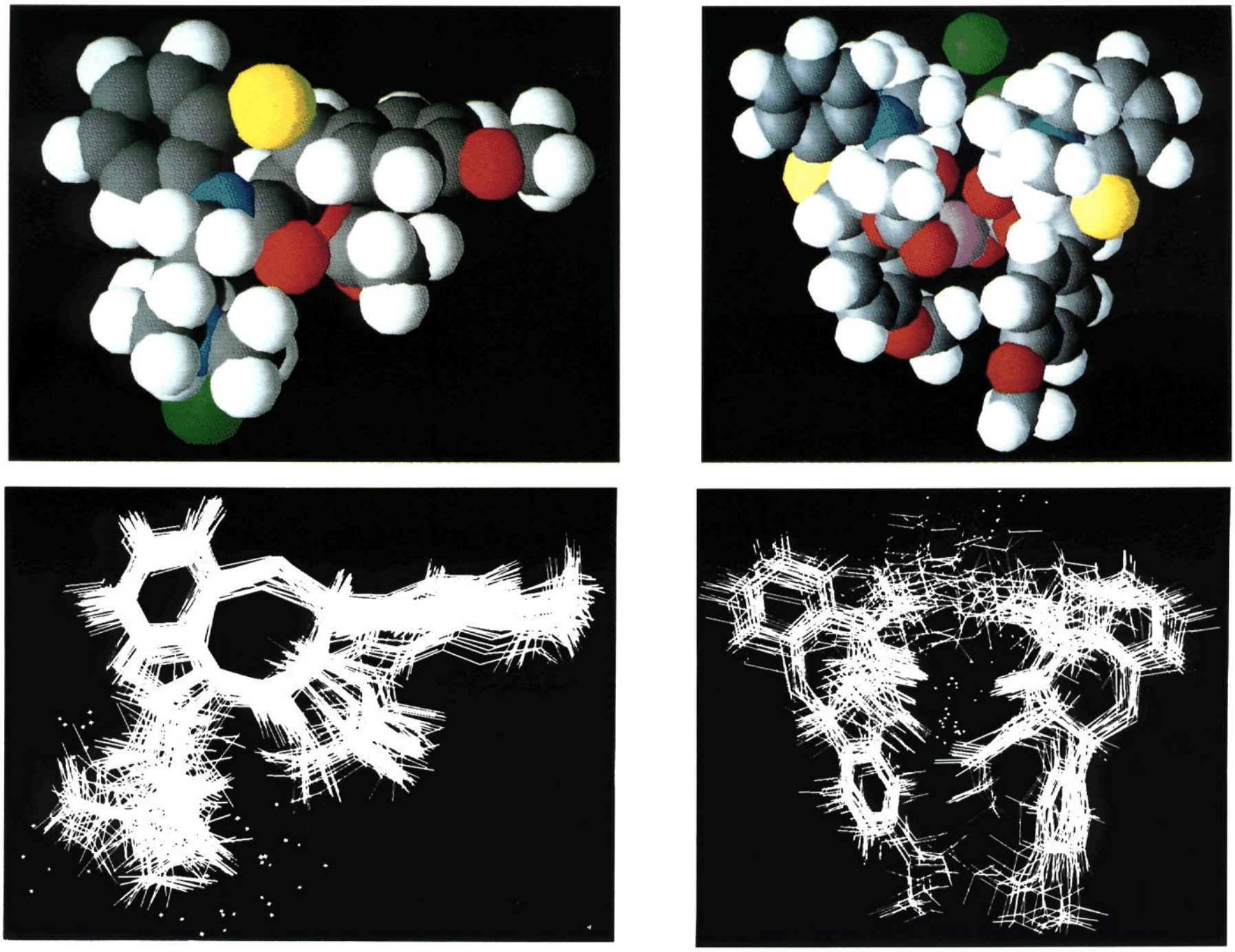

Figure 5. Molecular models of diltiazem and its 2:1 drug–Ca^{2+} (sandwich) complex obtained from Monte Carlo and energy-minimization protocols using NOESY-derived constraints. A (top left) and C (top right) show space-filled models of the minimum-energy conformers of the free drug and the Ca^{2+} complex, respectively; Ca^{2+} ion is shown in purple and Cl^- ion in green. B (bottom left) and D (bottom right) show a superimposition of the minimum-energy conformer and 20 of the low-energy conformers that lay within an rms deviation of 1.5 Å for the free drug and 2 Å for the Ca^{2+} complex from the minimum-energy conformer. The white dots indicate the locations of 1.5 the Ca^{2+} and Cl^- ions.

constant of 140 μM estimated from the analysis of the binding isotherms is an overestimate.) Parallels for such sandwich complex formation with Ca^{2+} exist in the case of other drugs such as verapamil[10,26] and some adrenergic drugs[27] as well as in the case of Ca^{2+} ionophores such as A23187[28] and chlortetracycline[29] and synthetic peptides.[23] (While diltiazem also shows affinity to Mg^{2+}, complexation with Ca^{2+} assumes special relevance in view of the drug's function as a calcium channel blocker, see below.)

