Interaction of Calcium Channel Antagonists with Calcium: Structural Studies on Verapamil and Its Ca^{2+} Complex

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The conformation of the calcium channel antagonist verapamil has been determined in acetonitrile, in the absence and presence of Ca^{2+}, using two-dimensional ^{1}H-NMR and molecular modeling techniques. Interproton connectivities in the drug molecule were identified from the observed NOESY cross peaks and interproton distances were estimated from the magnitudes of the volume integrals of the cross peaks. The molecular modeling program utilized the Monte Carlo simulation to generate a random ensemble of conformers complying with the NOESY-derived distance constraints. The energies of these conformers were subsequently computed. The minimum-energy structure of the free drug obtained in this manner exhibited some significant differences from the structure of verapamil determined by X-ray crystallography. In particular, the torsional angles in the middle region of the molecule containing the aliphatic "backbone" were such that the two aromatic rings at either end of the drug molecules were moved farther apart from each other in solution than in the crystal structure. The nearly perpendicular orientation of the aromatic rings seen in the crystal was, however, maintained in the solution structure as well. The addition of Ca^{2+} to a solution of verapamil in acetonitrile caused marked changes in the difference absorbance of the drug in the 200–300-nm region and in many of its ^{1}H-NMR resonances. The changes were most significant up to a mole ratio of about 0.5 Ca^{2+}:drug. Analysis of the binding data at 25 °C showed the presence of both 2:1 and 1:1 drug:Ca^{2+} complexes in equilibrium, the former "sandwich" complex being dominant at the lower cation concentrations with an estimated dissociation constant of about 300 μM. All of the NOESY cross peaks of the free drug remained on addition of 0.5 mol ratio of Ca^{2+} to verapamil in deuterated acetonitrile and only two new connectivities were observed. Using the interproton distances calculated from these NOESY data, molecular modeling of the 2:1 drug:Ca^{2+} complex was carried out to yield the minimum-energy conformer. In this conformer, Ca^{2+} was coordinated to two methoxy oxygens from each of the two drug molecules. The implications of the verapamil–Ca^{2+} interaction are discussed in terms of available experimental data on the binding of verapamil to the dihydropyridine-sensitive channel and in terms of a hypothesis on the formation of a drug–Ca^{2+}–receptor complex in the lipid bilayer environment.

Introduction

Verapamil [α-isopropyl-α-(*N*-methyl-*N*-homoveratryl-γ-aminopropyl)-3,4-dimethoxyphenylacetonitrile hydrochloride] belongs to the phenylalkylamine group of calcium channel antagonists and has been extensively used clinically in the treatment of several cardiovascular diseases.[1] Two other classes of calcium channel antagonists that have also been widely studied and used in the field of calcium channel blockade are the 1,4-dihydropyridines (DHPs, exemplified by nifedipine) and the benzothiazepines (exemplified by diltiazem). In spite of much effort,[2] understanding of the molecular basis of the action of these calcium channel drugs has fallen far behind their clinical studies and therapeutic uses. An important missing link in this context is the knowledge of the structure of the drug at the site of its interaction with the membrane-bound channel. Significant advances have recently been made in delineating the structure of the DHP-sensitive L-type calcium channel.[3] Besides binding nifedipine, this multi-subunit protein has allosterically-interacting binding sites for verapamil and diltiazem.[4] This, combined with the observed Ca^{2+} dependence of the allosteric interactions,[5] suggests the possibility of some common structural attribute(s) that may be shared by the different types of calcium channel blockers. We have recently explored this suggestion by examining whether the interaction of the calcium channel antagonists with Ca^{2+} in a nonpolar environment (such as that prevailing in a lipid membrane) could be a common characteristic among these drugs. Such an interaction, if present, would have important structural and functional consequences.[6] In a recent study,[7] we have characterized the interaction between Ca^{2+} and diltiazem in the lipid mimetic solvent acetonitrile (H_3CCN) and arrived at the structure of the free and Ca^{2+}-bound forms of this drug using CD and NMR spectral methods combined with molecular modeling. In this study, we present a similar characterization of verapamil–Ca^{2+} interaction in H_3CCN using difference absorption and NMR spectroscopy. We have taken advantage of two-dimensional nuclear Overhauser enhancement spectroscopy (NOESY) to obtain geometrical constraints which could be used in a molecular modeling procedure for arriving at the most probable conformation of the drug in the free and Ca^{2+}-bound forms. Our results show that the minimum-energy structure of the free drug differs significantly from the previously determined crystal structure of verapamil[8] and from the structure derived from NMR data in dimethyl sulfoxide (DMSO).[9] In the Ca^{2+}-bound form, two verapamil molecules are arranged with a 2-fold symmetry such that the two methoxy oxygens from each molecule act as ligands to the cation.

