Nicotinic Receptor Fourth Transmembrane Domain Hydrogen Bonding by Conserved Threonine Contributes to Channel Gating Kinetics

Cecilia Bouzat,* Francisco Barrantes,* and Steven Sine‡

From the *Instituto de Investigaciones Bioquímicas, Universidad Nacional del Sur-Consejo Nacional de Investigaciones Científicas y Técnicas, 8000 Bahía Blanca, Argentina; and ‡Receptor Biology Laboratory, Department of Physiology and Biophysics, Mayo Foundation, Rochester, Minnesota 55905

Abstract The fourth transmembrane domain (M4) of the nicotinic acetylcholine receptor (AChR) contributes to the kinetics of activation, yet its close association with the lipid bilayer makes it the outermost of the transmembrane domains. To investigate mechanistic and structural contributions of M4 to AChR activation, we systematically mutated ωT422, a conserved residue that has been labeled by hydrophobic probes, and evaluated changes in rate constants underlying ACh binding and channel gating steps. Aromatic and nonpolar mutations of ωT422 selectively affect the channel gating step, slowing the rate of opening two- to sevenfold, and speeding the rate of closing four- to ninefold. Additionally, kinetic modeling shows a second doubly liganded open state for aromatic and nonpolar mutations. In contrast, serine and asparagine mutations of ωT422 largely preserve the kinetics of the wild-type AChR. Thus, rapid and efficient gating of the AChR channel depends on a hydrogen bond involving the side chain at position 422 of the M4 transmembrane domain.

Key words: patch clamp • kinetic analysis • nicotinic acetylcholine receptor channel gating • fourth transmembrane domain • hydrogen bond

Introduction The nicotinic acetylcholine receptor (AChR) is a pentamer of homologous subunits with composition α2βγδ in fetal muscle and α2βεδ in the adult. Each subunit contains an amino-terminal extracellular domain of ~210 amino acids, four transmembrane domains (M1-M4), and a short extracellular carboxy-terminal tail. The M2 domain of each subunit contributes to the cation-selective channel, and agonist binding triggers motion of M2 to initiate ion flow (Unwin, 1995). The locations and functional roles of the M1, M3, and M4 transmembrane domains are not as well understood. The M1 domain appears closely associated with both the ion conducting pathway and the bilayer, as revealed by substituted cysteine accessibility and labeling by hydrophobic reagents (Akabas and Karlin, 1995; Blanton and Cohen, 1994). The M3 domain has been shown to have some contact with lipid (Blanton and Cohen, 1994) and to contribute to channel opening and closing rates (Wang et al., 1999). The M4 domain is the least conserved among the transmembrane domains, is the most hydrophobic, and has been extensively labeled by hydrophobic probes (Blanton and Cohen, 1992, 1994), suggesting that it is the outermost of the transmembrane domains. Labeling by the photoactivatable hydrophobic probe [3H]TID ([3H]3-(trifluoromethyl)3-m-(iodophenyl)diazirine) occurs at C412, M415, C418, T422, and V425 of the α subunit of Torpedo californica AChR, making these the best candidates for residues in contact with the lipid bilayer (Blanton and Cohen, 1994). Mutagenesis studies suggest that the M4 domain is essential for proper AChR activation (Lee et al., 1994; Ortiz-Miranda et al., 1997). By alanine scanning of the lipid-exposed residues in the M4 domain of the α subunit, we recently showed that T422 affects channel open duration (Bouzat et al., 1998).

Here we examine the structural and mechanistic contributions of the M4 domain to AChR activation by systematically mutating ωT422 of the mouse muscle AChR and evaluating changes in activation kinetics. Our results show that ωT422 contributes through a hydrogen bond to both opening and closing steps.

Materials and Methods

Construction of Mutant Subunits Mouse cDNAs were used subcloned into the cytomegalovirus-based expression vector pRBG4 (Sine, 1993). Mutant α subunits were constructed by bridging the naturally occurring sites BstX-1 and BspM-1 with synthetic double-stranded oligonucleotides (Bio-Synthesis Inc.), essentially as described previously (Bouzat et al., 1998). Single-stranded oligonucleotides were purified by PAGE.

