Analysis of cyclic and acyclic nicotinic cholinergic agonists using radioligand binding, single channel recording, and nuclear magnetic resonance spectroscopy

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ABSTRACT The relationship between the structure and function of a series of nicotinic cholinergic agonists has been studied using radioligand binding, single channel recording, and nuclear magnetic resonance spectroscopy. The cyclic compound 1,1-dimethyl-4-acetylpiperazinium iodide and its trifluoromethyl analogue (F_3-PIP) interact with nicotinic acetylcholine receptors (nAChRs) from both *Torpedo* electroplaque and BC_3H-1 cells at lower concentrations than the acyclic derivatives, *N,N,N,N'*-tetramethyl-*N'*-acetylethylenediamine iodide and its fluorinated analogue (F_3-TED). The magnitude of the difference in potencies depends on the type of measurement. In binding experiments, the differences between the two classes of compounds depends mainly on the conditions of the experiment. In measurements of the initial interaction with the nAChR, the PIP compounds have an affinity approximately one order of magnitude higher than that of the TED compounds. Longer incubations indicated that the PIP compounds were able to induce a time-dependent shift in receptor affinity consistent with desensitization, whereas the TED compounds were unable to induce such a shift. The activation of single channel currents by the cyclic compounds occurs at concentrations approximately two orders of magnitude lower than for the acyclic compounds, but the TED compounds exhibit a larger degree of channel blockade than the PIP compounds. Previous work (McGroddy, K. A., and R. E. Oswald. 1992. *Biophys. J.* 64:314–324) has shown that the TED compounds can exist in two energetically distinct conformational states related by an isomerization of the amide bond. ^{19}F nuclear magnetic resonance experiments suggest that the higher energy population of the TED compounds may interact preferentially with the ACh binding sites on the nAChRs and that a significant fraction of the difference between the initial affinity of the PIP and TED compounds may be accounted for by the predominance in solution of a conformational state less able to interact with the ACh binding sites on nAChRs.

INTRODUCTION

The cloning and sequencing of a large number of nicotinic acetylcholine receptor (nAChR)[1] subunits in recent years has led to a wealth of information concerning the specific amino acids thought to play important roles in the acetylcholine binding sites and the binding sites for channel blockers (for review, see Changeux, 1990). Both site-directed mutagenesis (e.g., Galzi et al., 1991; Tomaselli et al., 1991) and affinity labeling (e.g., Kao et al., 1984; Galzi et al., 1990) have identified specific aromatic amino acids that may comprise part of the cholinergic binding site in much the same way as the aromatic residues found in the active site of acetylcholinesterase (Sussman et al., 1991). Likewise, chemical modifications of agonist structure have been used to study the important structural and electronic features required for nicotinic cholinergic agonist activity (e.g., Papke et al., 1988; Spivak et al., 1989). The original work of Beers and Reich (1970) suggests that the requirements include a positive charge distribution centered ~5.9 Å from a hydrogen bond acceptor. In addition, the presence of a methyl group adjacent to the carbon atom bearing the hydrogen bond acceptor is important (Waters et al., 1988).

A commonly used approach to determine features important for binding involves the synthesis of compounds with slight variations in their chemical structure (for review, see Hansch, 1983). Comparison of the functional effects of newly synthesized compounds with structures obtained from either molecular modeling studies or nuclear magnetic resonance (NMR) spectroscopy may not lead to clear results, however, since the structures being studied in these cases may not resemble the bound state structure of the ligand (e.g., Behling et al., 1988; Ni et al., 1990). Also, in some cases, modifications of the chemical structure of a ligand can produce unexpected changes in its orientation within a binding site such that ligand affinity is changed in an unpredictable fashion (Ealick et al., 1991). Nevertheless, useful information has been obtained by this approach when the chemical structures are relatively rigid.

Two series of compounds that interact with nAChRs were synthesized, one that is sterically restrained by the presence of a piperazinium ring and the other that is acyclic and presumably able to access a greater variety of conformational states. The corresponding compounds in each series are chemically identical except for the presence or absence of one C—C bond. Within the series of cyclic compounds, the relationship between chemical structure and biological activity is reasonably well de-

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[1] *Abbreviations used in this paper:* αBgt, α-bungarotoxin; α[^{125}I]Bgt, ^{125}I-labeled α-bungarotoxin; F_3-PIP, 1,1-dimethyl-4-trifluoroacetylpiperazinium iodide; F_3-TED, *N,N,N,N'*-tetramethyl-*N'*-trifluoroacetylethylene-diamine iodide; HPIP, 1,1-dimethyl-4-acetylpiperazinium iodide; HTED, *N,N,N,N'*-tetramethyl-*N'*-acetylethylene-diamine iodide; MOPS, 3-[N-morpholino]propanesulfonic acid; nAChR, nicotinic acetylcholine receptor; NMR, nuclear magnetic resonance spectroscopy.

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fined (Carter et al., 1992). In this communication, we attempt to compare the functional activity with the structure of the cyclic versus acyclic compounds. Previous work (McGroddy and Oswald, 1992) has shown that the cyclic 1,1-dimethyl-4-trifluoroacetylpiperazinium iodide (PIP) compounds exist in two symmetric solution state structures on the NMR time scale, both of which undergo rapid chair/boat interconversion. The acyclic *N,N,N,N'*-tetramethyl-*N'*-trifluoroacetylethylenediamine iodide (TED) compounds exist in two different solution structures, one of which is favored by ~1 kcal/mol over the other. The interconversion between these two structures involves isomerization about an amide bond and is characterized by a high energy barrier (~19 kcal/mol) and an extremely slow rate constant at room temperature (k ~0.01 s^{-1}). Radioligand binding and single channel recording studies were used to investigate the ability of these compounds to bind to and activate the nAChR from *Torpedo* electric organ and from BC_3H-1 cells under several conditions. In addition, we show using fluorine (^{19}F) NMR spectroscopy that one of the two TED conformers can interact with the nAChR from *Torpedo* electroplaque. No binding of the other conformer was detected, although the presence of a tightly bound species cannot be definitively ruled out. These results suggest that the solution structure of the TED compounds could play an important role in determining the binding affinity and, as will be discussed, can provide suggestions as to the bound conformation of the compound.

MATERIALS AND METHODS

Materials

Deuterium oxide was purchased from Aldrich Chemical Co. (Milwaukee, WI) at 99.9% purity. $\alpha[^{125}I]$Bungarotoxin ($\alpha[^{125}I]$Bgt; 70–140 Ci/mmol) was purchased from New England Nuclear (Wilmington, DE). Frozen *Torpedo nobiliana* electroplaque was obtained from Biofish Associates (Georgetown, MA). The BC_3H-1 cell line was from American Type Culture Collection (Rockville, MD). 1,1-Dimethyl-4-acetylpiperazinium iodide (HPIP), F_3-PIP, *N,N,N,N'*-tetramethyl-*N'*-acetylethylene-diamine iodide (HTED), and F_3-TED were synthesized as described previously (McGroddy and Oswald, 1992). α-Bungarotoxin (αBgt) was purified from the venom of *Bungarus multicinctus* as described previously (Freeman et al., 1980), except that Mono Q and Superose 12 columns were used in a Pharmacia (Gaithersburg, MD) FPLC. The remaining chemicals were from Sigma Chemical Co. (St. Louis, MO). Because of the slow hydrolysis in solution ($t_{1/2}$ of ~2 h, measured by ^{19}F and ^1H NMR spectroscopy), F_3-PIP was dissolved immediately before use, and experiments using this compound were completed within 25 min or less. F_3-TED shows no sign of hydrolysis for up to 12 h in solution under the conditions used in these experiments.

Membrane preparation and cell culture

Torpedo electroplaque

Membranes rich in nAChRs were prepared from frozen *Torpedo nobiliana* as described previously (Saitoh et al., 1980) and were stored in liquid nitrogen at 10–15 μM (expressed in αBgt binding sites) until needed. The concentration of $\alpha[^{125}I]$Bgt sites was measured by the DE81 filter disc assay (Schmidt and Raftery, 1973) using 0.1% w/v Triton X-100 in 10 mM tris(hydroxymethyl)-aminomethane (pH 7.2) as the buffer. The protein content of samples or bovine serum albumin standards were determined using a Bicinchoninic acid Protein Assay Kit from Pierce (Rockford, IL).

