Solution structure and dynamics of cyclic and acyclic cholinergic agonists

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ABSTRACT Two classes of nicotinic cholinergic agonists, which vary in flexibility and electronegativity, have been synthesized, and their structural and dynamic properties have been studied with nuclear magnetic resonance (NMR) spectroscopy. Although the compounds are chemically identical except for the presence or absence of one cyclicizing C—C bond, single channel recording and radioligand binding studies have shown that the cyclic compounds are considerably more potent than the acyclic derivatives (McGroddy, K. A., A. A. Carter, M. M. Tubbert, and R. E. Oswald. 1993. *Biophys. J.* 64:325–338). Using one- and two-dimensional NMR spectroscopy, we have shown that these molecules exist in two distinct stable conformers, which differ in the orientation of the amide bond. The cyclic 1,1-dimethyl-4-trifluoroacetyl-piperazinium iodide and its trifluoromethyl derivative compounds are symmetric, and the two conformers are of equal energy. The acyclic *N,N,N,N'*-tetramethyl-*N'*-acetylethylene-diamine iodide (TED) and its trifluoromethyl derivative derivatives, however, populate two energetically unequal solution conformations.

Using variable temperature NMR spectroscopy on these molecules and their uncharged precursors, we have characterized the energetics of amide bond isomerization and have distinguished steric and electrostatic contributions to the equilibrium between the two conformers. The more populated TED conformer has the amide methyl group *trans* to the carbonyl oxygen, and it is stabilized by an electrostatic attraction between the partially negative carbonyl oxygen and the positively charged quaternary amine nitrogen. As discussed in the accompanying paper (McGroddy, K. A., A. A. Carter, M. M. Tubbert, and R. E. Oswald. 1993. *Biophys. J.* 64:325–338), the differences in the stable solution structures of the TED derivatives and their interconversion kinetics may be of biological significance.

INTRODUCTION

The nicotinic acetylcholine receptors (nAChRs)[1] are the most studied and best characterized of the neurotransmitter receptors. Despite all of the work that has been done in this area, the mechanism by which the binding of an agonist leads to ion channel opening remains unclear. To determine the details of the molecular interactions involved in ion channel gating, a high resolution picture of the receptor protein and its dynamics are required as well as a complete understanding of the structure and dynamics of the activating ligands.

One important approach to determining the structural and electronic features critical for the interaction of a drug with the nAChRs is to compare the binding and ion channel activation of a series of compounds with systematic structural differences. Such an approach, when combined with independent measures of agonist conformation, has proven to be very useful in the design of new agonists and in the definition of properties involved in agonist activity. These studies have, in general, supported the original proposal by Beers and Reich (1970) that an agonist must contain a cationic center and a hydrogen bond acceptor separated by 5.9 Å. This template has been shown to be necessary but not sufficient to understand and predict the potencies of new synthetic compounds (Spivak et al., 1989). In addition, this approach is complicated by potential differences between agonist conformations in the bound and solution states (Behling et al., 1988).

We have synthesized two classes of cholinergic ligands that differ from each other by the presence or absence of a cyclicizing C—C bond (Fig. 1). The solution state structures and internal dynamics of these molecules have been compared using one- and two-dimensional nuclear magnetic resonance (NMR) spectroscopy. Single channel recording has shown that both sets of compounds are agonists, but the acyclic *N,N,N,N'*-tetramethyl-*N'*-acetylethylene-diamine iodide (TED) derivatives are approximately two orders of magnitude less potent than the cyclic 1,1-dimethyl-4-acetyl-piperazinium iodide (PIP) compounds. Inhibition of [^{125}I]-α-bungarotoxin binding also suggests that the PIP compounds are much more potent than the TED compounds (McGroddy et al., 1992). We show here that both the PIP and TED molecules exist in two stable solution structures related by rotation about the amide bond. The PIP derivatives are symmetric with respect to this rotation, so both conformers should have the same biological activity. In the case of the TED compounds, however, the *trans* conformer (Fig. 1) is more stable than the *cis* conformer. The distance between the van der Waals ex-

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[1] *Abbreviations used in this paper:* F_3-PIP, 1,1-dimethyl-4-trifluoroacetyl-piperazinium iodide; F_3-TED, *N,N,N,N'*-tetramethyl-*N'*-trifluoroacetylethylene-diamine iodide; HETCOR, heteronuclear correlated NMR spectroscopy; HPIP, 1,1-dimethyl-4-acetylpiperazinium iodide; HTED, *N,N,N,N'*-tetramethyl-*N'*-acetylethylenediamine iodide; nAChR, nicotinic acetylcholine receptor; NaOD, sodium deuteroxide; NAPZ, *N*-acetylpiperazine; NMR, nuclear magnetic resonance; NOE, nuclear Overhauser effect; NOESY, nuclear Overhauser effect spectroscopy; pre-HTED, *N,N,N'*-trimethyl-*N'*-acetylethylenediamine.

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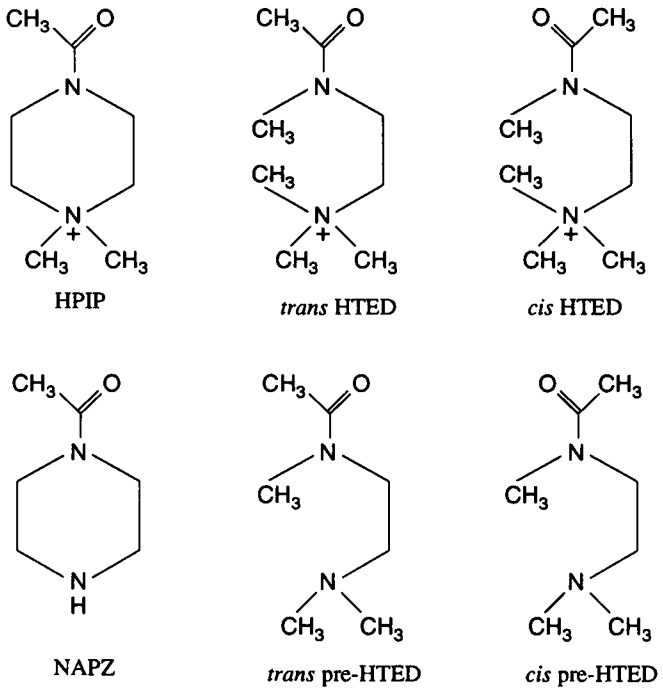

FIGURE 1 Structures of the cholinergic agonist 1,1-dimethyl-4-acetylpiperazinium iodide (HPIP), its open-chain derivative, *N,N,N,N'*-tetramethyl-*N'*-acetylethylenediamine iodide (HTED) in its *cis* and *trans* forms, *N*-acetylpiperazine (NAPZ; the precursor of HPIP), and *N,N,N'*-trimethyl-*N'*-acetylethylenediamine (pre-HTED; the precursor of HTED). F_3-TED and F_3-PIP have a trifluoromethyl group in place of the methyl group bonded to the carbonyl carbon.

tension of the carbonyl oxygen and the quaternary amine nitrogen is considerably shorter than 5.9 Å for the *trans* conformer but can approach 5.9 Å when the amide bond is in the *cis* orientation. The relative stability of the two conformers suggests that the predominance of the *trans* conformation in solution could decrease the concentration of the TED derivative that is available for binding, thus decreasing the apparent affinity of the molecule.