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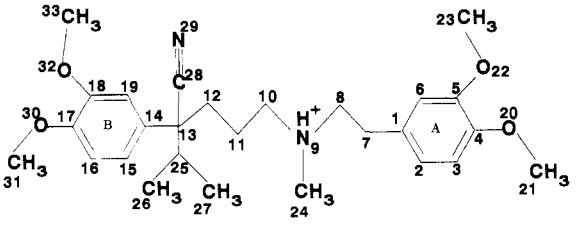
Experimental Section

Materials. Verapamil hydrochloride was purchased from Sigma Chemical Co. (St. Louis, MO), deuterated acetonitrile (D_3-CCN) was from MSD Isotopes (Montreal, Quebec), and acetonitrile (H_3CCN) and $Mg(ClO4)_2$ were from Fisher Scientific (Toronto, Ontario). $Ca(ClO_4)_2$ and $Zn(ClO_4)_2$ were, respectively, from VWR Scientific (Ontario, Canada) and GFS Chemicals (Cleveland, OH). H_3CCN was dried by treatment with molecular sieves (4 Å) and was kept under argon. The perchlorate salts were lyophilized overnight to remove any trace of water.

Methods. UV Spectrophotometry. UV spectra were recorded using a Perkin-Elmer Lambda 6 Spectrophotometer equipped with a microprocessor for spectral accumulation and data manipulation. Spectra were recorded at room temperature (22 ± 1 °C) in dry H_3CCN using a quartz cell of 1-cm pathlength. Drug concentration used was 200 μM.

NMR Spectroscopy. Bruker AC-200 and AM-500 instruments were used to record ^1H and ^{13}C spectra. The Bruker AC-200 instrument was used in two-dimensional correlation spectroscopy (COSY) and NOESY experiments. All experiments were performed at room temperature (22 ± 1 °C). Drug concentration was 7 and 17 mM in D_3CCN for ^1H and ^{13}C experiments, respectively. ^1H spectral linewidths did not show significant changes in the concentration range 5–20 mM, implying that aggregation of the drug is negligible. The resonance of the residual proton of H_3CCN was used as an internal reference (a quintuplet set at 1.93 ppm). Most of the proton assignments of verapamil were obtained after an analysis of the one-dimensional spectrum. Confirmation was provided by the COSY spectral data (not shown). The standard COSY sequence was used.[10] In the Ca^{2+} titration experiments, small aliquots of a stock solution of $Ca(ClO4)_2$ in D_3CCN were made and the resulting changes in the proton resonances were monitored as a function of increasing Ca^{2+}/verapamil mole ratios. Less than 5% dilution of the drug was introduced during the complete titration. In NOESY runs, 112 scans were obtained for each of the 256 t_1 increments used. A 3-μs initial evolution time was followed by subsequent increments of 666 μs, resulting in an effective aquisition time of 170.5 ms in the F_1 dimension. In order to minimize any contributions of NOE cross peaks stemming from coherent magnitization transfer between scalar-coupled nuclei, we introduced a random variation of 20 ms (independent of the mixing time used). Spectral data collected at different mixing times (from 0.2 to 1.2 s) revealed that the highest volume integrals were obtained at a value of 0.6 s. COSY-type (spin–spin diffusion) cross peaks were also minimal at this mixing time. This value was therefore selected as the optimum mixing time for routine measurements. Two dummy scans preceded each experiment with a relaxation delay of 2 s between each scan. The two-dimensional NOESY plots were based on a 512 × 512 matrix and are shown in the absolute value mode after zero filling twice in the F_1 dimension and multiplying with a shifted sine-bell offset of $\pi/2$ in both dimensions. To evaluate interproton distances from the volume integrals of the NOE cross peaks, the 1/(distance)^6 relationship[11] was used to represent the extent of transfer of magnitization between spin systems during the NOE mixing period; the distance between protons H-25 and CH_3-26 was used as the reference.

Molecular Modeling. Modeling of verapamil was conducted on a Personal Iris computer using the Biograf version 2.2 software (Molecular Simulations, Inc.). The Monte Carlo[12] method was used to generate a set of random conformers for verapamil in the presence and absence of Ca^{2+}. The energies of these conformers were minimized using the DREIDING generic force field.[13] A conjugate gradient method was introduced so as to achieve a convergence to 0.001 kcal/mol per Å. Energy minization was performed with and without incorporating the interproton distances derived from the NOESY data. The distance constraints were input in the form of a harmonic force field with a force constant of 25 kcal/mol. We also applied a distance cutoff of 9 Å for nonbonded interactions. A dielectric constant of 37.5 was used in these calculations to mimic the solvent, H_3CCN. Ca^{2+} ion-specific parameters of Hori et al.[14] were integrated into the Biograf software when modeling the Ca^{2+} complex of verapamil.



Verapamil

Figure 1. Chemical structure of verapamil showing the numbering of protons in the molecule used in labeling the NMR chemical shifts. The numbering scheme is identical to that used in the verapamil crystal structure study (ref 8).