BC_3H-1 cells

BC_3H-1 cells were grown and maintained in Dulbecco's modified Eagle medium with 10% fetal calf serum at 37°C in 10% CO_2 and passed weekly. After enzymatic dissociation of the stock cultures, an aliquot of the cells was resuspended in growth medium and plated in T-150 flasks. Twenty-four hours later, the cells were rinsed in low serum medium (0.5% fetal calf serum) and subsequently maintained in low serum medium with medium changes every 4–5 d (Olsen et al., 1983). Cells were used for single channel recording or harvested for binding assays 8–10 d after serum change. To harvest the cells, 20 ml per flask of 20 mM 3-[N-morpholino]propanesulfonic N acid (MOPS)-NaOH with 1 mM EGTA (pH 7.4) were added to the cells for 30 min at 4°C. The cells were then collected in the same buffer and homogenized three times in a Brinkman (Westbury, NY) Polytron (setting 6) for 10 s each. The homogenate was centrifuged at 20,000 g for 30 min, and the pellet was resuspended to 0.4 mg protein/ml in 50 mM MOPS-NaOH with 1 mM EGTA (pH 7.5). This homogenate was used for subsequent experiments.

Binding Measurements

The inhibition of the initial rate of $\alpha[^{125}I]$Bgt binding by HPIP, F_3-PIP, HTED, and F_3-TED was measured in membrane fragments from both *Torpedo* electroplaque and BC_3H-1 cells at 4°C. Two protocols were used: simultaneous addition and prior addition. In both cases, the buffer was 50 mM MOPS-NaOH with 5 mM EGTA (pH 7.5). The $\alpha[^{125}I]$Bgt concentration was 5 nM for both tissue types. *Torpedo* membranes were diluted to 2.5 nM (expressed in $\alpha[^{125}I]$Bgt sites), and BC_3H-1 membranes were used at a final dilution of 0.2 mg protein/ml. The simultaneous addition protocol consisted of mixing the PIP or TED derivative with $\alpha[^{125}I]$Bgt and then adding this mixture to the membrane fragments. This provides an indication of the ability of the compound to inhibit $\alpha[^{125}I]$Bgt binding before the completion of the slow phase of desensitization (Weber et al., 1975; Weiland et al., 1976). The prior addition protocol consists of incubating the PIP or TED derivatives with membrane fragments for 5–15 min, followed by the addition of $\alpha[^{125}I]$Bgt. This provides an indication of the ability of the compound to bind to and desensitize the nAChR. The protection constant (K_p) was determined for each data set by a nonlinear least-squares fit (Marquardt-Levenberg algorithm; PLOT, Gradient Software, Inc., Ithaca, NY) to the following equation:

$$\frac{k_T^L}{k_T} = \frac{1}{1 + \left(\frac{L}{K_P}\right)^{n_H}}, \qquad (1)$$

where L is the ligand concentration, n_H is the Hill coefficient, and k_T^L and k_T are the initial rates of $\alpha[^{125}I]$Bgt binding in the presence and absence of competing ligand. In all cases, n_H was not significantly different from 1, so that n_H was fixed to a value of 1 in all subsequent analyses. All computations were performed on a Sun (Mountain View, CA) 4/330 workstation.

Single channel recording

Single channels were recorded from nAChRs on BC_3H-1 cells using the cell attached configuration with the agonist in the pipette as described by Hamill et al. (1981). Both the bathing medium and the medium inside the pipette consisted of 147 mM NaCl, 5.4 mM KCl, 1 mM $MgCl_2$, and 10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethane sulfonic acid, pH 7.4. All data were collected using a Dagan patch clamp ampli-

fier at 90 mV hyperpolarized with respect to resting potential, with the temperature maintained at 15 ± 0.3°C using a Medical Systems (Greenvale, NY) TC-202 temperature controller. The data were filtered at 5 kHz using an 8 pole Bessel filter (Frequency Devices, Haverhill, MA) and transferred at 44 kHz to a Macintosh IIci computer. The data were then transferred to a VAXStation II computer (Digital Equipment Corp., Maynard, MA) for analysis. Semiautomated channel detection software with a threshold crossing algorithm and with user-verification of all channels was used (software developed in the laboratory).

Data analysis was performed on a Sun 4/330 or a Silicon Graphics (Mountain View, CA) 4D/220 GTX workstation using software developed in the laboratory, except where otherwise indicated. Dwell times were fit to multiple exponentials using maximum likelihood analysis (Colquhoun and Sigworth, 1983) and a Simplex algorithm (Caceci and Cacheris, 1984). Rate constants were estimated using three procedures: (*a*) at low agonist concentrations where relatively simple correlations between rate constants and exponential components of a dwell time distribution could be made, the rates were estimated from the presumed exponential components (exponential fitting); (*b*) data were fit by comparing the predicted *pdf* for a given set of rate constants (determined using the techniques described by Colquhoun and Hawkes, 1981) with the observed data, and the constants were optimized using a Simplex algorithm and the maximum likelihood criterion (matrix method); and (*c*) rate constants were determined by combinatorial analysis as described by Horn and Lange (1983) using software generously provided by Dr. Anthony Auerbach (which includes subroutines provided by Dr. R. Horn). Two-dimensional dwell time distributions (Magleby and Weiss, 1990) were displayed using Transform and Format (Spyglass, Champaign, IL) on a Macintosh II computer.

NMR experiments

Sample preparation

For the NMR samples, *Torpedo* membranes were thawed and incubated with 1 mM diisopropyl fluorophosphate for 15 min to inactivate residual acetylcholinesterase activity. The diisopropyl fluorophosphate-treated membranes were then diluted to 2.5 mg protein/ml with buffer I (20 mM MOPS, 400 mM NaCl, 50 mM KCl, 1 mM EDTA, 1 mM EGTA, pH 7.4). The receptor was solubilized by adding 20% recrystallized Na cholate to a final concentration of 2%, incubating at 4°C with stirring for 1 h, and then centrifuging in a rotor (model Ti50; Beckman Instruments, Fullerton, CA) at 110,000 *g* for 30 min. The supernatant was placed in Spectrapor dialysis tubing (mol wt cutoff 12,000–14,000), and Sephadex G-200 was applied to the outside of the tubing to concentrate the sample. The concentration procedure took ~48 h at 4°C with an initial volume of 14–15 ml and a final sample volume typically 0.4–0.6 ml.

Data acquisition

The fluorine NMR experiments were performed on a spectrometer (model XL-400; Varian, Sunnyvale, CA) using a proton probe tuned to 376.105 MHz and a spectral width of 5400 Hz centered between the two major peaks in the spectrum. Between 3,600 and 6,000 free induction decays were averaged in each case. All analysis was performed using FELIX (Hare Research, Inc, Woodinville, WA) after transfer to a Silicon Graphics 4D/220GTX computer. The time domain signal was apodized with a matched exponential function. Samples consisted of 10–40 μM acetylcholine binding sites (as determined by $\alpha[^{125}I]$Bgt binding assays) and 200–250 μM F_3-TED in a 20% D_2O solution. Trifluoroacetic anhydride at a concentration of 200 μM was used as an internal reference. When used, an excess concentration of HPIP (typically 5–6 mM) was added to displace F_3-TED from its binding sites. Control experiments were also performed in the presence of 80 μM αBgt.

RESULTS

Radioligand binding measurements

General considerations

Two distinct states of agonist affinity are apparent using the simultaneous addition protocol and the prior addition protocol. As demonstrated previously (Weber et al., 1975; Weiland et al., 1976, 1977), the affinity for agonists increases on the time scale of seconds to minutes after agonist addition. This state transition is indicative of receptor desensitization in that the functional activation of the channel decreases in parallel with the increase in affinity (Sine and Taylor, 1979). To a first approximation, data from equilibrium competition experiments can be modeled as follows:

$$\begin{array}{ccc} L + R & \overset{K_L}{\rightleftharpoons} & LR \\ {\scriptstyle M}\uparrow\downarrow & {\scriptstyle K'_L} & \\ L + R' & \rightleftharpoons & LR' \end{array}, \qquad \text{(A)}$$

where R is the resting, nondesensitized receptor, R' is the desensitized receptor, L is the ligand, M is the allosteric constant reflecting the ratio of R'/R, K_L is the equilibrium dissociation constant of the ligand for the desensitized state. This model has been used extensively for the analysis of binding data (e.g., Sine and Taylor, 1979; Weber et al., 1975; Weiland et al., 1976, 1977); however, time-dependent shifts in receptor affinity occur on time scales that cannot be resolved by this technique (Boyd and Cohen, 1980; Dunn et al., 1980; Heidmann and Changeux, 1980). The protection constant for the simultaneous addition protocol (K_P^{simul}) provides an upper limit of the affinity for the nondesensitized state (i.e., lower limit on the numerical value of K_L) in that the process of desensitization (accompanied by an increase in affinity) is occurring during the measurement process. The protection constant for the prior addition condition (K_P^{prior}) is related to the affinity for the desensitized state (K'_L) by the following relationship:

$$K'_L = \frac{MK_P^{prior}}{\left(1 + M - \frac{K_P^{prior}}{K_L}\right)}. \qquad (2)$$

K_L cannot be determined directly but will be greater than or equal to K_P^{simul} due to a time-dependent increase in affinity. This means that the K_P^{prior}/K_L ratio is significantly less than one for agents that bind preferentially to R' and approximately equal to one for those that bind nonselectively. In the case of potent cholinergic agonists, the K_P^{prior}/K_L ratio is small, reducing the equation to:

$$K'_L = \frac{MK_P^{prior}}{(1 + M)}. \qquad (3)$$

M is ~0.1 (Weiland et al., 1976) for *Torpedo* electroplaque and 0.017 for BC_3H-1 cells (Sine and Taylor, 1979).