MATERIALS AND METHODS

Compounds

1,1-Dimethyl-4-acetylpiperazinium iodide (HPIP) and its trifluoromethyl analogue (F_3-PIP) were synthesized according to procedures described by Spivak et al. (1986) and Gund et al. (1986). *N,N,N,N'*-tetramethyl-*N'*-acetylethylenediamine iodide (HTED) and its trifluoromethyl analogue (F_3-TED) were synthesized using the same procedure except that 1-methylpiperazine was replaced by *N,N,N'*-trimethylethylenediamine in the first step. *N,N,N'*-trimethyl-*N'*-acetylethylenediamine (pre-HTED) was synthesized by performing the first step of the HTED synthesis. *N*-acetylpiperazine (NAPZ) was purchased from Aldrich Chemical Company (Milwaukee, WI) and was used without further purification. (See Fig. 1 for the structures of these compounds.) Deuterium oxide was purchased from Aldrich at 99.9% purity. Titrations were performed using dilute D_2O solutions of either NaOD or DCl, both purchased from Aldrich.

Variable temperature NMR experiments and assignments

The variable temperature ^1H NMR experiments were performed on a 400 MHz spectrometer (model XL-400; Varian, Sunnyvale, CA) with 10-mM samples of each compound in 0.5 ml D_2O. A series of 128 free induction decays was recorded at each temperature with a recycle time of 10 s. The temperature was calibrated using a sample of neat ethylene glycol with an expected error within 0.3°C (Van Geet, 1968). After changing the temperature, the sample was allowed to equilibrate for ≥10 min at the new setting before any data were collected. Assignments of the peaks were confirmed using one-dimensional decoupling experiments and also through the use of a two-dimensional heteronuclear correlated (HETCOR) NMR experiment. This allows for the assignment of ^1H and ^{13}C resonances that are related through scalar coupling. The HETCOR experiments were also performed on the Varian XL-400 spectrometer using a broadband probe with a ^{13}C resonance frequency of 100 MHz. The sample concentrations ranged from 100 to 300 mM in 0.5 ml D_2O.

Lineshape analysis

For the variable temperature experiments, a series of spectra were taken at eight or nine different temperatures and then transferred to a Digital MicroVax II computer (Digital Equipment Corp., Maynard, MA) where detailed dynamic NMR simulations were performed with programs written in the laboratory using PLOT (Gradient Software, Inc., Ithaca, NY). The Bloch equations were solved for the case of simple two-site exchange with a variable equilibrium constant to generate a dynamic function that gave the signal shape (McConnell, 1958). This dynamic function was then convolved with the appropriate static Lorentzian lineshape equations to give the desired lineshape, including effects of nonexchange broadening and scalar coupling. A complete lineshape analysis of each spectrum was obtained by fitting the static chemical shifts, couplings, and linewidths of the relevant peaks at a temperature below the onset of exchange. This leaves the exchange rate and an inhomogeneous broadening parameter as the only variables. An iterative routine was then used to minimize the rms difference between the simulated curve and the data. Once the rate constant for rotation (k) was known for each temperature, $\Delta G^{\ddagger}$, the Gibbs free energy of activation for this isomerization, was calculated using the Eyring equation (Eyring, 1935):

$$ln\,(k/T) = -\Delta H^{\ddagger}/RT + \Delta S^{\ddagger}/R + ln\,(k_B/h), \quad (1)$$

$$\Delta G^{\ddagger} = \Delta H^{\ddagger} - T\Delta S^{\ddagger}, \quad (2)$$

where k_B is the Boltzmann constant, h is Planck's constant, and R is the universal gas constant. Plots of $ln\,(k/T)$ vs. $1/T$ were constructed. Values of $\Delta H^{\ddagger}$, the enthalpy of activation, and $\Delta S^{\ddagger}$, the entropy of activation, were then obtained from the slope and y-intercept of each Eyring plot and were used to calculate $\Delta G^{\ddagger}$ at a given temperature.

Nuclear Overhauser effect spectroscopy (NOESY)

The phase-sensitive two-dimensional nuclear Overhauser effect (NOE) experiment was performed at the University of Leicester Centre for Biological NMR on a spectrometer (model AM-500; Bruker Instruments, Billerica, MA) using a spectral window of 1.75 kHz. The mixing time was 1.5 s, and the recycle time was 8 s. The data size was 1,024 complex points in the t_2 dimension and 128 hypercomplex points in the t_1 dimension. The data set was transferred to a Silicon Graphics (Mountain View, CA) Iris 4D/220GTX computer for processing with FELIX (Hare Research, Inc., Woodinville, WA). The processed spectrum was zero-filled to give a 2K by 2K real spectrum.

Titrations

A complete titration was performed for each compound using between 9 and 13 samples each, with pH values ranging from 4 to 13. ^1H NMR

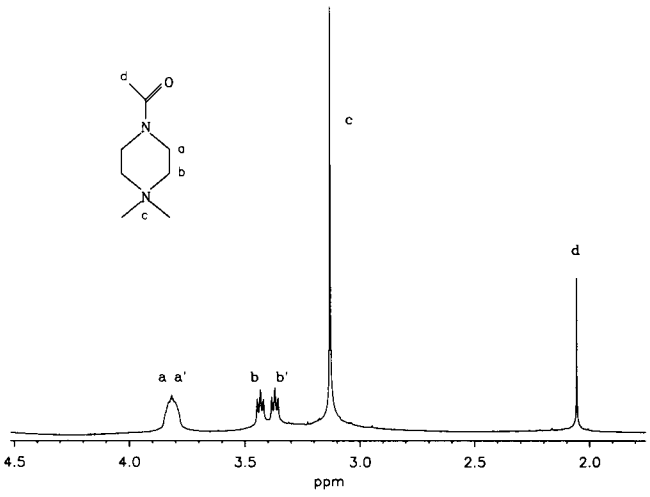

FIGURE 2 A 400 MHz ^{1}H spectrum of HPIP in D_2O at 23°C. The assignments are based on a HETCOR experiment and on one-dimensional decoupling experiments as discussed in the text. The methylene protons on the piperazinium ring are magnetically nonequivalent due to the slow rotation about the amide bond.

spectra at 400 MHz were collected at 23°C. The deuterium ion concentration was determined using an H^+/glass electrode. All pD values are reported as actual pH meter readings without correction for the deuterium isotope effect. A series of one-dimensional spectra was obtained for each molecule, and the chemical shifts were referenced to internal water soluble 3-(trimethylsilyl)-1-propanesulfonic acid (TMS). The relative populations of charged and neutral molecules were determined using Eq. 3:

$$\nu_{obs} = P_{(+)}\nu_{+} + P_{(neu)}\nu_{neu}, \quad (3)$$

$$P_{(+)} + P_{(neu)} = 1, \quad (4)$$

where ν_{obs} is the measured chemical shift, and ν_{+} and ν_{neu} are the chemical shifts at low and high pH, respectively (when no further changes are observed). This information was then used to obtain a pK_A value for the titration. The relative amounts of *cis* and *trans* pre-HTED were determined by integrating all pairs of proton resonances in each spectrum.