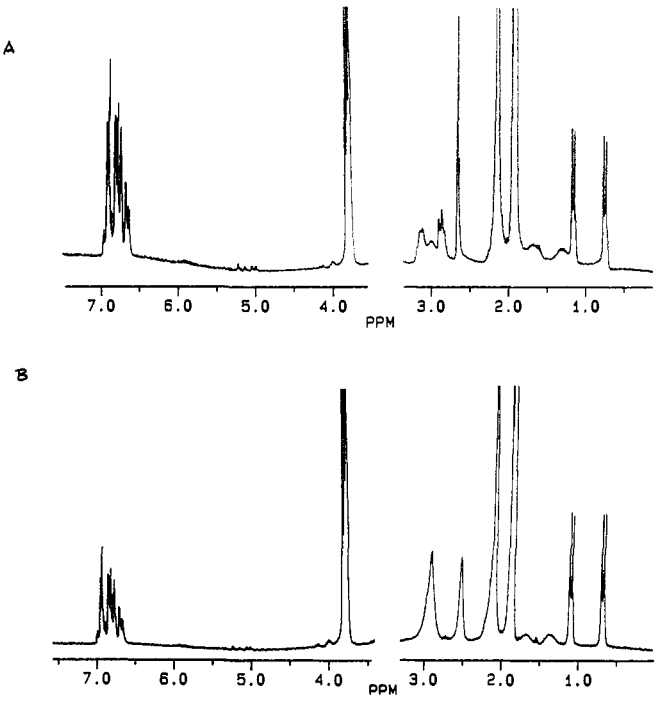


Figure 2. ^1H-NMR spectrum of verapamil in D_3CCN at 22 ± 1 °C: panel A, drug + 0.5 molar equiv Ca^{2+}; panel B, drug alone.

Results

The labeling scheme of verapamil used in the interpretation of the results is shown in Figure 1.

Structure of Free Drug: NMR Data. Assignment of most of the ^1H resonances in verapamil in H_3CCN-d_3 was made from chemical shift data of related compounds and from multiplicities and coupling constants derived from the one-dimensional spectrum (Figure 2). The assignments are presented in Table I. These correlated well with those of verapamil obtained in DMSO.[9] We were successful in assigning protons H-21, H-23, H-31, and H-33 by using COSY and ^{13}C–^1H correlation spectroscopy (data not shown). Two-dimensional NOESY spectrum of verapamil in D_3CCN is shown in Figure 3. The NOE cross peaks, which correspond to interproton connectivities, are shown in Table II along with a classification of their relative intensities. The latter were used to estimate the interproton distances which were subsequently used as geometrical constraints in the modeling procedures. Some of the more interesting cross peaks involved protons H-24, H-26; H-24, H-27; H-26, H-31; H-27, H-33; H-12, H-33; and H-12, H-31. These suggest that verapamil adopts a relatively compact structure.

Table I. Assignments of ^1H Chemical Shifts for Verapamil in D_3CCN at 22 °C

proton	chemical shift (ppm) free drug	chemical shift (ppm) with 0.5 mol ratio Ca^{2+}
H-2	6.67	6.7
H-3	6.8	6.83
H-6	6.76	6.79
H-7	2.63	2.88
H-8	2.49	2.74
H-10	2.36	2.7
H-11	1.42, 1.07	1.53, 1.18
H-12	2.07, 1.89	2.09, 1.90
H-15	6.92	6.95
H-16	6.9	6.93
H-19	6.85	6.85
CH_3-21	3.73	3.75
CH_3-23	3.75	3.77
CH_3-24	2.16	2.45
H-25	2.12	2.16
CH_3-26	0.72	0.73
CH_3-27	1.11	1.13
CH_3-31	3.78	3.79
CH_3-33	3.80	3.81

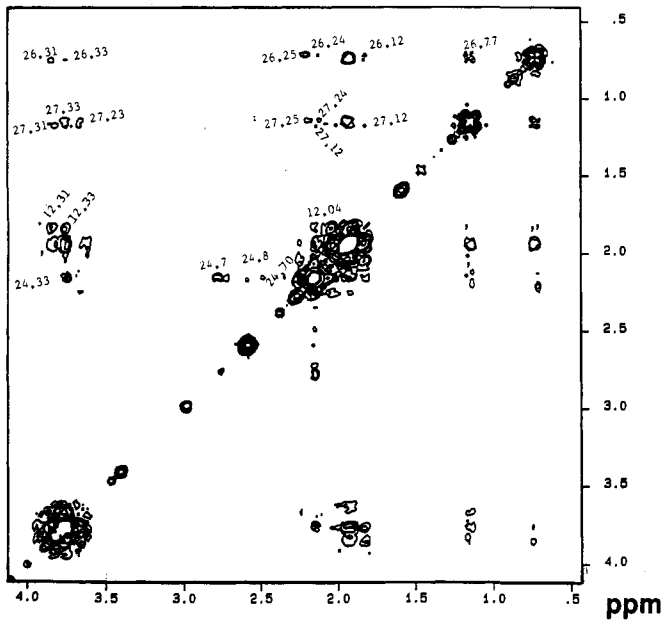


Figure 3. Two-dimensional ^1H-NOESY spectra of verapamil in D_3CCN at 22 ± 1 °C. The numbers on the cross peaks denote those of the NOE-connected protons labeled according to Figure 1.