Address correspondence to Cecilia Bouzat, Instituto de Investigaciones Bioquímicas, UNS-CONICET, Camino La Carrindanga Km 7, 8000 Bahía Blanca, Argentina. Fax: 54-291-4861201; E-mail: inbouzat@criba.edu.ar

Abbreviations used in this paper: AChR, nicotinic acetylcholine receptor; M4, fourth transmembrane domain.
Expression of AChR

Human embryonic kidney cells (HEK293) were transfected with α (wild-type or mutant), β, δ, and ε cDNAs using calcium phosphate precipitation at a subunit ratio of 2:1:1:1 for α:β:δ:ε, respectively, essentially as described previously (Bouzat et al., 1994, 1998). For transfections, cells at 40–50% confluence were incubated for 8–12 h at 37°C with the calcium phosphate precipitate containing the cDNAs in DMEM plus 10% fetal bovine serum. Cells were used for single-channel measurements 1 or 2 d after transfection.

Patch-Clamp Recordings

Recordings were obtained in the cell-attached configuration (Hamill et al., 1981) at a membrane potential of −70 mV and at 20°C. The bath and pipette solutions contained 142 mM KCl, 5.4 mM NaCl, 1.8 mM CaCl₂, 1.7 mM MgCl₂, and 10 mM HEPES, pH 7.4. Patch pipettes were pulled from 7052 capillary tubes (Garner Glass), to produce final resistances of 5–7 MΩ, and were coated with Sygard (Dow Corning Corp.). Pipette concentrations of ACh ranged from 10 to 1000 μM. Single channel currents were recorded using an Axopatch 200 B patch-clamp amplifier (Axon Instruments, Inc.) and digitized at 94 kHz with a PCM adapter (VR-10B; Instrutech). Data were transferred to a computer using the program Acquire (Bruxton Corp.) and detected by the half-amplitude threshold criterion using the program TAC 3.0 (Bruxton Corp.) at a final bandwidth of 10 kHz. Open- and closed-time histograms were plotted using a logarithmic abscissa and a square root ordinate (Sigworth and Sine, 1987) and fitted to the sum of exponentials by maximum likelihood using the program TACFit (Bruxton Corp.). For each patch corresponding to a given ACh concentration, the number of opening events ranged from 4,000 to 10,000.

Open probability within clusters (P_open) was experimentally determined at each ACh concentration by calculating the mean fraction of time the channel is open within a cluster. The experimental P_open determinations were compared with theoretical dose–response curves calculated from either Scheme I or II, using the fitted rate constants in Table II.

Kinetic Analysis

At ACh concentrations >10 μM, clusters of openings corresponding to a single channel were identified as a series of closely spaced events preceded and followed by closed intervals greater than a specified duration; this duration was taken as the point of intersection of the predominant closed-time component and the succeeding component in the closed-time histogram. To minimize errors in assigning cluster boundaries at the lower ACh concentrations, we analyzed only recordings from patches with low channel activity. Only clusters containing >10 openings were considered for further analysis. In addition, any clusters showing double openings were rejected. For each recording, kinetically homogeneous clusters were selected based on their mean channel open duration and open probability distributions (Wang et al., 1997). For each cluster within a recording, we calculated the P_open and mean open duration and plotted their distributions. Histograms were constructed and fitted by a Gaussian function, which yielded a mean and a standard deviation for each distribution. Typically, the distributions contained a dominant, approximately Gaussian component and minor contributions of clusters with different properties. Clusters showing mean open duration and open probability values within 2 SD of the mean of the major component were selected and retained for the kinetic analysis. Typically, >80% of the clusters were selected. For each recording, mean values of open probability, and mean open and closed durations changed little after the selection procedure (see examples of selection results in Table I). The similarity of the evaluated parameters before and after selection indicates no qualitative changes in the dwell-time distributions due to the selection procedure. The resulting open and closed intervals, from single patches at several ACh concentrations, were analyzed according to a kinetic scheme using the program MIL (QuB suite, www.qub.buffalo.edu). In brief, MIL simultaneously fits recordings obtained at different ACh concentrations, and estimates rate constants in a kinetic scheme using a maximum likelihood method that corrects for missed events (Qin et al., 1996). We typically included recordings obtained at 10, 30, 50, 100, 200, and 300 μM ACh in the analysis of each mutant AChR. The dead time was typically 30 μs, but the fitted rate constants were stable over a range of dead times ranging from 18 to 40 μs (data not shown). Probability density functions of open and closed durations were calculated from the fitted rate constants and instrumentation dead time and superimposed on the experimental dwell-time histogram, as described by Qin et al. (1996). Calculated rate constants were accepted only if the resulting probability density functions reasonably superimposed upon the experimental open- and closed-duration histograms at all ACh concentrations. To check the final set of fitted rate constants, open and closed intervals were simulated according to Scheme I or II, using the program SIMU (QuB suite). Simulated channel events were analyzed and the resulting dwell times were fitted with the program MIL. The original rate constants were recovered with 10–15% accuracy. Comparison of the fits to different schemes was evaluated by the log likelihood ratio (LLR). For example, a LLR of 50 indicates that the probability of the model with the higher LL is e50 times more than that of the scheme with the lower LL (Salamone et al., 1999).