Molecular modeling

All of the molecular modeling was performed using CHARMm (vol. 21.2; Polygen Corp., Waltham, MA) on a supercomputer (model 3090-600J; IBM Corp., Danbury, CT) at the Cornell National Supercomputer Facility and CHARMm (vol. 21.2; Polygen) and Quanta (vol. 3.0; Polygen) on a Silicon Graphics Iris 4D/220GTX. The parameters used were from the Polygen Parm30 set, and atomic charges were obtained from the work of Jorgensen and Swenson (1985) and Jorgensen and Gao (1986) on small model compounds.

RESULTS

Resonance assignments

The room temperature ^{1}H spectrum of HPIP (Fig. 2) consists of two singlet resonances at 2.16 and 3.23 ppm, corresponding to the equivalent protons of the acetyl methyl group and the ammonium methyls, respectively. In addition, two sets of exchanging triplets are present between 3.3 and 4.0 ppm. One set (**a** and **a'**) is very broadened, whereas the other set (**b** and **b'**) is clearly defined and of equal amplitude. A HETCOR experiment confirmed that the broadened triplets (**a** and **a'**) arise from the methylene protons ortho to the amide and that **b** and **b'** correspond to the methylene protons meta to the amide nitrogen. A homonuclear decoupling experiment in which the left side of the **a**/**a'** peak was irradiated converted the **b** triplet to a singlet. Likewise, irradiating the right side of the **a**/**a'** peak converted the **b'** triplet to a singlet. A similar result was found on irradiation of the **b** and **b'** peaks. This indicates that each **b** (and likewise **b'**) arises from protons on a single methylene carbon. Since protons in the axial and equatorial positions are unlikely to have the same chemical shift, the conversion between the axial and equatorial positions for the **b** and **b'** protons must be in the fast exchange regime (symmetrical exchange rate $\gg$ 56 Hz). A possible explanation for the observed broadening could be a long-range scalar coupling to the acetyl methyl protons; however, this possibility was eliminated by a one-dimensional decoupling experiment in which the acetyl methyl protons were irradiated and no change in broadening of the **a**/**a'** triplets was observed. Another possibility would be that the broadening of the **a** and **a'** protons may be a result of slowed conversion between the axial and equatorial positions, placing them in the intermediate exchange regime, although it is not clear from preliminary molecular dynamics simulations that this is the case (McGroddy, K. A., unpublished results). The equivalent chemical shifts for the two protons on the **b** carbon and the **b'** carbon suggest that as expected, the amide bond is planar and that the differences in chemical shift between **b** and **b'** are due to the shielding and deshielding arising from the position of the carbonyl bond. The chemical shifts of these protons can thus be used as a sensitive measure of amide bond rotation. The two well-resolved triplets suggest that bond rotation is in the slow exchange regime at 23°C (symmetrical exchange rate $\ll$ 56 Hz), and as expected from the symmetry of the molecule, the two isomers are equally populated.

The trifluoromethyl derivative of HPIP, F_3-PIP, gives rise to a ^{1}H NMR spectrum similar to that of HPIP. Only one singlet is present in the spectrum due to the trifluoromethyl substitution on the acetyl moiety, and the chemical shift differences between the two sets of exchanging triplets are smaller than in HPIP. The less shielded pair of triplets is again significantly broadened when compared with the more shielded pair and the equilibrium constant is one, as observed in HPIP.

These experiments were then repeated for the TED compounds, and very different results were obtained. A proton spectrum of HTED in D_2O is shown in Fig. 3. Two sets of resonances of unequal intensity are observed for each group of magnetically equivalent protons in the molecule. This indicates that two energetically different solution state structures exist for the TED compounds

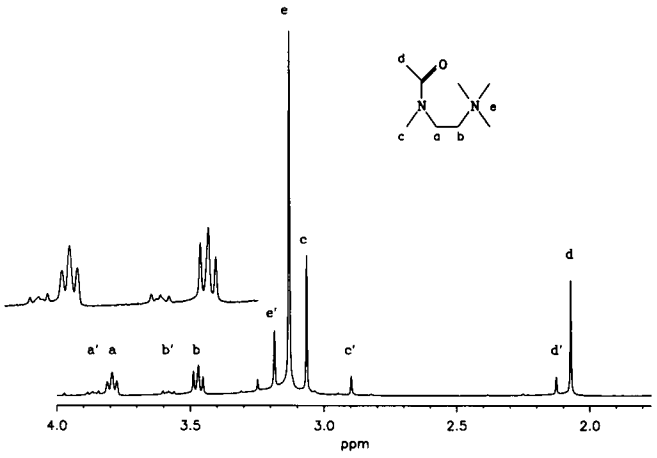

FIGURE 3 A 400 MHz ^1H spectrum of HTED in D_2O at 23°C. Two conformers of unequal energy with an equilibrium constant of 6.13 are observed. The singlets **d** and **d'** correspond to the acetyl methyl protons, **c** and **c'** to the amide methyl protons, and **e** and **e'** to the quaternary amine methyls. The two sets of exchanging triplets again correspond to the methylene protons with the less shielded pair (**a**/**a'**) assigned to the methylene group ortho to the amide nitrogen.

(as opposed to the two symmetric structures of the PIP derivatives), each with a different orientation with respect to the amide bond. All of the minor conformer proton resonances are deshielded with respect to the major conformer resonances except for the amide methyl resonances that have the opposite orientation. This could indicate that all of the protons in the molecule except for the amide methyl protons might have a similar orientation with respect to the carbonyl oxygen. Several theoretical and experimental treatments have been used to attempt to predict assignments in amides, with varying success. It has frequently been observed that the methyl or methylene group *cis* to the carbonyl oxygen resonates at higher magnetic field than the group *trans* due to the diamagnetic anisotropy of the double bond and that methyl or methylene groups that are *trans* to each other across the amide bond exhibit a stronger spin–spin coupling than those that are *cis* to each other (Stewart and Siddall, 1970). The chemical shift argument could be used to suggest that the larger population of the TED derivatives exists with the amide methyl *trans* to the carbonyl oxygen. However, no long-range coupling was observed between the methyl or methylene protons ortho to the amide and the acetyl methyl protons in any of these compounds. As described below, NOE spectroscopy was used to assign the resonances unambiguously.

Since the exchange in HTED was not symmetrical, each pair of resonances was integrated to determine the equilibrium constant for the isomerization. A value of 6.13 ± 1.24 was found for HTED and 4.41 ± 1.30 for F_3-TED (averaging over all sets of resonances for three data sets collected between 25 and 40°C). This corresponds to a free energy difference ΔG^0 of 1.09 kcal/mol between the two conformations of HTED and 0.89 kcal/mol for F_3-TED. The free energy differences for both compounds were temperature and concentration independent over the ranges studied (10–250 mM and 20–60°C).