Structure of the Drug–Ca^{2+} Complex: Absorption Spectroscopy. Using difference absorption spectroscopy in the 200–300-nm region, we followed conformational changes caused by progressive addition of small aliquots of $Ca(ClO_4)_2$ to a solution of verapamil in H_3CCN. The spectral data are shown in Figure 4. The change in difference absorbance caused by Ca^{2+} addition was saturable to a Ca^{2+}:drug mole ratio of about 0.5. The change in the difference absorbance was used to construct the binding isotherm at 25 °C shown in Figure 5. As in the case of diltazem,[7] the binding curve was analyzed for the presence of both 1:1 and 2:1 drug:Ca^{2+} complexes using the protocol of Reuben.[15] The results of this analysis showed that both the above types of complexes are present in equilibrium, their relative populations depending on the Ca^{2+}:drug ratio. This is shown in Figure 5. The 2:1 drug:ion complex, which is also known as the ion sandwich complex,[16] predominates at lower ion/drug ratios. The dissociation constant for this complex was estimated to be around 300 μM. Binding studies were also carried out with Mg^{2+} and Zn^{2+} and the data obtained are shown in Figure 6. Like Ca^{2+}, Mg^{2+} showed a saturable binding to the drug, but its calculated dissociation constant was about 10 times higher than that of Ca^{2+}. In the case of Zn^{2+}, no saturable binding was observed (Figure 6) and the data were not analyzed further. Monovalent ions such as Na^+ exhibited no spectral changes even up to a cation:drug mole ratio of 4. The formation of a 2:1 drug:Ca^{2+} complex in the low-dielectric solvent is compatible with the data obtained from a study of the translocation of Ca^{2+} by verapamil across the lipid bilayer in model liposomes; analysis of the transport kinetics showed the 2:1 drug:Ca^{2+} sandwich complex to be the ion-carrying species.[17]

Table II. Proton Connectivities in Verapamil and Its Ca^{2+} Complex[a] Derived from NOESY Data in D_3CCN at 22 °C

connected protons	NOE[b] free	NOE[b] with Ca^{2+}	connected protons	NOE[b] free	NOE[b] with Ca^{2+}
26, 27	+++[b]	+++	24, 10	+	++
26, 12	+++	–[c]	24, 8	+	++
26, 25	+++	+++	24, 7	++	++
26, 24	+	++	24, 33	++	++
26, 33	++	++	25,31		+
26, 31	++	++	26, 15	++	+
27, 12	+		27, 15	+	+
27, 25	+++	+++	27, 16	+	
27, 24	++	++	26, 19		+
27, 23	++	++	33, 19	+	+
27, 33	++	++	31, 16	++	++
27, 31	++	++	23, 6	++	+
12, 24	+	*c*	21, 3	+	+
12, 33	++	*c*			
12, 31	++	*c*			

[a] Drug Ca^{2+} ratio is 2:1. [b] On the basis of the magnitudes of volume integrals of cross peaks: +++, 1.5–3.0 Å; ++, 2.5–4.0 Å; +, 3.5–5.1 Å. [c] Ca^{2+} addition caused H-12 to move closer to the reference peak of H_3CCN, so that any possible NOE cross peak(s) involving this proton is likely to be masked by the t_1 trail noise of residual nondeuterated H_3CCN.

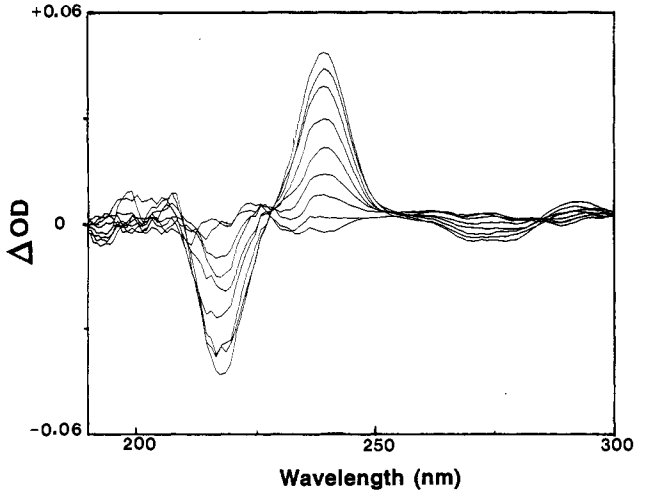


Figure 4. Difference spectra of verapamil produced by addition of $Ca(ClO_4)_2$ in H_3CCN at 25 °C. Ca^{2+} concentration increases from zero for the bottom curve to 0.8 molar equivalent for the top curve in increments of 0.1 molar equiv of the cation to drug.