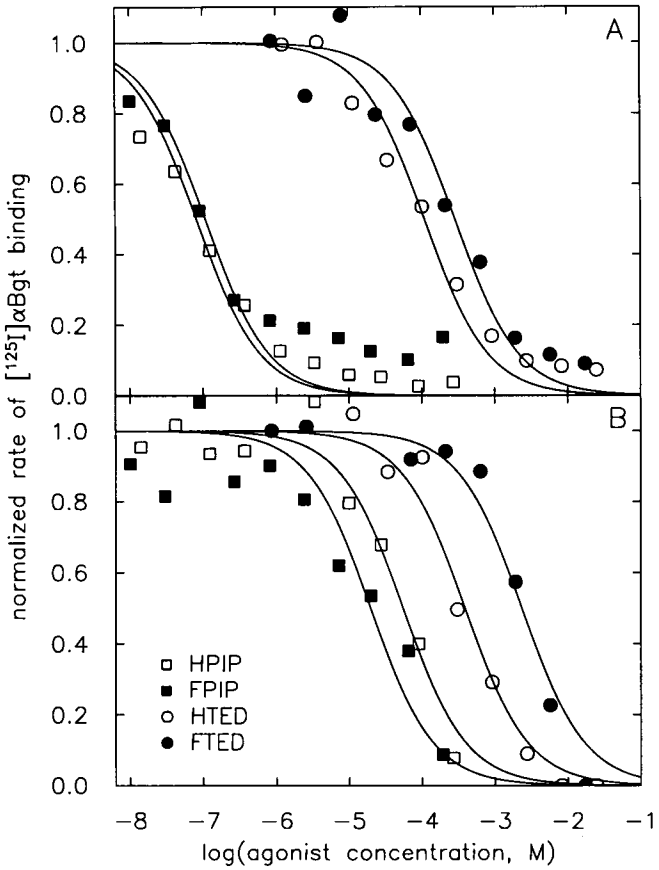

FIGURE 1 The inhibition of the initial rate of $\alpha[^{125}I]$Bgt binding to the nAChR from *Torpedo* electroplaque membranes by PIP and TED compounds. Representative experiments are shown, and the points are averages of duplicate determinations. For the prior addition condition (*A*), membranes were incubated with the test compound for 5 min before the addition of $\alpha[^{125}I]$Bgt. A small percentage of the receptors was in the low affinity state as shown by the deviation from simple mass action inhibition, particularly for FPIP. Shown is the fit to the high affinity portion of the binding curve. Longer incubations converted essentially all of the receptor population to the high affinity state. For the simultaneous addition condition (*B*), the compound to be tested was added to the membrane at the same time as $\alpha[^{125}I]$Bgt, and the initial rate of binding was measured. Binding was linear up to 30 s. Data were normalized to binding in the absence of PIP and TED compounds, and nonspecific binding was defined by incubation in the presence of 1 mM carbamylcholine. The solid lines were generated using Eq. 1 with $n_H = 1$.

Torpedo electroplaque

The ability of the PIP and TED compounds to inhibit the binding of $\alpha[^{125}I]$Bgt to nAChR from *Torpedo* membranes was measured under two different conditions. In the simultaneous addition protocol, the PIP compounds inhibit $\alpha[^{125}I]$Bgt binding 10- to 50-fold more potently than the TED compounds (Fig. 1 *B*). The comparison between the PIPs and TEDs changes dramatically in the prior addition condition (Fig. 1 *A*). In this case, the difference in protection constants is more than three orders of magnitude (Table 1). This difference is due almost entirely to a large increase in apparent affinity of the PIPs after desensitization (300-fold increase). This indicates that the PIP compounds promote a transition to the desensitized state with much greater efficacy than the TED compounds. As discussed above, the affinity measured in the simultaneous addition protocol can be considered a maximum affinity for the nondesensitized state. Since the ratio of protection constants (${}^{TED}K_P^{simul}/{}^{PIP}K_P^{simul}$) between the TED and PIP compounds diverges with desensitization, the ratio of their affinities for R can also be considered an upper limit of the true ratio. Given the large shift in affinity for the PIP compounds, the affinity for R' can be estimated from Eq. 3 and is given in Table 1. In the case of the TED compounds, the minimal shift in affinity with time suggests that the K_P^{prior}/K_L ratio is significant and that the affinity for R' would be lower than that estimated by Eq. 3. This suggests that the ${}^{TED}K_L'/{}^{PIP}K_L'$ ratio is greater than the ratio of the protection constants (${}^{TED}K_P^{prior}/{}^{PIP}K_P^{prior}$).

BC_3H-1 cells

Similar experiments were performed on membranes from BC_3H-1 cells, and the results are shown in Fig. 2. Again, the PIP compounds were more potent in the prior addition experiment than in the simultaneous addition assay, whereas the binding of the TED compounds was similar under the two different conditions. The ratio of the affinity of PIPs to TEDs was 10- to 40-fold in the simultaneous addition condition. As reported previously (Sine and Taylor, 1979), the shift in apparent affinity with desensitization is considerably less in BC_3H-1 cells than is observed in *Torpedo* electroplaque. The PIP compounds exhibited a 10- to 40-fold increase in apparent affinity; however, the TED compounds exhibited little change in apparent affinity. Given that M is an order of magnitude lower in BC_3H-1 cells than in *Torpedo* electroplaque, the K_L' for the PIP compounds are of the same order of magnitude in BC_3H-1 cells as in *Torpedo* electroplaque (see Table 1). Like *Torpedo,* the ${}^{TED}K_P^{simul}/{}^{PIP}K_P^{simul}$ ratio is an upper limit of the ${}^{TED}K_L/{}^{PIP}K_L$ ratio and the ${}^{TED}K_P^{prior}/{}^{PIP}K_P^{prior}$ ratio is a lower limit of the ${}^{TED}K_L'/{}^{PIP}K_L'$ ratio.

Single channel recording

The single channel kinetics of BC_3H-1 acetylcholine receptors exhibit a considerable amount of detail. This has been interpreted with some success both as four to eight discrete states (Sine and Steinbach, 1986, 1987; Papke and Oswald, 1989) and as a discrete diffusion process involving a large number of energetically related states but a small number of rate constants ("Diffusion Model") (Oswald et al., 1991; Millhauser et al., 1992). The purpose of this communication is to compare the properties of four related agonists, and a detailed description of the kinetics of this complex system will not be attempted. For this reason, we will consider first a subset of the full kinetic scheme that is common both to

TABLE 1 **PIP and TED binding to nAChRs from BC_3H-1 cells and *Torpedo* electroplaque**

	Torpedo				BC_3H-1			
	K_P^{prior}	K_P^{simul}	K_P^{prior}/K_P^{simul}	K'_L	K_P^{prior}	K_P^{simul}	K_P^{prior}/K_P^{imul}	K'_L
HPIP	0.08*	43	530	0.0075	1.5	60	41	0.024
F_3-PIP	0.11	43	390	0.01	3.3	34	10	0.055
HTED	120	430	3.4	12	330	700	1.8	6.5
F_3-TED	340	3,300	6.5	31	1,100	1,500	1.4	18

	HTED/HPIP	F_3-TED/F_3-PIP
Torpedo K_P^{prior}	1,560	3,090
Torpedo K_P^{simul}	10	52
BC_3H-1 K_P^{prior}	266	323
BC_3H-1 K_P^{simul}	11.7	41

* Values are given in μM and represent the average of two to three experiments.

the Diffusion Model (Millhauser et al., 1992) and models involving a smaller number of discrete states:

$$\begin{array}{ccccc} R + L \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} & RL & + L \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} & RL_2 & \\ & \beta' \downarrow\uparrow \alpha' & & \beta \downarrow\uparrow \alpha & . \\ & RL^* & & RL_2^* & \end{array} \qquad \text{(B)}$$