Solution structure of HTED

To assign the major and minor peaks of the HTED spectrum to either the *trans* or *cis* configuration (see Fig. 1), a two-dimensional phase sensitive NOESY experiment was performed. A phase-sensitive NOESY experiment on a molecule of this size allows the separation of chemical exchange from dipolar interactions. Cross-peaks arising from exchange dynamics will be negative, whereas those due to through-space dipolar interactions will be positive (Sanders and Hunter, 1988). Fig. 4 shows a 500-MHz phase-sensitive NOESY spectrum of a sample of 250 mM HTED in D_2O. All of the exchange cross-peaks can be observed (*dotted lines*) as well as several NOE

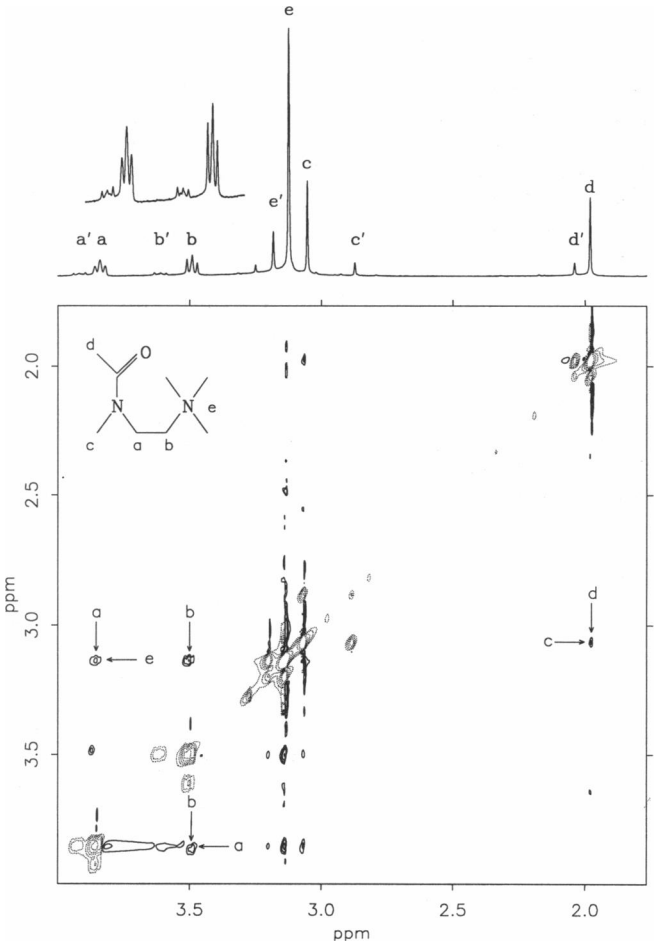

FIGURE 4 A 500 MHz phase sensitive NOESY experiment of 250 mM HTED in D_2O. Cross-peaks due to exchange are shown with dotted lines (negative contours), and NOE cross-peaks are shown as solid lines (positive contours). The corresponding one-dimensional spectrum is shown for comparison.

cross-peaks (*solid lines*). As expected, NOEs are observed between the two sets of methylene protons as well as between these resonances and the quaternary amine methyl proton resonances. In addition, a strong NOE cross-peak is observed between the amide methyl protons and the acetyl methyl protons, whereas none is observed between the acetyl methyl protons and the methylene protons ortho to the amide nitrogen. Also, no cross-peak is observed between the amide methyl protons and the quaternary amine methyl protons, indicating that the latter group must have the same orientation with respect to the carbonyl oxygen as the methylene protons. This is also consistent with the relative chemical shifts of the large and small methylene and quaternary amine methyl resonances. Calculations based on volume integration of the cross-peaks indicate that the average separation distance between the acetyl methyl group and amide methyl group is ~1.9 Å.

To define more clearly the solution state structure of HTED, energy minimization was performed using CHARMm (Polygen). Each of the two orientations of the amide bond was used as a starting structure and then minimized. The relative populations obtained were dependent on the dielectric constant used; however, in all cases the *trans* structure was lower in energy than the *cis* conformation (Fig. 1). Inspection of these minimum energy structures of HTED indicated that the NMR distance constraints could only be obeyed if the major conformer of HTED in D_2O were *trans*, with the quaternary amine methyls rotated toward the carbonyl oxygen. By the similar positions of the chemical shifts, this must also be true for F_3-TED. This conformer is energetically favored either due to the electrostatic attraction between the quaternary ammonium group and the carbonyl oxygen or by a reduction in steric repulsion that might occur between the amide methyl protons and the quaternary amine methyls.

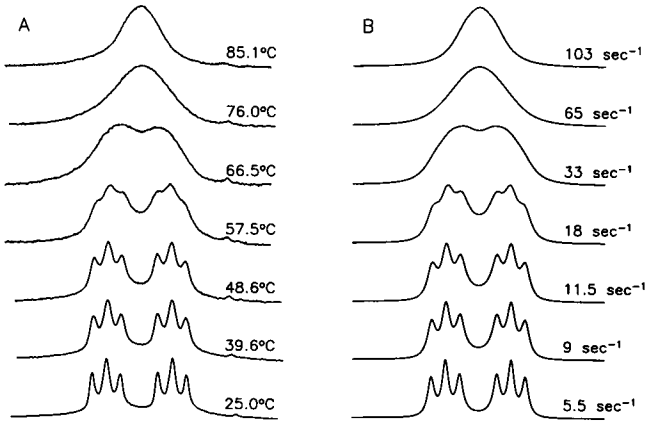

FIGURE 5 Experimental (*A*) and simulated spectra (*B*) for the **b**/**b**′ methylene protons of HPIP with temperatures and rate constants calculated as indicated.

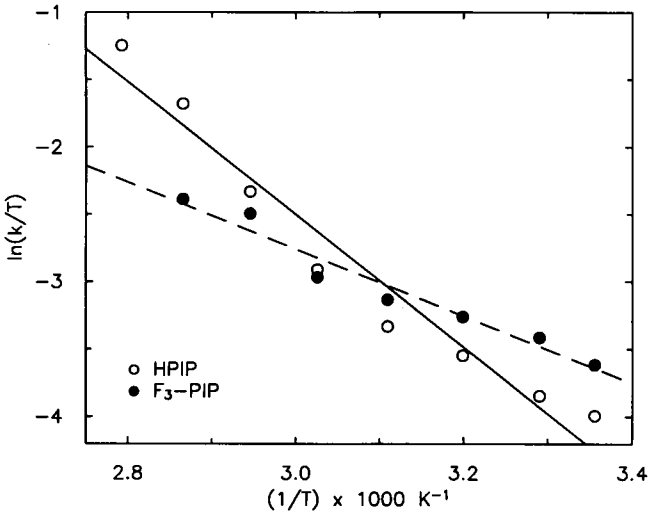

FIGURE 6 Eyring plot for HPIP (○) and F_3-PIP (●) in D_2O calculated from the temperatures and rate constants obtained from the lineshape analysis. The free energy of activation, $\Delta G^{\ddagger}$, is 16.6 kcal/mol at 300 K with enthalpic and entropic components of 9.78 kcal/mol and −22.9 cal/mol-K for HPIP. Values of 16.3 kcal/mol for $\Delta G^{\ddagger}$, 4.3 kcal/mol for $\Delta H^{\ddagger}$, and −37.9 cal/mol-K for $\Delta S^{\ddagger}$ were obtained for F_3-PIP.