NMR Data. Considerable changes in the ^1H spectra of verapamil were observed on addition of $Ca(ClO4)_2$ in D_3CCN up to a Ca^{2+}:drug mole ratio of about 0.7. Binding curves depicting the changes in the chemical shifts of H-7, H-8, H-10, and H-11, which were the most sensitive to Ca^{2+} addition, are shown in Figure 7. The ^{13}C resonances showed only minor changes upon Ca^{2+} addition (data not

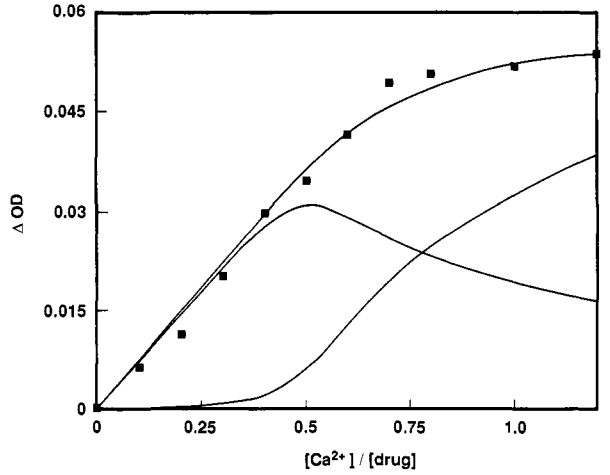


Figure 5. Ca^{2+} binding curves for verapamil in H_3CCN at 25 °C: (■), experimental data derived from difference absorbance changes at 240 nm. Computed curves correspond, from top to bottom, to the sum of 2:1 and 1:1 drug:Ca^{2+} complexes, the 2:1 drug:Ca^{2+} complex, and the 1:1 drug:Ca^{2+} complex. The method of Reuben[15] was used to obtain the computed curves.

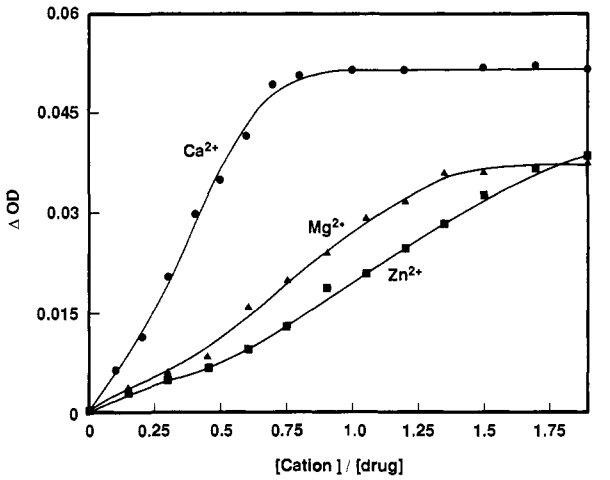


Figure 6. Plot of the difference absorbance of verapamil in H_3CCN at 240 nm as a function of concentrations of Ca^{2+}, Mg^{2+}, and Zn^{2+}.

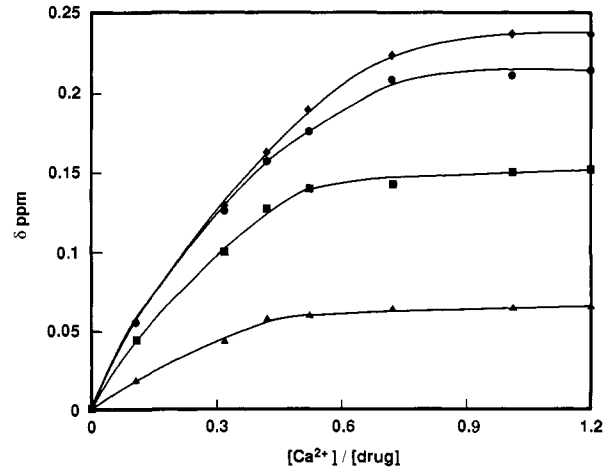


Figure 7. Plot of ^1H NMR chemical shifts versus Ca^{2+}:verapamil mode ratio for (♦) H-8, (●) H-10, (■) H-11, and (△) H-7.

shown). To know more about the conformational characteristics of the verapamil:Ca^{2+} complex, we obtained the NOESY spectrum of the drug in presence of 0.5 mole ratio of the cation to maximize the population of the 2:1