In this scheme, R, RL, and RL_2 are unliganded, singly liganded, and doubly liganded closed states and RL^* and RL_2^* are singly and doubly liganded open states. Two exponentials (corresponding to the closure of singly liganded and doubly liganded channels) would be predicted in the open dwell time distribution, and the ratio between the two exponentials should vary with agonist concentration. This has been observed with acetylcholine as the agonist in BC_3H-1 cells (Papke and Oswald, 1989), with the exponential having the longer time constant corresponding to the doubly liganded openings. The PIP compounds exhibit behavior similar to acetylcholine as shown in the two-dimensional dwell time distributions (Fig. 3). At concentrations below 1 μM, the open dwell time distribution is dominated by an exponential component with a time constant of <1 ms. As the concentration is increased, the percentage of the longer component increases. The two-dimensional dwell time distributions demonstrate that the shorter openings are consistently correlated with long channel closures, whereas the longer openings are correlated with the full range of closed times. Using a burst terminator of 1 ms, the burst lengths also exhibit two exponentials, with the longer events comprising more of the distribution at higher concentrations than at lower concentrations, increasing to a plateau of ~85% (Fig. 4 *A*). The reason for the plateau seen in Fig. 4 *A* is not apparent, as Model B predicts 100% long bursts at high concentrations; however, similar results have been observed with acetylcholine for BC_3H-1 cells (Sine and Steinbach, 1987; Papke and Oswald, 1989), for rat muscle cells (Colquhoun and Sakmann, 1985), and for purified nAChR from *Torpedo* electroplaque (Labarca et al., 1985). Because significant channel blockade is present at 100 μM and above, measurements of open channel lifetime were restricted to lower concentrations for the TED compounds, and the lifetimes were 25–50% of that observed for the PIP compounds. The percentage of long bursts (burst terminator of 1 ms) increased with increasing concentration to 65–75%.

At low concentrations of agonist, the closed time distribution clearly exhibits an exponential component that approaches the time resolution of the system as well as additional components (Fig. 5). This rapid component is consistent with dwells in the RL_2 state, and the time constant is equal to the reciprocal of $\beta + k_{-2}$. It is not dependent on the ligand concentration and is not significantly different between the four agonists, having a time constant of 0.04–0.07 ms. The number of closures within bursts represented by transitions between RL_2 and RL_2^* determines the β/k_{-2} ratio. These measurements cannot be made in the presence of rapid channel blockade because the number of closures within a burst can be increased (sometimes dramatically) due to the association and dissociation of the agonist with a binding site inside the channel. Nevertheless, at low concentrations of agonist where channel blockade is not significant, estimates of β and k_{-2} can be made (Table 2). Again, the differences between the compounds are not great.

At low agonist concentrations, one of the three eigenvalues of the closed state matrix is approximated by $-k_{-1}$ (Colquhoun and Hawkes, 1981; Sine et al., 1990). If k_{-1} is of the same order of magnitude as β and k_{-2}, then an exponential component arising from k_{-1} would not be easily distinguishable from β and k_{-2}. On the other hand, if k_{-1} is significantly slower, then a discrete component could be observed in the closed time distribution at low concentrations. Such a component has been observed for *Torpedo* nAChR (Sine et al., 1990) and has been taken as evidence for negative cooperativity in the ago-

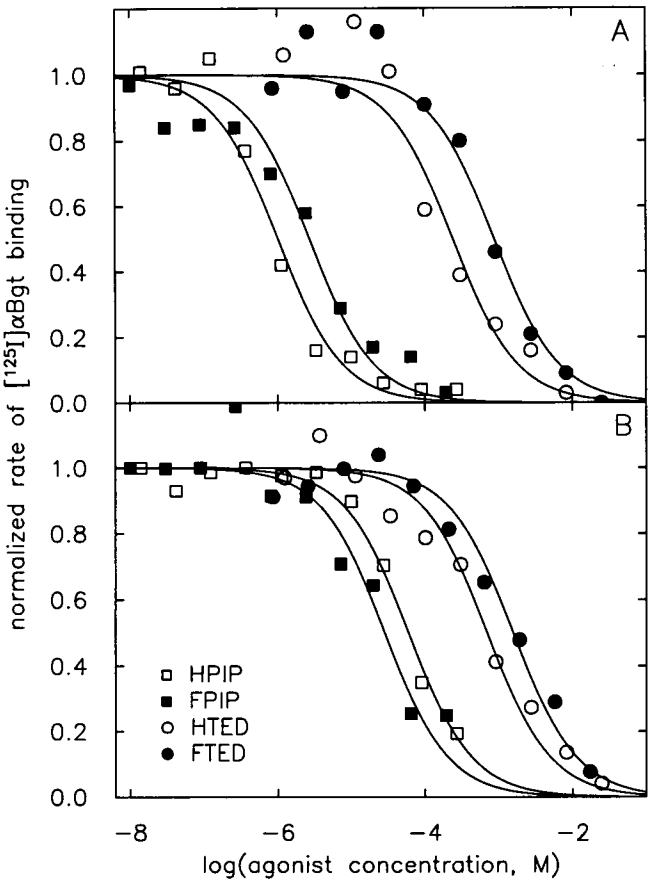

FIGURE 2 The inhibition of the initial rate of $\alpha[^{125}I]$Bgt binding to the nAChR from BC_3H-1 cell membranes by PIP and TED compounds. Representative experiments are shown, and the points are averages of duplicate determinations. For the prior addition condition (*A*), membranes were incubated with the test compound for 30 min before the addition of $\alpha[^{125}I]$Bgt. For the simultaneous addition condition (*B*), the compound to be tested was added to the membrane at the same time as $\alpha[^{125}I]$Bgt, and the initial rate of binding was measured. Binding was linear up to 60 s. Data were normalized to binding in the absence of PIP and TED compounds, and nonspecific binding was defined by incubation in the presence of 1 mM carbamylcholine.

nist binding steps (Jackson, 1988; Sine et al., 1990) (note, however, that a sequential model of induced fit binding without negative cooperativity is also consistent with these results; Auerbach, 1992). Despite negative cooperativity in the binding steps, overall channel activation would be positively cooperative due to the large β/α ratio. As shown in Fig. 5, a presumably exponential component with a time constant of 1–2 ms (i.e., greater than that ascribed to $\beta + k_{-2}$) is present in the closed time distribution of HPIP and F_3-PIP at 100 nM and 1 μM. The component with approximately the same time constant is less apparent for the TED compounds, possibly because of the low levels of channel activation, but can be detected at 10 μM. These results are not inconsistent with a strong negative cooperativity in binding and do not reveal a clear difference between the PIP and TED compounds.

To estimate the remainder of the constants in this scheme, the open and closed dwell time distributions were fit simultaneously with Model B using maximum likelihood analysis. Because Model B is incomplete in the sense that desensitization and channel block, which dominate the closed time distribution at high concentrations, are not included, fits were performed with the lowest concentration(s) that gave adequate channel activation (100 nM and 1 μM for HPIP, 1 μM for F_3-PIP, and 10 μM for HTED and F_3-TED). The estimates for the remainder of the parameters are given in Table 2, and examples of the fit of these parameters with Model B are shown in Fig. 5. According to this scheme, the large differences between the compounds are in the association rates. With the large number of parameters that are involved in such a fitting procedure, the exact values of the calculated rate constants have to be interpreted with some caution. In particular, the assumption is made that most of the closed time events included in the analysis are intervals between the closure of one channel and the opening of that same channel. This is probably a good assumption for the PIP compounds, particularly at 100 nM and 1 μM, because most of the events in the closed time distribution correspond to closures within a cluster of openings separated by long closed times. Thus, in the case of the PIP compounds, clusters of channel openings were clearly defined, and the combinatorial analysis as described by Horn and Lange (1983) was directly applicable. For this analysis, the events list was edited to include only clusters of openings, which effectively removed events that have been ascribed to singly liganded channels, so that Model B is used without the *RL** state. The results obtained (given in Table 2) are not dramatically different from those obtained using the matrix approach. Because most of the closed time distribution of the TED compounds consisted of intervals > 10 ms at concentrations of 10 μM and less, many of the intervals may represent the time between the closure of one channel and the opening of another. For this reason, the association rate constants are probably overestimates of the true rates. These results confirm that the TED compounds are poorer agonists than the PIP compounds, with the difference arising largely from the association rate constants.