The physiological relevance for the Ca^{2+}-bound structure of diltiazem may be sought in terms of the role of Ca^{2+} in the drug's interaction with the receptor (namely, the Ca^{2+} channel[1e]). Although we have characterized the drug–Ca^{2+} complex in CH_3CN, evidence for the presence of the 2:1 sandwich complex in an aqueous buffer solution has been presented in experiments illustrating the Ca^{2+} translocating ability of diltiazem using an artificial liposome.[30] That study suggested that the drug is capable of entering the lipid bilayer with the cation bound in a 2:1 drug–Ca^{2+} complex. We had visualized that the drug could therefore interact at the receptor site, presumed to be hydrophobic from the temperature-dependent binding data,[9b,31] in the Ca^{2+}-bound form. If the drug binding site is proximal to the Ca^{2+} binding site of the channel, as in the dihydropyridine receptor,[32] one might further visualize a ternary complex formed of the drug, Ca^{2+}, and receptor.[10b] This might, in turn, populate the inactive or closed state of the receptor and lead to the observed channel blockade.[33] The possibility of the drug–Ca^{2+}-channel ternary complex formation has also been suggested from theoretical calculations.[34] While more detailed studies on the drug–receptor interaction are needed to verify our proposal, the data presented in this paper point to the possibility of an important role for Ca^{2+} in governing the bioactive conformation of diltiazem. Available pharmacological data emphasize the role of Ca^{2+} on the drug's binding to the receptor.[9b] However, the Ca^{2+} effect seems to be concentration-dependent[1d,31] and EDTA which is used to remove extracellular Ca^{2+} might itself inhibit the channel.[35] These data are therefore not easily interpretable at present in terms of any particular model.

Conclusions. The present study represents, to the best of our knowledge, the first detailed study on the conformation of diltiazem in a nonpolar milieu.[36] While this may be useful in understanding the structure–activity relationship in diltiazem and its analogs, it is also likely that the structure of the Ca^{2+}-bound form of the drug determined in this study may be closer to its bioactive

Table III. Torsion Angles (deg) of Diltiazem and Its Ca^{2+} Complex[a]

	free	Ca^{2+} complex[b]	X-ray[c]
Benzothiazepine Ring			
S(1)–C(2)–C(3)–C(4)	−55.6	−46.6	−42.1
C(2)–C(3)–C(4)–N(5)	71.4	79.3	90.6
C(3)–C(4)–N(5)–C(11)	10.8	−2.1	−13.3
C(4)–N(5)–C(11)–C(10)	−55.6	−52.9	−48.0
N(5)–C(11)–C(10)–S(1)	−2.0	1.0	0.6
C(11)–C(10)–S(1)–C(2)	56.1	61.8	69.4
C(10)–S(1)–C(2)–C(3)	−17.4	−29.8	−42.0
Substituents			
S(1)–C(2)–C(12)–C(13)	56.1	53.8	49.5
S(1)–C(2)–C(3)–O(2)	163.8	−173.5	−163.7
C(2)–C(3)–O(2)–C(19)	73.9	−141.2	−175.6
C(3)–O(2)–C(19)–C(20)	1.1	11.4	−179.6
C(3)–O(2)–C(19)–O(3)	−178.9	−167.9	−0.2
C(4)–N(5)–C(21)–C(22)	−78.2	78.8	−79.6
N(5)–C(21)–C(22)–N(6)	145.8	61.1	169.7
C(21)–C(22)–N(6)–C(23)	−72.9	−170.6	−76.6
C(21)–C(22)–N(6)–C(24)	56.5	63.6	159.3
C(12)–C(2)–C(3)–O(2)	−70.5	−45.5	−37.2
C(12)–C(2)–C(3)–C(4)	70.6	81.4	82.3
C(13)–C(12)–C(2)–C(3)	−72.3	−75.5	−77.6

[a] Drug:Ca^{2+} ratio is 2:1. [b] Torsion angles shown are for one of the two drug molecules in the Ca^{2+} complex; torsion angles for the second molecule were very close but not identical to the first. [c] For comparison, the torsion angles of the X-ray structure of the free drug[5] are also shown. The numbering of atoms is as per the X-ray structure.

structure. Ca^{2+} binding would increase the overall hydrophobicity of the drug by "neutralizing" the polar oxygen-bearing groups. In the 2:1 drug–Ca^{2+} sandwich complex, these groups will be in the interior and the nonpolar groups will be on the exterior of the molecule so that interaction with the lipid membrane, and possibly with the receptor, may be facilitated by the interaction of diltiazem with Ca^{2+}.

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(19) Ca^{2+} addition to diltiazem also caused substantial changes in the ^{13}C chemical shifts as monitored on the 200-MHz instrument (data not shown). One of two carbonyl carbons at 168 ppm moved closer to that of the other at 170 ppm (indicating that their environment is more similar) on the addition of Ca^{2+}; the chemical shifts of the methylene carbons also underwent significant alterations. These ^{13}C data suggest the involvement of carbonyl oxygens in Ca^{2+} chelation and alterations in the environment of the methylene groups.

(20) About 7.5% of the total number of conformers had energies within 2 kcal/mol and 18% within 5 kcal/mol of the minimum-energy conformer.

(21) This observation could be taken as a measure of the quality of fit between the computed final structure and the experimental NOE data.

(22) About 4% of the total number of conformers had energies within 2 kcal/mol and 21% within 5 kcal/mol of the minimum-energy conformer.

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(36) **Note Added in Proof.** An earlier paper (Glaser, R.; Sklarz, B. Stereochemistry and Conformation in Solution of Diltiazem Hydrochloride, a 1,5-Benzothiazepine Coronary Vasodilator. *J. Chem. Soc., Perkin Trans. 2* **1989**, 1031–1036) was missed by us. The conformation of the drug in $(CD_3)_2SO$ and CD_2Cl_2 arrived at by these authors by NMR and molecular mechanics is similar to ours in CD_3CN except for the orientation of the acetoxy group.