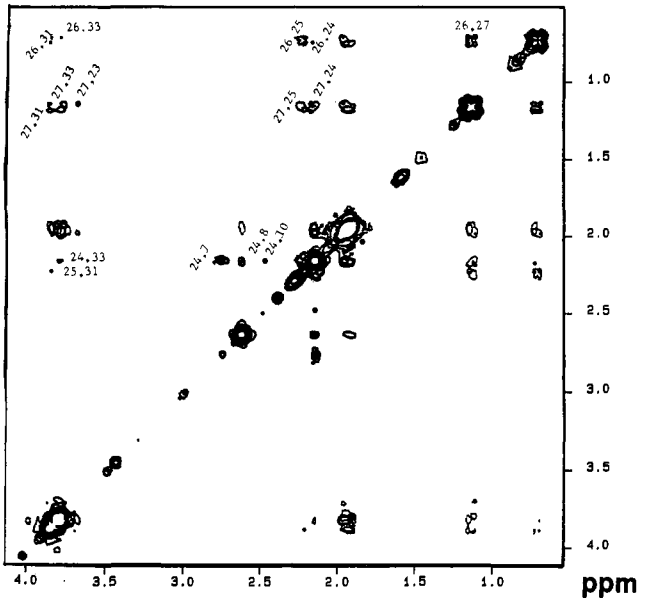


Figure 8. Two-dimensional ^1H-NOESY spectra of verapamil treated with 0.5 molar equiv of Ca^{2+} in H_3CCN-d_3 at 22 ± 1 °C. The H-19, H-26 cross peak listed in Table II is not seen in this figure due to the relatively higher NOE cutoff used.

sandwich complex (see Figure 5). Except for the appearance of two new cross peaks corresponding to interactions between H-25 and H-31, and H-19 and H-26, the rest of the NOE cross peaks of the free drug were also seen in the Ca^{2+} complex with some alterations in their intensities (Figure 8 and Table II; the H-19, H-26 cross peak is not presented in Figure 8, see the figure caption). It is, however, possible that some of the observed connectivities that are *intra*molecular in the free drug may actually correspond to *inter*molecular connectivities in the sandwich complex. Also, some additional intermolecular connectivities in the complex may have gone undetected due to the cutoff limit for cross peak identification.

Molecular Modeling

The results of the NOESY experiments for verapamil in H_3CCN in the free and Ca^{2+} sandwich complex were used in a molecular modeling procedure described in the Experimental Section, so as to obtain the energetically most favorable conformer. Molecular simulations for the free drug were conducted with the crystal structure of verapamil as the starting point[8] and allowing for random variations of this structure within the distance constraints estimated from the volume integrals for NOESY cross peaks (Table II). After minimizing the energies of the ensemble of 450 conformers, the minimum-energy conformer was identified. This is shown in Figure 9A. All the observed NOESY connectivities and distance constraints were accounted for in this structure. About 6% of the total number of generated conformers had a root-mean-square (rms) deviation of within 1 Å of the minimum energy structure. Figure 9B shows the superimposition in some of these conformers. The conformation of verapamil in H_3CCN solution thus obtained exhibited some significant differences when compared with the reported crystal structure,[8] as indicated by comparison of the torsional angle data of the two structures presented in Table III. Most noticeable is the difference in the disposition of the two aromatic rings at either end of the drug molecule, which arises from differences in the