At high concentrations, the PIP and TED compounds exhibit another important difference. This is illustrated in the two-dimensional dwell time distributions shown in Figure 3. The TED compounds are clearly potent channel blockers as demonstrated by the fact that most of the density is located in the lower left quadrant of the plot, particularly at 1 mM (i.e., short closures are correlated with short openings). As shown in Fig. 4 *C*, F_3-PIP produces significant channel blockade at 1 mM, whereas very little channel blockade is observed for HPIP. The "flickering" observed at high concentrations for the TED compounds and F_3-PIP is consistent with sequen-

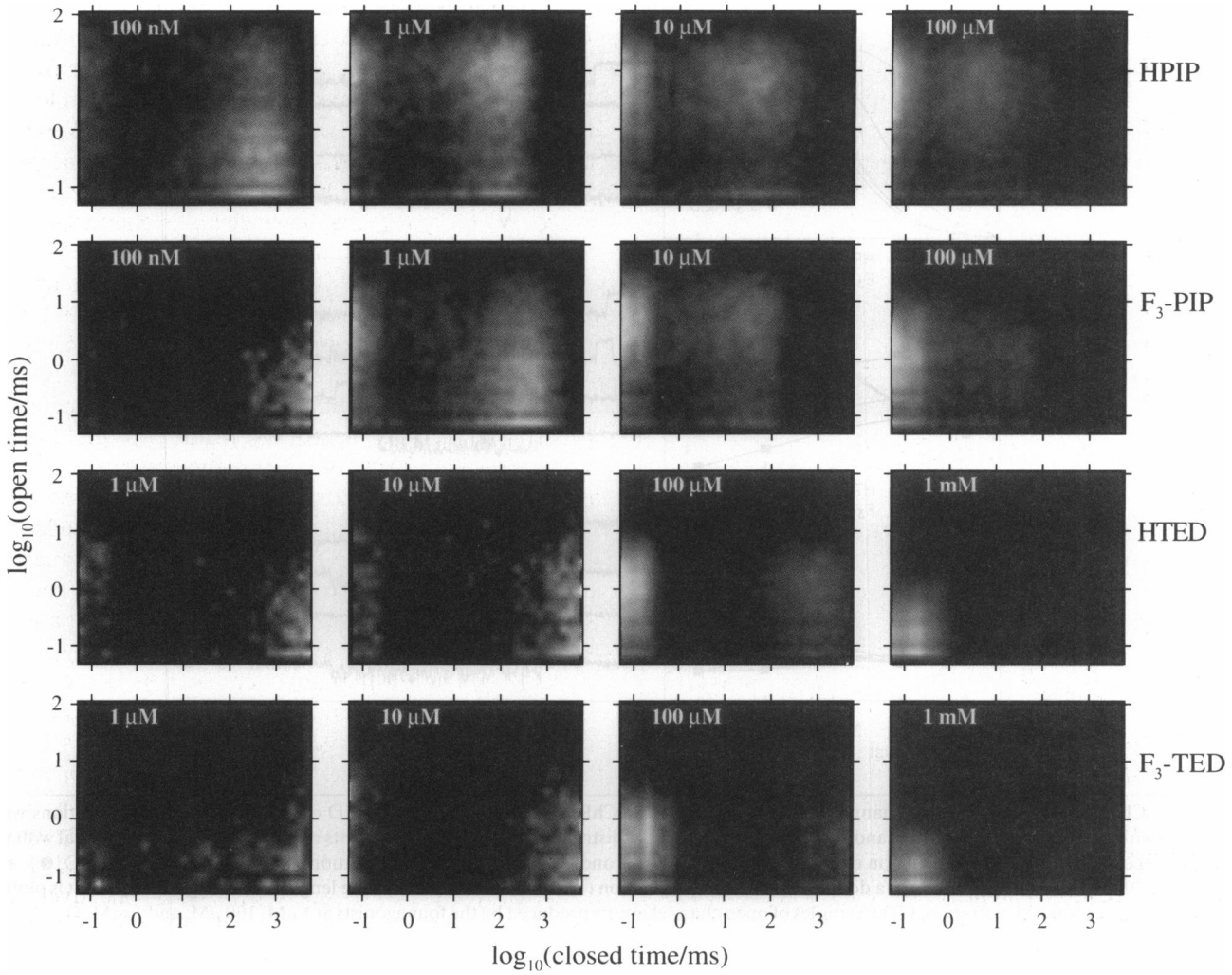

FIGURE 3 Two-dimensional dwell time histograms for the PIP and TED compounds at four concentrations of each compound. The histograms were generated by incrementing the value along the z-axis in the appropriate logarithmic two-dimensional bin (x = closed time; y = open time). The histograms are shown as normalized, interpolated gray-scale plots with the z axis representing the square root of the number of events in each bin (increasing from black to white). Before interpolation, each histogram had 25 bins along the open time axis and 30 bins along the closed time axis. Each bin of a square root-log open time distribution (Sigworth and Sine, 1987) can be generated from these data by $\sqrt{\Sigma\,(\text{row}^2)}$. The closed time distribution can be generated in an analogous fashion.

tial channel blockade (Neher and Steinbach, 1978) in that the open time decreases and the burst time increases with increasing concentration of the blocking compound (Fig. 4). The increased open time within a burst is not strictly consistent with sequential channel blockade. However, here both the agonist and the blocker are the same molecule so that increasing the agonist concentration increases the blocker concentration. Since we require concentrations on the order of 0.1–1 mM to produce channel blockade, dwells in the closed states cannot always be distinguished temporally from dwells in the blocked state, which means that the open time per burst will increase with concentration. The large increase in the burst length for HPIP at 1 mM is probably a reflection of the high rate of association of HPIP at this concentration rather than a dramatic increase in channel blockade.

^{1}H and ^{19}F NMR spectroscopy

As discussed previously (McGroddy and Oswald, 1992), 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 stable solution state structures exist on the NMR time scale, 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, which have the opposite orientation. This is due to their positions with respect to the anisotropic carbonyl oxygen. Previous work (McGroddy and Oswald, 1992) demonstrated that the major solution conformer of the TED compounds exists with the carbonyl oxygen *trans* to the amide methyl protons and constrained near the quaternary amine methyl groups due to electrostatic at-

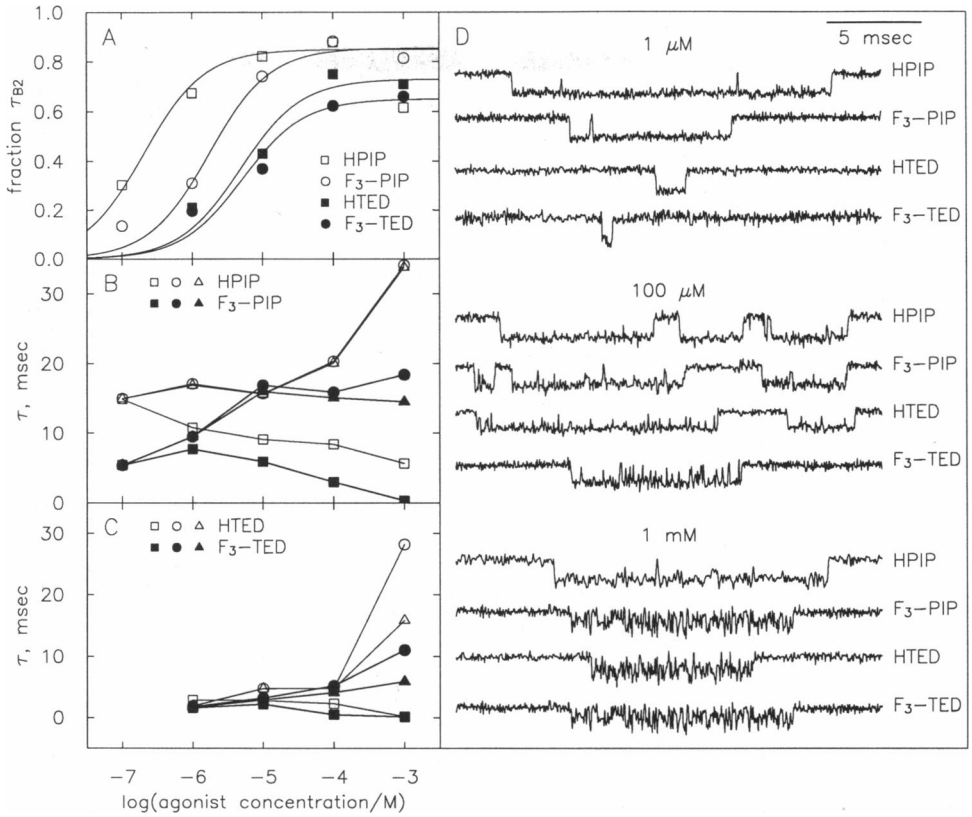

FIGURE 4 Characteristics of the open channel behavior of BC_3H-1 nAChRs activated by PIP and TED compounds. (*A*) Burst durations were calculated with a burst terminator of 1 ms and fit to a double exponential distribution. The fraction of events represented by the exponential with the longer time constant is plotted as a function of agonist concentration. (*B* and *C*) The open channel durations (□, ■), burst durations (○, ●), and open time within a burst (△, ▲) were fit to a double exponential distribution (1-ms burst terminator). The length of the longer component is plotted as a function of agonist concentration. (*D*) Examples of open channel events produced by the four agonists at 1 μM, 100 μM, and 1 mM. Downward deflections represent channel openings.

traction. The carbonyl oxygen is *cis* to the amide methyl protons in the minor conformer. The free energy of activation for interconversion between the two states at 300 K is 18.8 kcal/mol, with the majority of the barrier attributed to enthalpy (17.5 kcal/mol). The equilibrium constant for the two conformers is 4.41, which corresponds to a free energy difference of 0.89 kcal/mol between the two states. This difference is somewhat larger (6.13 and 1.09 kcal/mol) for HTED.