RESULTS

We previously examined the M4 transmembrane domain by alanine-scanning mutagenesis, and found that αT422 was unique in that its mutation markedly affected the rate of channel closing (Bouzat et al., 1998). To further examine the structural contribution of αT422 to AChR activation, we replaced it by polar (serine and asparagine), aromatic (tryptophan and tyrosine), and nonpolar amino acids (alanine, valine, and cysteine). Then we transfected HEK293 cells with either wild-type or mutant α plus wild-type β, ε, and δ subunit cDNAs and recorded single channel currents.

AChR channels were activated by a range of desensitizing concentrations of ACh (10–1,000 μM) to produce clear clusters of events corresponding to a single channel (Sakmann et al., 1980). Wild-type as well as mutant AChRs open in clusters of well-defined activation episodes at ACh concentrations >10 μM. Each activation episode begins with the transition of a single receptor from the desensitized to the activatable state and terminates by returning to the desensitized state (Fig. 1). For both wild-type and mutant AChRs, closed intervals within clusters become progressively briefer with increasing ACh concentration (Fig. 2). However,
compared with wild-type, most of the mutant AChRs show both briefer openings and prolonged intracluster closings (Fig. 1).

For both wild-type and mutant AChRs, the predominant peak of the closed-time histograms moves to shorter duration with increasing ACh concentration (Fig. 2). However, for mutant AChRs containing alanine, closed intervals are clearly longer than those of wild-type AChRs at all ACh concentrations (Fig. 2). Similar results were obtained for receptors containing nonpolar (valine and cysteine) or aromatic (tryptophan and tyrosine) substitutions. The predominant open-time component of either wild-type or mutant AChRs does not change with changes in ACh concentration (Fig. 2). However for \( \alpha T422A \) (Fig. 2), as well as for \( \alpha T422V, \alpha T422W, \alpha T422Y, \) and \( \alpha T422C \) AChRs (not shown), open-time distributions shift to briefer durations compared with wild-type AChRs. In contrast, when T422 is replaced by serine (Fig. 2) or asparagine (not shown), open- and closed-duration histograms are similar to those of wild type.

The observed changes in the kinetics of AChR activation could be due to changes in rate constants underlying either ACh binding or channel gating steps. To identify the kinetic steps affected by each M4 mutation, we fitted kinetic schemes to the open and closed dwell times. For wild-type AChRs, we used the classical activation scheme (Scheme I), where two agonists (A) bind to the receptor (R) in the resting state with association rates \( k_{11} \) and \( k_{12} \) and dissociate with rates \( k_{-1} \) and \( k_{-2} \). Receptors occupied by one agonist open with rate \( \beta_1 \) and close with rate \( \alpha_1 \), and AChRs occupied by two agonist molecules open with rate \( \beta_2 \) and close with rate \( \alpha_2 \). At high agonist concentrations (>100 \( \mu M \) ACh) channel blockade is evident, requiring addition of the blocked state \( A_2B \).