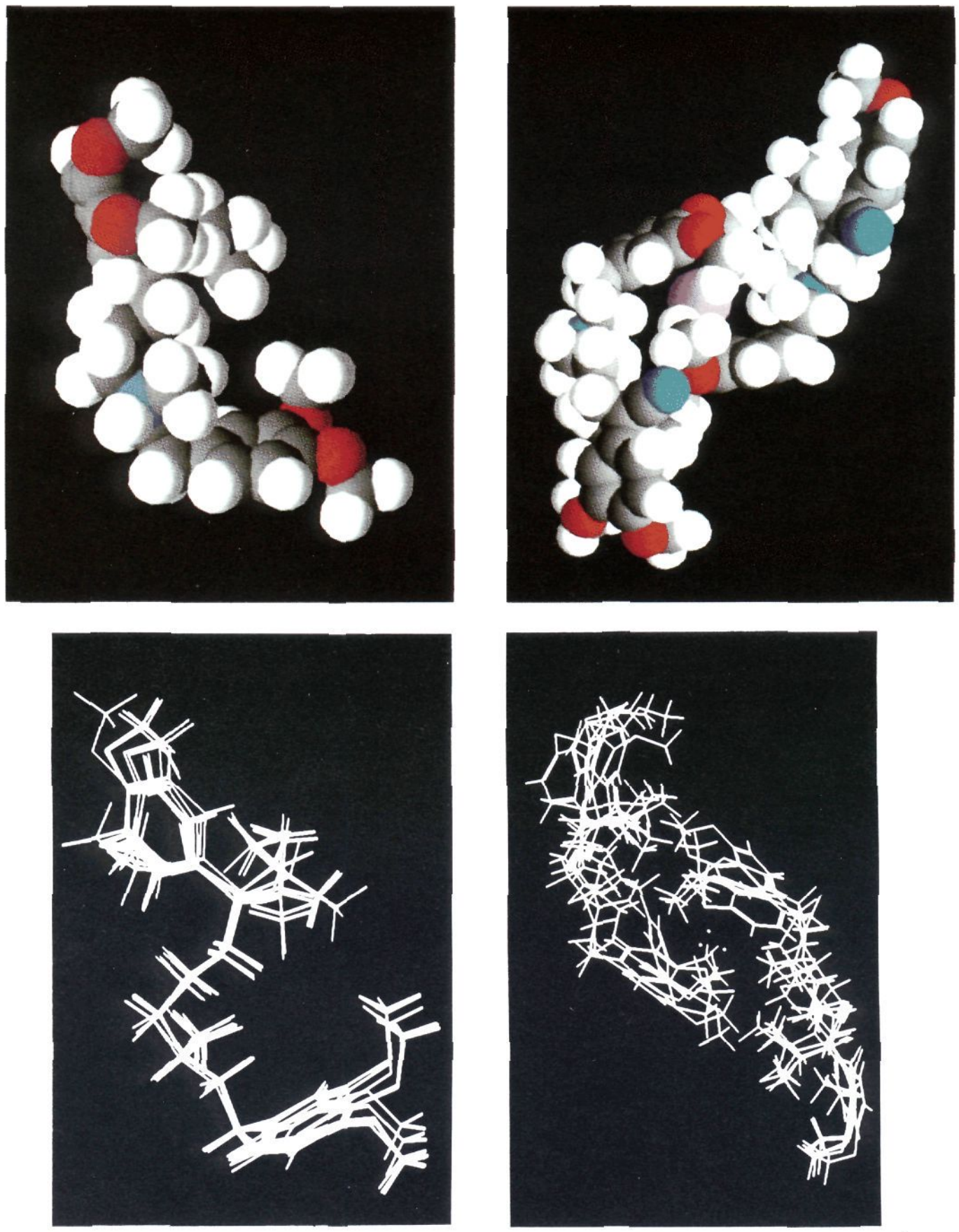


Figure 9. Molecular models of verapamil and its 2:1 drug:Ca^{2+} (sandwich) complex obtained from Monte Carlo and energy minimization protocols using NOESY-derived constraints: A (top left) and C (top right) show models of the minimum-energy conformers of the free drug and the Ca^{2+} complex, respectively. The Ca^{2+} ion is shown in purple B (bottom left) and D (bottom right) show a superimposition of the minimum-energy conformer and five of the low energy conformers, for the free drug as well as the Ca^{2+} complex, that lie within an rms deviation of 1 Å from the minimum-energy conformer. The white dots in the middle of the complex denote the positions of the Ca^{2+} ion.

torsional angles of the aliphatic backbone in the region between C(7) and C(11) (Table III). In the computed solution structure, the two phenyl rings are much farther apart from each other than in the crystal[8] so that the drug molecules appears to be more "open" in solution. The relative orientation of the phenyl rings with respect to each other is, however, similar in both the structures (angle between the ring planes is ≈90° in solution and ≈70° in the crystal).[8] A recent study by Gaggelli and co-workers[9] of the solution structure of verapamil in deuterated DMSO using one-dimensional ^1H- and ^{13}C-NMR data led to a preferred conformation for the drug where both the phenyl rings are out of the plane of the aliphatic backbone.[9] Also, the deduced overall structure of the molecule in DMSO was more extended[9] when compared with the structure in H_3CCN or the crystal structure.[8] A conformational analysis of the closely related methoxyverapamil by Brasseur et al.[18] yielded a family of low-energy conformers that show some similarities with the structure of verapamil shown in Figure 9A.

Modeling of the Ca^{2+}-bound sandwich form of verapamil was more difficult due to lack of sufficient intermolecular NOESY connectivities. On the basis of the 2:1 drug:Ca^{2+} stoichiometry of the complex derived from the binding data, two molecules of verapamil were allowed to coordinate the Ca^{2+} ion. The strategy was to use the available geometrical constraints deduced from the NOESY cross peaks as input, position the cation arbitrarily between the two verapamil molecules, and minimize the energy of the resulting structure. Several procedures were necessary to