A ^{19}F spectrum of 235 μM F_3-TED in D_2O is shown in Fig. 6. Two singlet peaks, corresponding to the trifluoroacetylmethyl substituents on F_3-TED in two different conformations, are observed. Their relative intensities, chemical shifts, and linewidths are potentially sensitive measures of an interaction with the nAChR. Fig. 7 *A* shows a spectrum of 235 μM F_3-TED in the presence of 22 μM nAChR. The chemical shift difference between the two resonances is decreased from 338.6 Hz (0.902 ppm) to 330.8 Hz (0.880 ppm). This is primarily due to a deshielding of the smaller resonance by 9.4 Hz (0.025 ppm), but is unlikely to be related to an interaction with the nAChR as described below. In addition, although both resonances are somewhat broadened in comparison to the D_2O spectrum, the less intense *cis* F_3-TED peak is significantly broadened in comparison to both the larger *trans* F_3-TED peak and the reference trifluoroacetic anhydride peak, suggesting that the bound and free forms of *cis* F_3-TED are in intermediate exchange. On the addition of 5.5 mM HPIP to the sample (Fig. 7 *B*), the broadening of the less-intense resonance disappeared without an effect on the linewidth of the other resonance. The increase in linewidth due to exchange was 2.3–5 Hz in the presence of 22 μM nAChR and ~1 Hz in the presence of 9.3 μM nAChR. The chemical shift difference between the two F_3-TED peaks remained the same. The experiment was repeated in the presence of 80 μM αBgt to block any specific interaction of F_3-TED with the nAChR. This spectrum is shown in Fig. 7 *C*. The chemical shift difference between the two peaks was essentially unchanged from either of the previous two experiments, and no broadening of the small F_3-TED resonance was observed. The spectrum of the sample containing αBgt remained unchanged on the addition of an excess of HPIP (data not shown). These results are consistent with a preferential interaction of the *cis* conformer with the acetylcholine binding sites of the *Tor-*

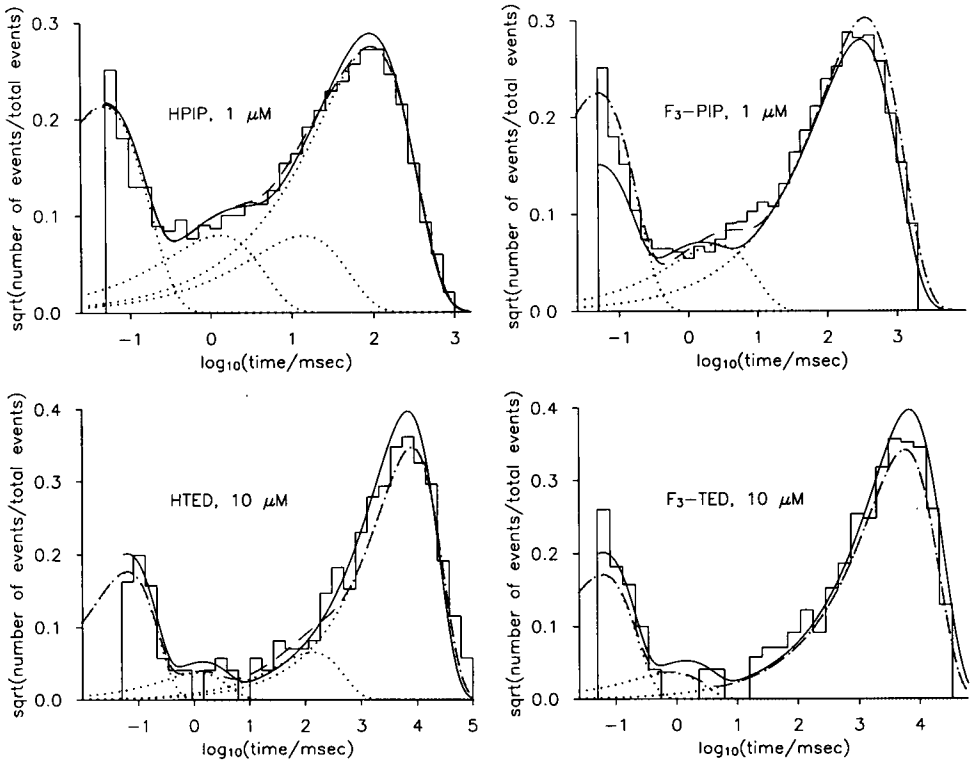

FIGURE 5 Characteristics of the closed time intervals of BC_3H-1 nAChRs activated by PIP and TED compounds. The distributions are shown as square root-log plots (Sigworth and Sine, 1987) and the fit to three or four exponentials is shown (*dashed line*) as well as the fit to Model B using the constants given in Table 2 generated by the matrix method (*solid line*). The individual exponential components that comprise the exponential fits are shown as dotted lines.

pedo nAChR. The possibility exists, however, that the *trans* conformer could be in slow exchange with a highly immobilized bound species for which no resonance would be detected due to a very large linewidth. If this were the case, then the integral of the *trans* peak would increase with the addition of excess HPIP by an amount consistent with the number of binding sites in the preparation (i.e., ~10%). Likewise, the ratio of the integral of the *trans* peak to the *cis* peak should increase with the addition of HPIP (i.e., since the *cis* peak is in intermediate exchange, its integral is not expected to change). In all cases measured, the *trans:cis* ratio was essentially unchanged (or even slightly decreased) on the addition of HPIP ($trans_{HPIP}/cis_{HPIP}:trans_{NoHPIP}/cis_{NoHPIP} = 0.97 \pm 0.02$). Although this negative evidence cannot completely rule out an interaction of the *trans* conformer with the nAChR, the existence of an interaction of the *trans* isomer with the nAChR is unlikely given these data.

DISCUSSION

The interaction of two classes of compounds with nAChRs from *Torpedo nobiliana* and BC_3H-1 cell membranes has been studied. In all cases, the PIP compounds were more potent than the TED molecules in binding to and activating the nAChRs. A number of distinctions between these two classes of compounds were apparent: (*a*) the PIP compounds bound with 10–50-fold higher affinity to "resting" nAChRs; (*b*) the PIP compounds produced an affinity shift consistent with receptor desensitization, whereas the TED compounds induced little desensitization; (*c*) the single channel recording studies suggest that the association rates of the PIP compounds are significantly greater than the TED compounds; and (*d*) the TED compounds exhibited channel blockade at concentrations of 100 μM and above, F_3-PIP exhibited channel blockade at 1 mM, and no significant channel blockade was observed for HPIP.

The accompanying article (McGroddy and Oswald, 1992) demonstrates that both classes of compounds can exist in two distinct and stable (on the NMR time scale) solution structures related by an isomerization of the amide bond. In the case of the PIP compounds, the two isomers are identical because of the symmetry of the compound, and they would be expected to have identical biological activities. On the other hand, because the TED compounds are not symmetric, the two isomers are not identical. In fact, proton NMR studies (McGroddy and Oswald, 1992) demonstrated that the two isomers were also energetically inequivalent, with the lower energy *trans* structure favored over the higher energy *cis* structure by four- to sixfold. The NMR studies and energy minimization using the CHARMm force field indi-

TABLE 2 **Rate constants for channel activation**

Parameters	HPIP (1 μM)	F_3-PIP (1 μM)	HTED (10 μM)	F_3-TED (10 μM)
Exponential fitting				
k_{-1} (s^{-1})	780	380	850	760
k_{-2} (s^{-1})	13,000	12,000	12,000	11,000
β (s^{-1})	7,600	7,800	3,400	4,977
τ_1 (ms)*	0.06	0.05	0.06	0.06
Matrix method‡				
k_1 (M^{-1} s^{-1})	1.2×10^8	6.3×10^7	8.8×10^5	3.3×10^6
k_{-1} (s^{-1})	550	700	810	880
k_2 (M^{-1} s^{-1})	1.0×10^8	4.4×10^7	1.1×10^6	7.0×10^6
k_{-2} (s^{-1})	11,000	12,000	10,000	12,000
β (s^{-1})	9,000	7,800	6,500	5,000
α (s^{-1})	91	210	340	491
β' (s^{-1})	22	24	7	3
α' (s^{-1})	4,000	3,500	5,100	6,300
K_{app} (M)§	2.3×10^{-6}	5.0×10^{-5}	6.4×10^{-4}	2.0×10^{-4}
Combinatorial analysis‖				
k_1 (M^{-1} s^{-1})	1.3×10^8	1.4×10^8		
k_{-1} (s^{-1})	1,186	360		
k_2 (M^{-1} s^{-1})	1.7×10^8	2.7×10^7		
k_{-2} (s^{-1})	7,800	6,500		
β (s^{-1})	15,000	12,000		
α (s^{-1})	160	220		

* Value of the shortest time constant from a three or four exponential fit.