Internal dynamics

Fig. 5 shows the spectra and simulations of the **b**/**b**′ methylene protons of HPIP at seven temperatures, with the rate constants generated by the lineshape analysis. The resonances initially broadened as the temperature was increased and then shifted toward each other, finally coalescing at their average chemical shift position. The coalescence is due to an increased rate of amide bond rotation that is a result of the averaging of the two chemical environments when $k > \partial\nu$, i.e., when the exchange frequency is greater than the static frequency of separation between the two peaks (the fast exchange regime). The resulting linear Eyring plot for both PIP derivatives is shown in Fig. 6 and yields an overall free energy of activation, $\Delta G^{\ddagger}$, of 16.8 kcal/mol at 300 K. The enthalpic and entropic components $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$ were 10.5 kcal/mol and −21.1 cal/mol-K, respectively, for HPIP. The variable temperature experiments and lineshape analysis gave a free energy of activation of 16.3 kcal/mol at 300 K for F_3-PIP with enthalpic and entropic components of 5.1 kcal/mol and −37.4 cal/mol-K, respectively.

In the case of both HTED and F_3-TED, no exchange broadening was observed until a temperature of ~60°C, indicating that the onset of bond rotation occurs at a much higher temperature than for the PIP derivatives. All of the resonances in the molecule are sensitive to the exchange. The Eyring plot for the two TED derivatives is shown in Fig. 7. The overall free energies of activation, $\Delta G^{\ddagger}$, were much higher than observed for the PIP molecules: 19.6 kcal/mol for HTED and 18.9 kcal/mol for F_3-TED (*trans*-to-*cis* conversion). The enthalpies and

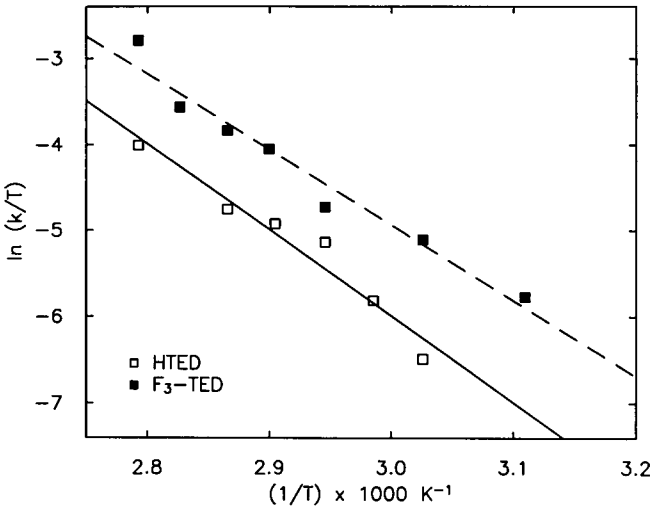

FIGURE 7 Eyring plot for HTED (□) and F_3-TED (■) in D_2O calculated from the temperatures and rate constants obtained from the lineshape analysis. The free energies of activation obtained for HTED and F_3-TED were 19.7 and 18.9 kcal/mol, respectively, and were dominated by the enthalpic components (19.9 kcal/mol for HTED and 17.5 kcal/mol for F_3-TED).

entropies (*trans*-to-*cis* conversion) of activation at 300 K, $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$, were 19.7 kcal/mol and 0.39 cal/mol-K for HTED and 17.1 kcal/mol and −6.4 cal/mol-K for F_3-TED. Thus, the free energy of activation for amide bond rotation for the TED compounds is dominated by enthalpy, whereas entropy makes a significant contribution in the case of the PIP molecules.

Studies of precursors

To determine the contribution of electrostatics to the conformations and dynamics of these compounds, similar experiments were performed on the tertiary amine analogues of the PIP and TED derivatives (NAPZ and pre-HTED).

Effect of electrostatics on solution structures

Titration experiments were performed for NAPZ, an uncharged analogue of HPIP and compared with results obtained for HPIP. Fig. 8 *A* shows titration curves for a group of protons from each of these molecules. The pH changes had no effect on any chemical shifts in HPIP over the range studied (4.7–11). However, chemical shift changes were observed in NAPZ on titration. All of the protons in the molecule incurred a chemical shift change with a pK_A value of 8.2. This resulted in a deshielding of all of the resonances as the pH was lowered. The greatest changes were observed on the methylene protons meta to the amide nitrogen (0.54 ppm), and the smallest shift was observed for the acetyl methyl resonance (0.06 ppm). This indicates that the site of protonation is the quaternary amine nitrogen and not the carbonyl oxygen, which also could have been susceptible to protonation. No population changes were observed in either of these molecules over the pH ranges studied.

A ^1H NMR spectrum of pre-HTED (pH 10) contained two resonances for each group of equivalent protons in the molecule, but the equilibrium constant for the two conformers in D_2O (with no titration) was 1.55 instead of 6.13, the value obtained for HTED. This gives a free energy difference, ΔG^0, of only 0.26 kcal/mol between the *cis* and *trans* conformations instead of the 1.09 kcal/mol observed between the two conformations of HTED. This difference might be explained by either steric or electrostatic factors. That is, either the bulk of the additional methyl group in HTED destabilizes the *cis* conformer or the addition of a positive charge stabilizes the *trans* conformer. To determine the cause of the observed shift in population, a pH titration experiment was