To estimate the set of rate constants, Scheme I was fitted to the data using the program MIL (Qin et al., 1996). MIL computes the likelihood of the experimental series of open and closed times given a set of trial constants, and then changes the rate constants to maximize the likelihood. We simultaneously analyzed recordings obtained at multiple ACh concentrations (10–300 \( \mu M \)), with the aim of representing all the states in Scheme I in the fitting. The procedure for kinetic analysis requires selection of clusters to produce kinetically homogeneous data (MIL, QUB suite; Salamone et al., 1999). For each recording, kinetic homogeneity was determined by computing the mean channel open duration and open probability of each cluster, and then plotting their distributions. Only clusters showing \( P_{open} \) and mean open duration values within the mean ±2 SD were included in the subsequent analysis. Comparison of distributions before and after selection (see examples in Table I) indicates that the selected clusters are representative of the entire population of events for each recording. In addition, open- and closed-time histograms constructed with selected data are very similar to those constructed with the original events (not shown). Data of open and closed intervals corresponding to the selected clusters were subsequently used to fit Scheme I.

For wild-type AChR, \( \beta_2 \) was constrained to its known value (Sine et al., 1995; Wang et al., 1997; Salamone et
(al., 1999) because brief closings due to gating and channel blocking became indistinguishable at high ACh concentrations (Wang et al., 1997; Salamone et al., 1999). When $\beta_2$ was allowed to vary freely, MIL failed to converge to a well-defined set of rate constants and approached a value of $\sim 100,000 \text{ s}^{-1}$ (data not shown). Similar observations were recently reported by Salamone et al. (1999). Although channel blocking is too brief to be completely resolved with our instrumentation dead time of 30 $\mu$s, we retain channel block in Scheme I because significant numbers of blockages are resolved at our highest ACh concentrations of 300 $\mu$M. Moreover, the unblocking rate constant obtained under the present conditions is similar to values previously reported (Maconochie and Steinbach, 1995; Wang et al., 1997). Finally, association and dissociation rate constants were assumed to be equal at both binding sites, as previously described for adult mouse AChRs (Akk and Auerbach, 1996; Wang et al., 1997; Salamone et al., 1999).

Rate constant estimates obtained for wild-type AChR, shown in Table II, agree with those previously reported for mouse AChR (Akk and Auerbach, 1996; Wang et al., 1997; Salamone et al., 1999). The fitting analysis confirms that singly occupied AChRs open slowly and with low efficiency, but doubly occupied receptors open rapidly and efficiently.

We next applied kinetic analysis to AChRs containing mutations of $\alpha$T422 using clusters selected as described for wild-type AChRs (Table I). Scheme I could not adequately describe data obtained from AChRs containing...
In fitting Scheme II to data from AChRs containing hydrophobic or aromatic mutations, the two binding sites were assumed to have equal microscopic rate constants, as found for wild-type AChR. In contrast to fitting Scheme I to data from the wild type, it was possible to allow $\beta_2$ to vary freely. For the hydrophobic and aromatic mutations, the unblocking rate constant ($k_u$) was constrained to the value used for wild-type AChRs because M4 does not contribute to the channel pore (Blanton and Cohen, 1994), and because we found the channel conductance to remain constant for all M4 mutations. The resulting fitting analysis establishes that the T422V, T422A, T422Y, T422W, and T422C mutations markedly affect the kinetics of channel gating (Table I). The steps primarily affected are channel opening and closing, with changes of two- to sevenfold in the opening rate ($\beta_2$ in Scheme II) and four- to ninefold in the closing rate ($\alpha_2$ (Table II). Therefore, the doubly occupied mutant AChR opens with greater latency and reduced efficiency compared with wild-type AChRs. However, after opening, the mutant channels rapidly return to the closed state or, with low probability, reach a more stable open state, represented by $A_2R^{**}$ in Scheme II.