‡ Model B was used.

§ The K_{app} is determined from the saturation function for model B:

$$\bar{Y} = \frac{\frac{L}{K_1}\left(\left[1 + \frac{1}{K_4}\right] + 2\frac{L}{K_2}\left[1 + \frac{1}{K_3}\right]\right)}{2\left\{1 + \frac{L}{K_1}\left(\left[1 + \frac{1}{K_4}\right] + \frac{L}{K_2}\left[1 + \frac{1}{K_3}\right]\right)\right\}},$$

where $K_1 = k_{-1}/k_1$, $K_2 = k_{-2}/k_2$, $K_3 = \alpha/\beta$, and $K_4 = \alpha'/\beta'$.

‖ Model B was used except that singly liganded channel openings were omitted.

cate that the *trans* isomer favors a conformation in which the positively charged quaternary amine interacts electrostatically with the partially negative carbonyl oxygen. Thus, assuming that the cyclic PIP compounds are in a conformation favorable for binding to and activating the nAChR, the *cis* TED isomers would be capable of readily assuming this conformation. On the other hand, assumption of a conformation similar to PIP by *trans* TED would require disruption of the electrostatic interaction between the quaternary amine and the carbonyl oxygen. Assuming that the *cis* TED affinity is similar to that of the PIP conformers, *cis* TED might be expected to bind preferentially to the nAChR, and the composite affinity of the TED compounds would be lower relative to the PIP compounds by a factor of $1 + K_L$, where K_L is the ratio of the populations of *trans* TED to *cis* TED.

Studies of the interaction of F_3-TED with the *Torpedo* nAChR were undertaken to determine if in fact *cis* F_3-TED bound preferentially. Fluorine NMR was used because the signal from F_3-TED could be measured in the absence of a background signal from the receptor, detergent, and solvent. Since, in biological terms, NMR is a relatively insensitive technique, a large number of free induction decays had to be averaged to provide an adequate signal-to-noise ratio. However, the linewidth of the signal from *cis* F_3-TED was clearly larger in the absence than in the presence of agents that occupy the acetylcholine binding sites (HPIP and αBgt), whereas the *trans* F_3-TED resonance was unaffected by this treatment. These results indicate that *cis* F_3-TED is in intermediate exchange between the bound and free forms of the compound. Unfortunately, without knowledge of the chemical shift of the bound form, we cannot estimate the exchange kinetics. Nevertheless, we can conclude that *cis* F_3-TED interacts with nAChRs, whereas no evidence for the binding of *trans* F_3-TED could be detected. The possibility exists that *trans* F_3-TED interacts with the nAChR with the bound and free forms in slow exchange. No bound peak was observed, although it may have been undetectable due to severe line broadening as a result of immobilization on the surface of the receptor. Also, no change in the intensity of the *trans* resonance was observed, supporting the notion that the *trans* form does not interact with the nAChR. These experiments could not have detected an interaction of F_3-TED with the binding site mediating channel blockade because binding to this site is inhibited potently by detergents (Heidmann et al., 1983). Also, similar studies using F_3-

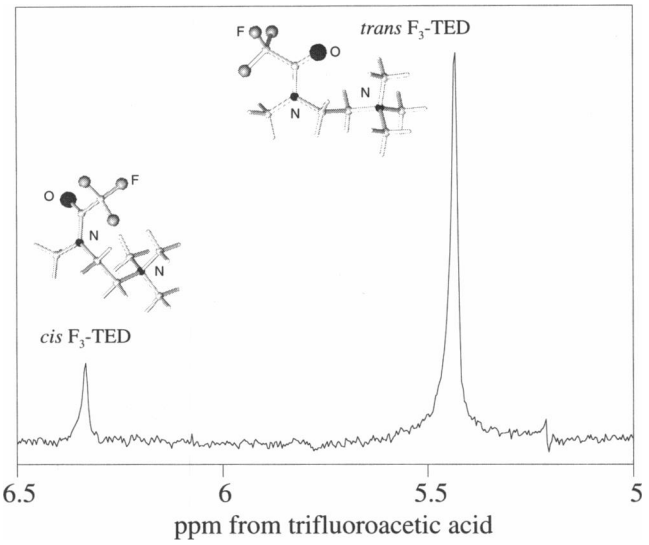

FIGURE 6 The ^{19}F spectrum of F_3-TED in D_2O. F_3-TED exists in two solution structures on the NMR time scale, with the more stable *trans* structure favored by a factor of 4.4 over the less stable *cis* structure. The structures shown are ball-and-stick representations generated using Quanta (Polygen/Molecular Simulations, Inc.) and represent the minimum energy structures from an adiabatic map generated in vacuum using CHARMm (Polygen/Molecular Simulations, Inc.).

PIP were not successful. The major reason for this is that F_3-PIP was not stable in the solutions used for the NMR studies, degrading with a half time of ~2 h. Also, it is likely that F_3-PIP binds more tightly to nAChRs such that it would be in slow exchange with the free form of the compound. If this were true, then the compound could be immobilized in the binding site to the extent that the resulting rotational correlation time would lead to a signal too broad to detect.

Thus, the NMR studies indicate that *cis* TED is the minor conformer and very likely binds preferentially to nAChRs. The simplistic assumption that *cis* TED would have the same affinity for the nAChR as PIP would suggest that HPIP would have an affinity sevenfold greater than HTED, and F_3-PIP would have an affinity 5.4-fold greater than F_3-TED. Considering first the binding experiments, previous results from a number of laboratories have indicated that the nAChR undergoes a shift in affinity for agonist, which at least in *Torpedo* electroplaque has two components: one on the subsecond time scale and the other on the time scale of several minutes. In both cases, the shift is to a higher affinity and is associated with desensitization. The binding experiments presented here can only detect the affinity shift that occurs in the seconds to minutes time scale. Nevertheless, a clear distinction between the PIP and TED compounds can be observed. The PIP compounds bind to the desensitized AChR from both *Torpedo* and BC_3H-1 cells with a 1,000-fold higher affinity than the TED compounds. Clearly, the solution structure of the TED compounds can play only a marginal role in this difference in affinity. Binding to the "resting" form of AChRs cannot be accurately estimated by these techniques, but since there is a time-dependent increase in affinity, the affinity measured with the simultaneous addition of the agonist with $\alpha[^{125}I]$Bgt provides an upper limit on the affinity. Under these conditions, the ratio of the PIP to TED affinities is on the order of 10–50. Since the PIP compounds tend to induce more of an affinity shift than the TED compounds, the true ratios are <10–50, suggesting that the largely unfavorable solution structure of TED compounds determines to a large extent the initial difference in affinity between the PIP and TED compounds. The