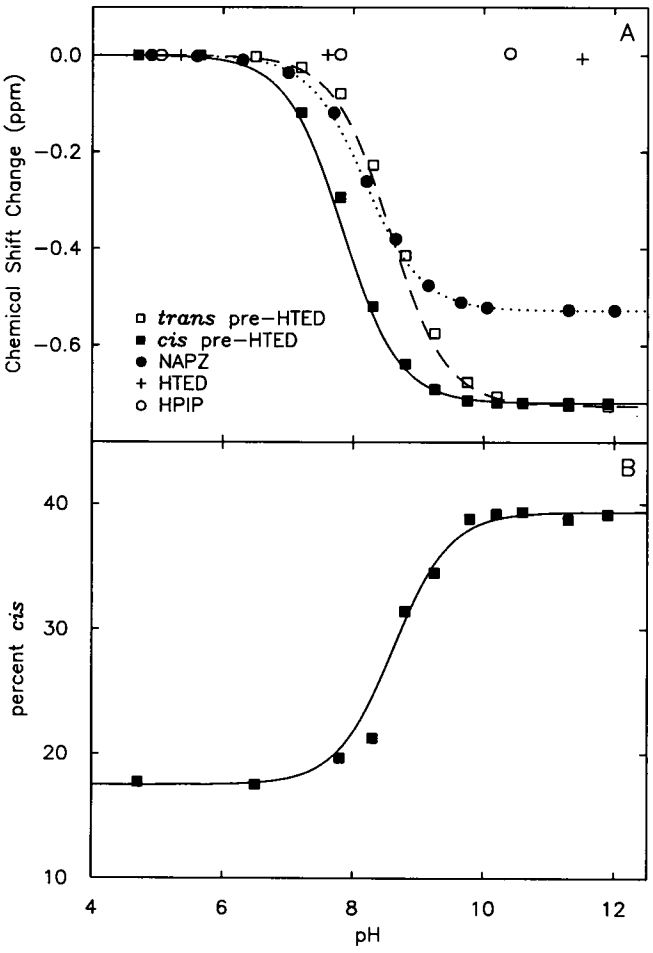

FIGURE 8 (*A*) pH dependence of the chemical shifts of the methylene protons meta to the amide nitrogen in NAPZ (●), the quaternary amine methyl protons from HPIP (○) and from HTED (+), and the *cis* (■) and *trans* (□) quaternary amine methyl protons in pre-HTED. The pK_A values obtained for these titrations were 8.2 for NAPZ, 8.6 for the *trans* pre-HTED, and 7.8 for the *cis* pre-HTED. (*B*) pH dependence of the equilibrium constant between the two conformers of pre-HTED. As more of the molecule becomes protonated, the amount of *trans* pre-HTED increases with a pK_A value of 8.6.

performed with pre-HTED and HTED. Samples of pre-HTED were observed by ^{1}H NMR at pH values ranging from 4.7 to 11.9. By analogy with ammonium ions, it was expected that the pK_A of the quaternary amine nitrogen should be ~9–10. At pH values much below this, most of the molecule should become protonated at the quaternary nitrogen position and therefore charged. If the addition of a positive charge at this position stabilizes the *trans* conformation, then a shift in population should be observed. This can be quantitated by integrating the pairs of proton resonances at the different pH values. A sample of pre-HTED at pH 11.9 gave an equilibrium constant of 1.55. However, the sample at pH 4.7 gave a very different spectrum. The quaternary nitrogen methyl resonances were deshielded from 2.22 and 2.25 ppm to 2.94 and 2.97 ppm on protonation at this nitrogen. The methylene resonances were also deshielded by the protonation, a shift of 0.28 ppm for the ortho methylene protons and 0.86 ppm for the meta methylene protons, which are adjacent to the site of protonation. In addition to these large chemical shift changes, the *trans*/*cis* ratio was markedly increased, giving a new equilibrium constant of 4.65. This corresponds to a free energy difference, ΔG^0, of ~0.92 kcal/mol between the *cis* and *trans* conformations in the protonated molecule and is a change of 0.66 kcal/mol from the deprotonated state. The pK_A values for the protonation were 8.6 for the *trans* conformer and 7.8 for the *cis* conformer. Fig. 8 *B* is a plot of the percentage of pre-HTED in the *cis* conformation at various pH values. The pK_A value obtained for this titration was 8.6. In contrast to these results, no chemical shift changes or population shifts were observed in HTED over the range studied (4.0–11.5).

Effect of electrostatics on internal dynamics

Variable temperature NMR experiments on both pre-HTED and NAPZ (precursor to HPIP) at different pH values were used to determine the contribution of electrostatics to the internal dynamics of the two classes of drugs. The rate constants for amide bond rotation were determined at various temperatures, and the activation parameters (*trans*-to-*cis* conversion; Table 1) were calculated from Eyring plots (Fig. 9). The free energy of activation, $\Delta G^{\ddagger}$, for amide bond isomerization in NAPZ at pH 11 was only 0.4 kcal/mol higher than at pH 7. The

TABLE 1 **Activation parameters for amide bond isomerization**

Compound	$\Delta G^{\ddagger}$	$\Delta H^{\ddagger}$	$\Delta S^{\ddagger}$	k
	kcal/mol	*kcal/mol*	*cal/mol K*	s^{-1}
HPIP	16.8 ± 0.1	10.5 ± 0.5	−21.1 ± 1.3	5.5
F_3-PIP	16.3 ± 0.03	5.1 ± 0.2	−37.4 ± 0.6	8.0
HTED	19.6 ± 0.2	19.7 ± 0.2	0.39 ± 0.1	0.019
F_3-TED	18.9 ± 0.1	17.1 ± 0.5	−4.68 ± 1.9	0.097
NAPZ (pH 7.0)	16.9	10.4	−21.9	2.5
NAPZ (pH 11.0)	17.3	10.7	−21.9	1.4
pre-HTED (pH 5.8)	19.1	18.6	−1.51	0.06
pre-HTED (pH 11.1)	20.1	21.8	5.99	0.01

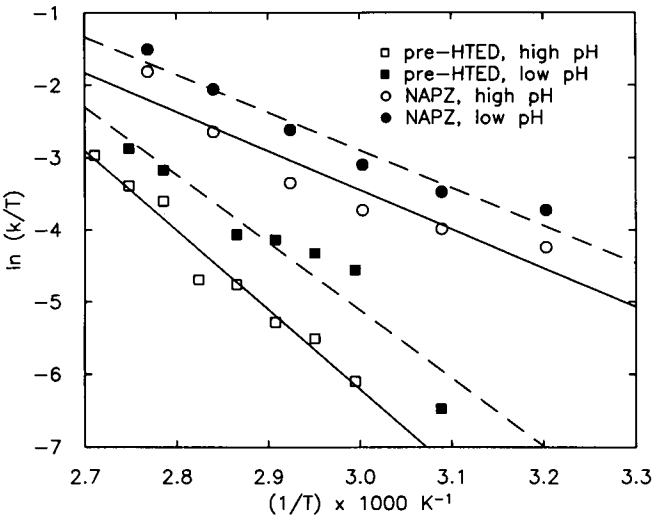

FIGURE 9 Eyring plot for NAPZ (○, pH 11.0, and ●, pH 7.0) and pre-HTED (□, pH 11.1, and ■, pH 5.8) at pH values above and below the pK_A values. The protonation has no significant effect on the free energy of activation for NAPZ but the enthalpy of activation is lowered by 3.2 kcal/mol on protonation of pre-HTED.

contributions to $\Delta G^{\ddagger}$ from enthalpy and entropy were essentially identical at the two pH values. The free energy of activation in pre-HTED was more strongly affected by protonation at the quaternary amine nitrogen. The enthalpic barrier to isomerization, $\Delta H^{\ddagger}$, is 3.2 kcal/mol higher in the unprotonated pH 11 sample than in the protonated pH 5.8 sample. The contribution of $-T\Delta S^{\ddagger}$ decreases the free energy of activation ($\Delta G^{\ddagger}$) in the unprotonated sample by 1.8 kcal/mol but increases the $\Delta G^{\ddagger}$ by 0.5 kcal/mol in the protonated sample. The difference in free energy of activation ($\Delta\Delta G^{\ddagger}$) between the two samples is ~1 kcal/mol, with the unprotonated sample having the higher $\Delta G^{\ddagger}$. Thus, although the free energy difference (ΔG^0) between the *cis* and *trans* forms is greater in the protonated form of pre-TED, the energy barrier to bond rotation is greater in the unprotonated form.