In contrast to the hydrophobic and aromatic mutations, AChRs containing the serine or asparagine mutations could be well fitted by Scheme I (Fig. 2). Using the same constraints as in wild-type AChRs ($\beta_2 = 49,000$ s$^{-1}$ and equivalent binding sites), the best-fit rate constants indicate that the primary effect of the serine and asparagine mutations is a twofold increase in the closing rate, $\alpha_2$ (Table II). Because, as described above for wild-type AChRs, it was necessary to constrain $\beta_2$ in fitting data from $\alpha T422 S$ and $\alpha T422 N$ AChRs, and because the hydrophobic and aromatic mutations affected $\beta_2$, we systematically varied $\beta_2$ from 19,000 to 49,000 s$^{-1}$. Based on likelihood, the best description of the $\alpha T422 S$ and $\alpha T422 N$ data was obtained when $\beta_2$ was 49,000 s$^{-1}$, as found for wild-type AChR. For example, for the $\alpha T422 S$ AChR, the fit using $\beta_2 = 49,000$ was $e^{24}$, $e^{81}$, and $e^{235}$ times more likely than with $\beta_2$ equal to 39,000, 29,000, and 19,000 s$^{-1}$, respectively.

Although dwell times from the hydrophobic and aromatic mutants were not well described by Scheme I, dwell times from wild-type, $\alpha T422 S$, and $\alpha T422 N$ AChRs could be well described by either Scheme I or Scheme II (Table II). To show that changes in rate constants due to the mutations are not scheme dependent, we also fitted Scheme II to data from wild-type as well as $\alpha T422 S$ and $\alpha T422 N$ AChRs (Table II). Comparison of log likelihoods, obtained after fitting both schemes to wild-type and $\alpha T422 N$ data, reveals that Scheme I provides the better description; Scheme I is $e^{75}$ and $e^{170}$ times more likely than Scheme II for wild-type and T422N AChRs, respectively. On the other hand, for the $\alpha T422 S$ mutation, Scheme II is $e^{72}$ more likely than Scheme I. Fitting Scheme II to data from the serine and asparagine mutations reveals only slight changes in the gating steps, similar to results obtained with

### Table I

| Subunit | Open Probability | Mean Open Duration | Mean Closed Duration |
|---------|------------------|--------------------|----------------------|
| T422A   | 0.34             | 0.12               | 0.12                 |
| T422V   | 0.34             | 0.12               | 0.12                 |
| T422W   | 0.34             | 0.12               | 0.12                 |
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Transitions.

T422 selectively affects channel opening and closing activation free energy for closing decreases (Table III).

Are substituted. When threonine is replaced by either
hydrogen bonding ability of the original threonine changes for opening and closing transitions (Table III).

Scheme I (Table II). Comparison of wild-type and M4 mutant rate constants, calculated on the basis of Scheme II, also shows that \( \alpha T422 \) contributes primarily to channel opening and closing, and that these steps are largely affected by the presence of hydrophobic or aromatic residues (Table II). Thus, residues preserving the hydrogen bonding ability of the original threonine support nearly normal gating kinetics.

The channel gating equilibrium constant, \( \theta_2 \), calculated as \( \beta_2 / \alpha_2 \), significantly decreases in most of the mutations (Table II). For each side chain at position 422, we calculated the net free energy change for the gating equilibrium, as well as the activation free energy changes for opening and closing transitions (Table III). Free energy changes of \( \approx 2 \text{ kcal/mol} \) in the gating equilibrium are observed when nonpolar or aromatic amino acids are substituted into position 422, whereas only slight changes occur when serine or asparagine are substituted. When threonine is replaced by either aromatic or nonpolar amino acids, the activation free energy for opening increases, whereas, for all mutants, activation free energy for closing decreases (Table III).

Rates of agonist association and dissociation (Table II) and dissociation equilibrium constants (Table IV) are not significantly affected by these mutations. Thus, T422 selectively affects channel opening and closing transitions.

To determine the overall consequences of each mutation for AChR activation, and to evaluate consistency of the fitted rate constants, we computed the open probability from the fitted rate constants and compared it