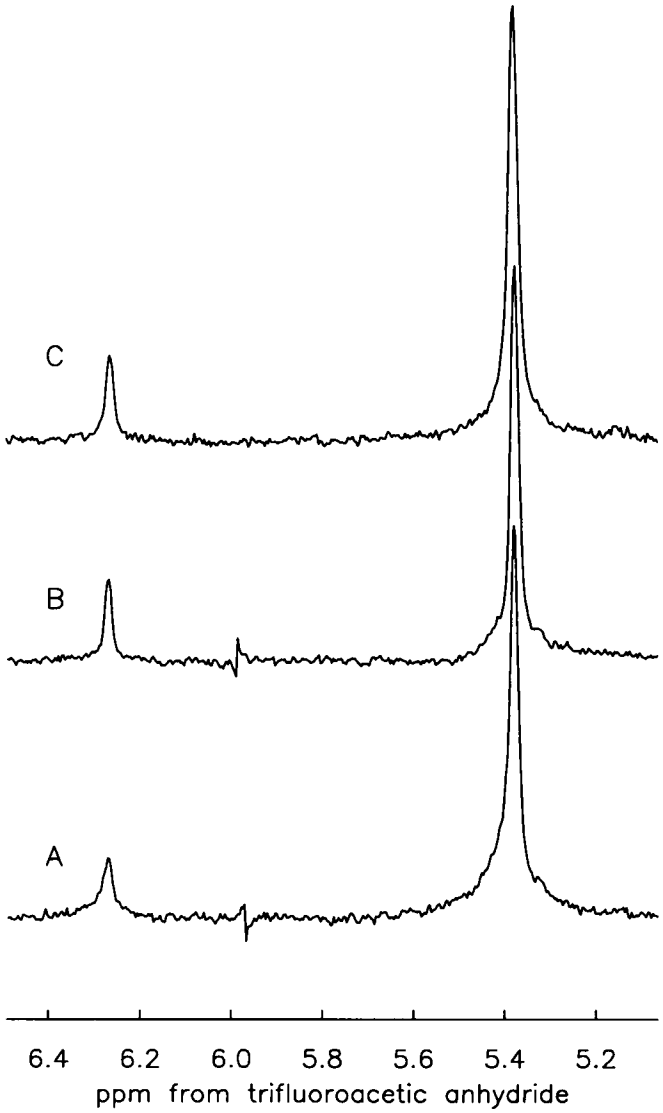

FIGURE 7 ^{19}F NMR spectrum of F_3-TED in the presence of solubilized nAChR from *Torpedo* electroplaque. Shown are the spectra with (*A*) solubilized nAChR, (*B*) solubilized nAChR with 5.5 mM HPIP, and (*C*) solubilized nAChR with 80 μM αBgt. In *A* and *C*, the nAChR concentration was 22 μM and the F_3-TED concentration was 235 μM. In *B*, the nAChR concentration was 18.3 μM and the F_3-TED concentration was 196 μM. The lower concentration of the reagents in *B* was due to a dilution on addition of HPIP.

affinity shift observed for the PIP compounds in *Torpedo* is somewhat larger than that observed for acetylcholine and is largely due to a lower apparent affinity in the simultaneous addition experiments (Weiland and Taylor, 1979).

The single channel recording experiments reveal additional complexities in the comparisons between the two compounds. Qualitatively, the TED compounds require 10–100-fold higher concentrations to activate nAChR channels than the PIP compounds and exhibit considerably more channel blockade. The models (Models B and C) used to analyze the data are clearly insufficient to account for all of the complexities of the nAChR behavior over a wide variation in agonist concentration. However, estimates of the rate constants can be made under some conditions. At low concentrations, the constants in Model B can be estimated. The dissociation rate constants (k_1 and k_2) and the opening rate (β) vary little between the PIP and TED compounds. On the other hand, the estimates of k_1 and k_2 suggest that the PIP and TED compounds differ mainly in association rate, the difference being approximately two orders of magnitude. In addition, a two- to threefold difference in closing rate (α) exists between the two classes of compounds, and TED compounds exhibit channel blocking activity at a lower concentration than PIP compounds.

Since the work of Beers and Reich (1970), considerable effort has been devoted to producing a "pharmacophore" capable of describing the structural and electronic features necessary for agonist activity at nicotinic acetylcholine receptors (e.g., Wasserman et al., 1979; Sheridan et al., 1986; Spivak et al., 1989). The overall structures of these models consist of a cationic center (e.g., the quaternary amine) and a hydrogen bond acceptor (e.g., the carbonyl oxygen), with a distance of ~5.9 Å between the quaternary nitrogen and the van der Waals extension of the carbonyl oxygen. Additionally, the N(+)—C—C—O backbone of acetylcholine is assumed to be in its GAUCHE (GAUCHE and TRANS refer to the N(+)—C—C—N or N(+)—C—C—O backbone of TED or acetylcholine; *cis* and *trans* refer to the amide bond) conformation (Sheridan et al., 1986). This has served as the basis for the synthesis of a number of very potent acetylcholine analogues, including the PIP derivatives used in the present study (e.g., Waters et al., 1988; Spivak et al., 1989). Behling et al. (1988), however, have suggested based on two-dimensional NMR experiments that the N(+)—C—C—O backbone of acetylcholine may be TRANS when bound to the desensitized nAChR of *Torpedo* electroplaque, whereas the acetylcholine in free solution was in the GAUCHE conformation. Comparisons between the PIP and TED compounds seem to be contrary to this conclusion. The TED compounds, which are capable of assuming either the TRANS or GAUCHE N(+)—C—C—N conformation, bind with lower affinities than the PIP compounds that are purely GAUCHE and are restricted from attaining a TRANS conformation. A conformational search in vacuum using CHARMm (Polygen, Inc., Waltham, MA) has shown that the preferred orientation of the N(+)—C—C—N backbone in HTED is TRANS, regardless of the orientation about the amide dihedral. The two GAUCHE forms are 2–3 kcal/mol higher in potential energy. In addition, only *cis* TED in the GAUCHE form exhibits a distance between the quaternary amine and carbonyl oxygen suitable for binding to the AChR (6.0 Å). This distance is considerably longer for *cis* TED in the TRANS form (7.4 Å) and shorter for *trans* TED in both the GAUCHE (5.0 Å) and TRANS (4.7 Å) forms. This could indicate that only a very small percentage of HTED and F_3-TED in solution is in the correct conformation to bind to the nAChR, and that isomerization about two dihedral angles (amide and N(+)—C—C—N) must occur before binding.

An additional difference between the PIP and TED compounds that may relate to the differences in binding affinity and association rate is the degree of hydration. Molecular dynamics simulations using CHARMm in water (499 TIP3P water molecules) indicate that all forms of HTED have an average of two to five more water molecules in the primary solvation shell than HPIP (McGroddy, K. A., unpublished results). Depending on how tightly the water molecules are bound, HTED may require more energy for desolvation before binding than HPIP, thus decreasing the affinity.

A number of studies using NMR spectroscopy (e.g., Ni et al., 1990; Wüthrich et al., 1991) and x-ray crystallography (e.g., Van Duyne et al., 1991) have shown that the bound structure of a ligand can vary considerably from its solution structure. This may indicate that the traditional notion of a lock and key complementarity of receptor-ligand interactions is considerably too simple in that the ligand may undergo a distortion to interact in an optimal manner with the receptor site (Jorgensen, 1991), although the preferential binding of a solution conformer similar to the structure in the bound state has been suggested (Altschuh et al., 1992). Our results suggest that the predominant conformation of the ligand in solution may not bind preferentially to the receptor. This is to some extent an unusual case in that the energy barrier for interconversion between the two stable solution structures of the TED molecules is very high. Consider the simplified mechanism shown below:

$$\begin{array}{ccc} & \overset{K_{L1}}{\rightleftharpoons} & \\ L_1 & & L_2 \\ + & & + \\ R & & R \\ {\scriptstyle K_{D1}}\downarrow\uparrow & {\scriptstyle K_{L2}} & \downarrow\uparrow{\scriptstyle K_{D2}} \\ RL_1 & \rightleftharpoons & RL_2 \end{array},$$

where L_1 is the lower energy conformer of the ligand, R is the receptor, K_{L1} and K_{L2} are isomerization constants (e.g., $K_{L1} = [L_1]/[L_2]$), and K_{D1} and K_{D2} are equilibrium dissociation constants. According to microscopic

reversibility, the relationship between K_{D1} and K_{D2} is given by:

$$K_{D2} = K_{D1} \frac{K_{L2}}{K_{L1}}.$$

If one assumes that RL_2 is the optimal conformation (i.e., lower energy) of the receptor-ligand complex, then K_{L2} is <1. Since by definition, K_{L1} is >1, then L_2 would bind preferentially since its affinity for the receptor would necessarily be greater than that of L_1 ($K_{D2} < K_{D1}$). This suggests that the preferential binding of one conformation of ligand is a direct consequence of a preferred bound conformation. Furthermore, the observation of a bound state structure that differs from the major solution conformation could be a result either of preferential binding of a small population of ligand in the preferred conformation or of the distortion of the ligand to fit the binding site.

In summary, experiments using NMR spectroscopy suggest that only one of the two stable solution structures of the acyclic TED compounds may interact with the nAChR. The equilibrium that favors the inactive conformer effectively decreases the concentration of active TED available for interaction with the nAChR. A significant portion of the difference in the initial affinity between the PIP and TED compounds can be ascribed to this solution behavior of the TED compounds. However, the binding experiments at equilibrium and single channel experiments demonstrate that additional factors may be important. These may include an unfavorable orientation of the N(+)—C—C—N backbone in solution and a greater hydration of all conformers of HTED relative to HPIP. These results illustrate the complexities involved in comparing the action of agonists with similar chemical structures and the importance of considering the contribution of the unbound solution structure to agonist affinity.

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