DISCUSSION

We have studied the solution structure and dynamics of two types of cholinergic agonists (the constrained PIP series and the potentially more flexible TED series) using ^{1}H and heteronuclear NMR spectroscopy. Both the equilibrium thermodynamics and the free energies of activation for amide bond isomerization are very different between the two classes of agonist. The cyclic PIP compounds exist in two symmetrical solution state structures on the NMR time scale, with only the chemical shifts of the methylene protons sensitive to the internal dynamics. These two structures correspond to a 180° amide bond isomerization. The free energy of activation, $\Delta G^{\ddagger}$, for these compounds has a large unfavorable entropic component but a fairly small enthalpic compo-

nent. In contrast, the acyclic TED derivatives have two solution state structures of unequal energy and the chemical shifts of all of the protons are sensitive to the internal dynamics. The barrier to amide bond isomerization is large and almost entirely enthalpic (see Table 1).

Solution structure of the compounds

The PIP compounds exist in two symmetric solution structures, differing only in the position of the carbonyl bond with respect to the rest of the molecule. Rotation about the amide bond is in the slow exchange regime at room temperature. A rapid interconversion between the axial and equatorial positions of the amide substituents seems to exist on the NMR time scale at room temperature. The ^{1}H NMR spectrum of the TED compounds suggests that the isomerization of the amide bond is in the slow exchange regime, with one isomer (carbonyl oxygen *trans* to the amide methyl group) energetically favored by 6.13-fold (1.09 kcal/mol) over the other isomer (carbonyl oxygen *cis* to the amide methyl group). Both the distance constraints determined from the two-dimensional NOESY experiment and energy minimization using CHARMm (Polygen) indicate that the *trans* isomer exists in a stable solution structure with the quaternary amine methyls rotated toward the carbonyl oxygen. The lower free energy of the *trans* conformation appears to be largely due to electrostatic attraction between the positively charged quaternary amine methyls and the partially negative carbonyl oxygen.

Effect of electrostatics

The pre-HTED titration experiments provide support for the above hypothesis. At high pH, the quaternary amine of pre-HTED is largely unprotonated and the *trans* form is favored by only 1.55-fold over the *cis* form. Addition of a positive charge by protonation at the quaternary amine nitrogen in pre-HTED stabilizes the *trans* conformation by 0.66 kcal/mol (*trans* form now favored over *cis* form by 4.65-fold). The stabilization of the *trans* conformation at low pH could potentially be explained either by electrostatic attraction or by the formation of a hydrogen bond between the quaternary amine nitrogen and the carbonyl oxygen. Studies of *N*-substituted peptides and dipeptides have shown an increased stabilization of the *trans* isomer on protonation that has been attributed it to an intramolecular hydrogen bond (Gerig, 1971; Evans and Rabenstein, 1974; Unkefer et al., 1983). The pK_A value for the *trans* isomer is 0.7 units higher than for the *cis* isomer, suggesting the presence of a hydrogen bond. However, since HTED is not capable of forming an intramolecular hydrogen bond and the structures of the two molecules appear to be very similar, electrostatic attraction appears to be sufficient to determine the conformations in both cases.

An additional explanation for the stability of a folded solution structure of HTED could be a hydrophobic hydration effect since a considerable portion of the molecule forms a hydrophobic surface. In some small molecules, a more compact conformation of a hydrophobic molecule in aqueous solution is energetically favored due to an increase in solvent entropy (Franks, 1975). Entropic stabilization can occur when less hydrophobic surface area is accessible to solvent. However, preliminary calculations using energy minimized structures (McGroddy, K. A., unpublished results using the CHARMm force field) show that the solvent accessible surface area is affected more by the orientation of the N(+)—C—C—N backbone dihedral than by the orientation of the amide dihedral. The hydrophilic solvent accessible surface area is slightly larger in both *cis* forms than in the two *trans* forms, most likely because the carbonyl oxygen points away from the N(+)—C—C—N backbone in the *cis* orientation. However, the hydrophobic solvent accessible surface area is determined more by the N(+)—C—C—N orientation than by the amide dihedral. For example, the TRANS (GAUCHE and TRANS refer to the N(+)—C—C—N backbone of TED; *cis* and *trans* refer to the amide bond) orientation of the N(+)—C—C—N backbone has significantly more hydrophobic surface area than the GAUCHE conformation (5 Å^2 more in the case of the *trans* conformer and 8 Å^2 more in the case of the *cis* conformer), whereas the difference between the *cis* and *trans* isomers both in the TRANS orientation is ~1 Å^2. The free energy of stabilization due to the hydrophobic interaction is thought to be approximately −24 to −47 cal/mol/Å^2 of buried surface area (Chothia, 1974; Sharp et al., 1991). This would lead to a stabilization of the two GAUCHE forms with respect to the two TRANS forms of 0.1–0.4 kcal/mol, and the *cis* amide forms would be 0.02–0.2 kcal/mol more stable than the *trans* amide forms. Thus, the hydrophobic hydration effect is probably not an important contributor to the difference between the *cis* and *trans* forms of the TED molecules.

Effect of halogenation

Substituting a trifluoromethyl group for the acetyl methyl group has little effect on the solution structure of the PIP molecules. The only difference appears to be a decrease in the chemical shift difference between the two methylene groups meta to the amide nitrogen. This in turn is probably due to either an increased rate of rotation about the amide bond at room temperature in F_3-PIP as compared with HPIP or to the differences in electronic distribution in the acetyl portion of the molecules due to the electronegative fluorine substituents. The same substitution in F_3-TED leads to a change in the equilibrium constant between the two conformers (from 6.13 in HTED to 4.41 in F_3-TED), corresponding to a smaller *cis*/*trans* free energy difference (0.89 kcal/mol for F_3-TED vs. 1.09 kcal/mol for HTED).

Internal dynamics

Energetics of amide bond isomerization

Table 1 shows the values obtained for the free energy of activation, $\Delta G^{\ddagger}$, for amide bond isomerization in each

of the molecules studied, along with the enthalpic and entropic components $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$. The differences are pronounced between the cyclic and acyclic molecules. The overall free energies of activation for amide bond isomerization are ≥ 2.1 kcal/mol higher for the TED derivatives than for the PIP molecules. This is mainly due to a much higher enthalpy of activation, $\Delta H^{\ddagger}$, for the TED compounds that is balanced somewhat by the large unfavorable contribution of entropy to the free energy barriers of the PIP molecules. Similar results are seen when the analogues pre-HTED and NAPZ are compared. The large entropic contribution to the free energy of activation for the PIP compounds indicates that the transition state is much more ordered than the ground state.