### Table I

| Scheme | \( k_{+1} \) | \( k_{-1} \) | \( k_{+2} \) | \( k_{-2} \) | \( \beta_1 \) | \( \beta_2 \) | \( \alpha_1 \) | \( \alpha_2 \) | \( \theta_2 \) | \( \beta_3 \) | \( \alpha_3 \) | \( k_{+5} \) | \( k_{-5} \) |
|--------|--------------|--------------|--------------|--------------|--------------|--------------|--------------|--------------|--------------|--------------|--------------|--------------|--------------|
| Wild type | 1 | 360 \pm 28700 \pm 180 \pm 57400 \pm 560 \pm 2300 \pm 49000 | 1400 \pm 9 \pm 79000 \pm 35 \pm 30 | 12 \pm 1200 \pm 6 \pm 2300 \pm 50 \pm 1400 \pm 30 | 10 \pm 2100 \pm 5 \pm 4200 \pm 50 \pm 580 | 100 | 1 \pm 1 \pm 1 |
| T422S | 1 | 260 \pm 36770 \pm 130 \pm 73500 \pm 690 \pm 9100 \pm 49000 | 3900 \pm 9 \pm 79000 \pm 12 \pm 30 | 10 \pm 2100 \pm 5 \pm 4200 \pm 50 \pm 580 | 100 | 1 | 1 |
| T422N | 1 | 220 \pm 16000 \pm 110 \pm 32000 \pm 3700 \pm 4600 \pm 49000 | 2900 \pm 5 \pm 79000 \pm 16 \pm 30 | 6 \pm 350 \pm 3 \pm 700 \pm 190 \pm 130 | 70 | 1 |
| T422V | 2 | 140 \pm 27400 \pm 70 \pm 54700 \pm 28100 \pm 8600 \pm 3.2 \pm 758 \pm 3400 \pm 30 \pm 79000 | 1700 \pm 75 \pm 130 \pm 300 \pm 4 | 10 \pm 1200 \pm 5 \pm 2400 | 1300 \pm 5 \pm 79000 |
| T422A | 2 | 250 \pm 27600 \pm 120 \pm 55300 \pm 12800 \pm 10400 \pm 1.2 \pm 1430 \pm 4900 \pm 57 \pm 79000 | 400 \pm 180 \pm 140 \pm 260 \pm 5 | 10 \pm 1300 \pm 5 \pm 2500 |
| T422W | 2 | 250 \pm 30300 \pm 120 \pm 60700 \pm 13600 \pm 11150 \pm 1.2 \pm 394 \pm 1680 \pm 166 \pm 79000 | 700 \pm 240 \pm 160 \pm 79000 |
| T422Y | 2 | 230 \pm 25000 \pm 120 \pm 50185 \pm 10550 \pm 14700 \pm 0.7 \pm 1470 \pm 5306 \pm 160 \pm 79000 |
| T422C | 2 | 260 \pm 16000 \pm 130 \pm 33000 | 6300 \pm 5300 \pm 1.2 \pm 2100 \pm 3300 \pm 18 \pm 79000 | 130 \pm 100 \pm 160 \pm 79000 |
| Wild type | 2 | 415 \pm 27800 \pm 210 \pm 56000 \pm 49000 | 2300 \pm 21 \pm 2000 \pm 4600 \pm 30 \pm 99000 | 9 \pm 800 \pm 8 \pm 1500 | 100 | 100 \pm 800 \pm 8 \pm 8000 |
| T422S | 2 | 260 \pm 31400 \pm 130 \pm 63000 \pm 49000 | 6200 \pm 8 \pm 850 \pm 2900 \pm 19 \pm 99000 | 8 \pm 1100 \pm 4 \pm 2300 | 150 \pm 140 \pm 300 \pm 4 |
| T422N | 2 | 380 \pm 16400 \pm 190 \pm 33000 \pm 49000 | 5700 \pm 9 \pm 610 \pm 1400 \pm 15 \pm 99000 | 6 \pm 320 \pm 4 \pm 700 | 100 \pm 60 \pm 90 \pm 2 |

Rate constants are in units of \( \mu \text{M}^{-1} \text{s}^{-1} \) for association rate constants and s\(^{-1}\) for all others. Values are results of a global fit of Schemes I and II to data obtained over the range 10-300 \( \mu \text{M} \) ACh, with standard errors. Data without showing standard error has been constrained to allow a better fit. The channel opening equilibrium constant, \( \theta_2 \), is the ratio of the opening to closing rate constants corresponding to the major open state (\( \beta_2 / \alpha_2 \)).