Table III. Torsional Angles (in deg) for the Computed and X-ray Structures of Verapamil

bond definition	free drug	with Ca^{2+}	X-ray[a]
C(6)–C(1)–C(8)	115	−114	na
C(2)–C(1)–C(7)–C(8)	−64	65	94
C(1)–C(7)–C(8)–C(9)	−77	78	188
C(7)–C(8)–C(9)–C(10)	−164	68	182
C(7)–C(8)–C(9)–C(24)	65	−69	−58
C(8)–C(9)–C(10)–C(11)	−51	−86	−56
C(9)–C(10)–C(11)–C(12)	−56	−122	−74
C(10)–C(11)–C(12)–C(13)	−152	178	168
C(11)–C(12)–C(13)–C(25)	−173	−66	172
C(11)–C(12)–C(13)–C(28)	−55	55	53
C(11)–C(12)–C(13)–C(14)	65	175	−66
C(12)–C(13)–C(14)–C(15)	−16	20	119
C(11)–C(12)–C(13)–C(19)	17	−162	na
C(12)–C(13)–C(25)–C(27)	58	170	−65
C(12)–C(13)–C(25)–C(26)	−70	−67	174
C(16)–C(17)–C(30)–C(31)	−80	92	na
C(19)–C(18)–C(32)–C(33)	77	−70	na

[a] The angles for the X-ray structure were those found in ref 8. na, not available.

determine the best possible arrangement of the two verapamil molecules with respect to the Ca^{2+} ion. For example, orientation of the two participating drug molecules such as to engage all eight methoxy oxygens in liganding Ca^{2+} (analogous to the sandwich complex of diltiazem[7]) led to an energetically unfavorable complex. The structure eventually arrived at satisfied all the NOE-derived constraints and had the lowest energy. This is shown in Figure 9C. In Figure 9D is shown a superimposition of some of the low-energy conformers that had an rms deviation of within 1 Å from the lowest energy conformer.

Discussion

Very little progress has so far been made toward understanding the molecular structural basis of the action of verapamil and its analogues by the use of traditional structure–activity relationships.[2] In this study, we have utilized two-dimensional NMR spectroscopy as a tool to extract geometrical constraints which could be used in a molecular modeling procedure that would yield an energetically favorable conformation of verapamil. Attention was also focused on the possible conformation that verapamil might adopt in presence of Ca^{2+} since this cation has been shown to influence the binding of verapamil to the calcium channel.[5] We had hypothesized that the Ca^{2+}-bound form of verapamil might be its biologically relevant conformation.[6,17] The three-dimensional structure of verapamil was first deduced from X-ray crystallography.[8] Crystal structures have, however, been shown to differ from the biologically more relevant solution structures in the case of drugs such as acetylcholine[19] and diltiazem.[7] In the case of verapamil also, significant differences are noticed between the solution structure derived from our data and the crystal structure. These arise mainly due to the variations in the aliphatic backbone which, in turn, alters the relative orientation of the aromatic moieties. The basis for the variations between the solution and crystal structure may well lie in the differences in the interactions unique to the respective systems, namely the solvent effect and crystal-packing forces. The solution structure deduced in the membrane mimetic solvent[20] D_3-CCN is also different from a previously reported structure deduced in the relatively more polar solvent DMSO, where the drug molecule showed a more extended aliphatic backbone and hence a more elongated structure for the drug with the phenyl rings being out of the plane of the aliphatic chain.[18] These differences in the structure of the drug in the two solvent systems are likely due to the higher solvation of the drug's polar groups by DMSO.

The case for Ca^{2+} as a necessary factor modulating verapamil's binding to its receptor is quite strong. Although early studies indicated an inhibitory effect of Ca^{2+} on the binding of phenylalkylamines to the receptor,[5a] there is also evidence that suggests that there could be a concentration dependence of the Ca^{2+} effect.[5b] Low Ca^{2+} concentration seems to favor binding while an excess of Ca^{2+} lowers the affinity of the channel for phenylalkylamine. Data obtained using a new fluorescent probe that interacts at the phenylalkylamine site reveal that removal of Ca^{2+} results in a lowering of the binding affinity for these drugs.[21] The binding site of phenylalkylamines is believed to be located in the vicinity of a putative Ca^{2+} binding EF-hand region on the cytoplasmic side of the $\alpha 1$ subunit of the receptor.[22,23] It is possible that Ca^{2+} interacts with the drug in this region, resulting in a ternary complex of drug–Ca^{2+}–receptor[24] as suggested by us earlier.[6] 1,4-Dihydropyridines have also been shown to label a binding site in the EF-hand region of the receptor.[25] Since benzothiazepines are thought to bind in a region closely linked to the 1,4-dihydropyridine binding site in the L-type calcium channel[6b] through allosteric interactions, it is possible that Ca^{2+} may play a similar role in the action of all calcium channel antagonists.[26]

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(26) **Note Added in Proof.** The modeling of the free and Ca^{2+}-bound forms of verapamil was done without taking into account the proton and chloride ion bound to N-9 in Figure 1. While this may not significantly alter the structure of the free drug, it could cause a major change in the Ca^{2+}-bound form. We are currently studying this possibility.