Rates of amide bond isomerization

The large difference in rates of amide bond isomerization between the PIP and TED compounds could possibly be explained by two different electrostatic effects. First, since the piperazinium ring constrains a positive charge near the amide nitrogen in the PIP molecules, this could potentially destabilize the double bond character of the amide bond and allow for easier internal rotation. The positive charge on average could be further away from the amide bond in the TED derivatives, allowing for more double bond character and a correspondingly higher free energy of activation to rotation. The rationale behind this hypothesis is that the amide moiety exists in a combination of two resonance forms: one with the double bond between the carbon and the oxygen that form the carbonyl and the other with the double bond between the nitrogen and the carbon. This latter form exists with a negative charge on the oxygen and a positive charge on the nitrogen. This resonance form would be destabilized by the presence of a positive charge distribution constrained to be near the amide nitrogen. Second, alternatively, the TED compounds could exist in a stable solution structure on the NMR timescale where the positive charge distribution of the quaternary amine is fixed in a position near in space to the partially negative carbonyl oxygen. This would have the effect of increasing the double bond character of the amide bond because of the increased partial negative charge on the carbonyl oxygen.

The first alternative can be ruled out based on the results of the titration experiment and dynamics studies on NAPZ. If the presence of a positive charge on the quaternary amine nitrogen does in fact lead to destabilization of the double bond character of the amide bond, protonation at this site in NAPZ should have led to a significant decrease in the enthalpic barrier to isomerization. Instead, no significant change was observed. The structure suggested by the second alternative was supported by all available evidence. In particular, the distance constraints from the two-dimensional NOESY experiment are consistent with the interaction of the carbonyl oxygen and the quaternary amine. However, the major differences observed in the breakdown of the energetics of amide bond isomerization appear to be between the two steric classes of molecules (cyclic and acyclic) rather than the due to the electrostatics. This suggests that steric effects play a significant role in modifying the energetics of amide bond isomerization in these molecules, whereas the effect of electrostatics on the internal dynamics is negligible. This is the opposite of what we observed for the equilibrium thermodynamics, where steric effects are much less important than electrostatics in determining the equilibrium *cis*/*trans* ratio.

Effect of protonation

Protonation at the quaternary amine nitrogen had no significant effect on the enthalpic or entropic contributions to $\Delta G^{\ddagger}$ in NAPZ. However, a decrease in the enthalpic barrier to isomerization of 3.22 kcal/mol was observed in pre-HTED on protonation. This may be due to a lowering of the free energy of the transition state. At low pH, the protonated quaternary amine nitrogen of pre-HTED can form two hydrogen bonds to the solvent, whereas the unprotonated pre-HTED molecule can form only one. This could cause the transition state of protonated pre-HTED to be 3–4 kcal/mol lower in energy (enthalpy) than the transition state of the unprotonated molecule, thus lowering the barrier for isomerization.

Both protonated pre-HTED and the TED compounds exhibit very small entropic contributions to the free energy barrier to amide bond isomerization, indicating that the transition state is not greatly ordered in comparison to the ground state in any of these molecules. This is probably due to the stabilization of the ground state structures by intermolecular electrostatic attraction and to the ordering of the transition state due to the interaction of the positively charged amine nitrogen with the solvent.

Effect of halogenation

Within each class of compound (PIP or TED), the overall free energy of activation obtained was fairly similar (within 1 kcal/mol), but the breakdown of the free energy into its constituent components varied depending on whether the substituents on the acetyl moiety were hydrogen or fluorine atoms. The substitution of electronegative fluorine atoms led to a reduction in the enthalpy of activation and to an increase in the contribution of the entropy of activation to the overall free energy barrier. This is probably due to a reduction in the double bond character of the amide bond caused by the presence of the trifluoromethyl group.

CONCLUSIONS

The dramatic differences in agonist potency between the PIP and TED derivatives can be partially explained by their very different solution structures and internal dy-

namics. Due to the steric hindrance of the piperazinium ring, the PIP compounds were thought to be restricted to a relatively rigid chair-like conformation, with an enthalpic barrier to *cis-trans* isomerization of ~2 kcal/mol in vacuum (Spivak et al., 1986). However, the spectroscopic results suggest that a rapid interconversion between chair and boat conformations occurs on the NMR time scale. As shown in Table 1, the enthalpic barrier to isomerization in D_2O is higher than the earlier vacuum calculations had predicted: 9.78 kcal/mol for HPIP and 4.93 kcal/mol for F_3-PIP, as compared with the calculated value of ~2 kcal/mol (Spivak et al., 1986). It was initially thought that the TED molecules could exist in a multitude of conformations and that the large loss of entropy on the binding of HTED to the nAChR was a primary cause for the difference in agonist behavior of HPIP and HTED. However, these NMR data show that HTED exists in two distinct stable conformations, the more favored of these consisting of a folded structure with the ammonium nitrogen held by electrostatic attraction near the carbonyl oxygen. Both the poor agonist behavior of the TED derivatives and their much slower rates of internal amide bond isomerization could be partially explained by this. If this structure is sufficiently stable, it will be energetically unfavorable for the molecule to unfold to interact with the binding site on the nAChR. In addition, isomerization about the N—C amide bond may be somewhat restricted because the interaction between the charged nitrogen and carbonyl oxygen will increase the double bond character of the amide bond due to the partial negative charge on the carbonyl oxygen.

It can be argued that the solution structure of a compound has no relevance to its actual bound state conformation since the local environment of the binding site will tend to be very different from bulk solvent. However, if a large conformational change is required for a binding event to occur, this event may be energetically unfavorable. If, in fact, the smaller population of HTED is already in or near the correct configuration for binding to the nAChR and the larger population must isomerize to bind, this could at least partially explain the differences in affinity and efficacy between the two classes of compounds (McGroddy et al., 1992). Using the Beers and Reich criterion as a predictor of agonist potency, it is expected that the TED molecules with the amide bond in the *cis* orientation will be more potent than the *trans* TED conformers. If an energy barrier of 18–20 kcal/mol must be surpassed to assume a conformation favorable for binding, then the probability of many interactions with the receptor will be low and internal dynamics may then play a large role in determining the ability of a molecule to isomerize into the correct configuration for binding. As described in the following article (McGroddy et al., 1992) using ^{19}F NMR spectroscopy, it is the minor population of the F_3-TED that appears to interact with the receptor. If the *trans* conformer does not bind to the receptor, approximately a sixfold difference in affinity would be predicted. Since the difference in affinity between HPIP and HTED is approximately one to two orders of magnitude, additional differences between the two compounds (e.g., entropic effects, the orientation about the N(+)—C—C—N backbone dihedral, or differences in the energetics of desolvation before binding) must account for the remainder of the differences.

In conclusion, we have shown that the acyclic TED derivatives exist in two energetically different solution state structures, related by isomerization of the amide bond. This bond rotation is characterized by a high energy barrier so that the rate of interconversion at room temperature is very slow. The kinetics of this interconversion may play a role in determining the apparent affinity of these molecules for the nAChRs. Molecular dynamics simulations are currently being performed on aqueous solutions of HPIP and HTED to study the time evolution of the molecules' solution structures and the differences in solvation of these compounds.

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