### Table II

| Mutation | Gating \( \Delta G \) | Opening \( \Delta G \) | Closing \( \Delta G \) |
|----------|----------------------|---------------------|---------------------|
| T442S    | 0.6 kcal/mol         | 0 kcal/mol          | -0.6 kcal/mol       |
| T442N    | 0.5 kcal/mol         | 0 kcal/mol          | -0.4 kcal/mol       |
| T442V    | 1.4 kcal/mol         | 0.3 kcal/mol        | -1.0 kcal/mol       |
| T442A    | 2.0 kcal/mol         | 0.7 kcal/mol        | -1.2 kcal/mol       |
| T442W    | 2.0 kcal/mol         | 0.7 kcal/mol        | -1.2 kcal/mol       |
| T442Y    | 2.3 kcal/mol         | 0.8 kcal/mol        | -1.4 kcal/mol       |
| T442C    | 2.0 kcal/mol         | 1.1 kcal/mol        | -0.8 kcal/mol       |

Gating \( \Delta G \) is the change between mutant (m) and wild-type (wt) AChRs in the energy of channel gating, calculated by \( \Delta G = -RT \ln (\theta_{m} / \theta_{wt}) \) (where R is the gas constant and T is the absolute temperature). \( \theta_2 \) is the initial channel opening equilibrium constant shown in Table II. \( \Delta G \) is the change between mutant and wild-type AChRs in the energy that the channel must overcome to make the transition from the biligated closed state to the main open state (Opening) and from this open to the closed state (Closing). The differences in free energies are calculated as \( \Delta G = -RT \ln (\beta_{m}/ \beta_{wt}) \) and \( -RT \ln (\alpha_{m}/ \alpha_{wt}) \) for opening and closing, respectively.
with the experimentally determined open probability (Fig. 3). For wild-type AChRs, open probability increases with increasing ACh concentration, reaching a maximum of \( \sim 0.9 \) at 100 \( \mu M \) and showing an EC\(_{50}\) of 40 \( \mu M \) (Fig. 3). For most mutant AChRs, both the EC\(_{50}\) and maximum open probability change. The open probability curves are shifted to higher ACh concentrations, owing to decreased efficiency of channel gating. For the mutants T422A, T422V, T422W, T422Y, and T422C, open probability is \(<0.6\) at 1,000 \( \mu M \), and the EC\(_{50}\) values range from 200 to 300 \( \mu M \) (Fig. 3). Thus, mutations of \( \alpha\)T422 affect both efficacy and EC\(_{50}\) for AChR activation. As expected, the profile for the T422N mutant is similar to that of wild-type AChR, and only slight changes are observed when threonine is replaced by serine. The EC\(_{50}\) and maximum open probability values for the T422S mutant are 80 \( \mu M \) and 0.8, respectively (Fig. 3). The dose–response curves computed from the fitted rate constants closely follow the experimentally determined open probability determinations, demonstrating overall consistency of the data.

**DISCUSSION**

T422 of the M4 domain of the \( \alpha \) subunit is presumed to be located at the lipid–protein interface, owing to its susceptibility to labeling by a hydrophobic probe (Blanot and Cohen, 1994). Although M4 is the least conserved of the four transmembrane domains, the high degree of conservation of T422 among subunits and species indicates that it might play a structural or functional role. We previously reported that among residues in M4, \( \alpha\)T422 is the most sensitive to mutation, with the alanine substitution decreasing the mean open time fivefold (Bouzat et al., 1998). Here we elucidated both mechanistic and structural bases for the contribution of \( \alpha\)T422 to channel gating. Kinetic analysis of mutant \( \alpha\)T422 AChRs discloses relatively small contributions to steps governing ACh binding to the resting, closed state of the AChR, but reveals significant contributions to steps underlying channel opening and closing.

Kinetic analysis requires that open and closed dwell times correspond to a single AChR channel. Clusters of opening events, corresponding to activation of the AChR between desensitized periods, were clearly distinguished at ACh concentrations \( >10 \) and 30 \( \mu M \) for wild-type and mutant AChRs, respectively. Because only clusters containing a single open conductance level were considered for kinetic analysis, it is possible to unequivocally assign the dwell times to sojourns in open and closed states of a single AChR. In addition, the clustering behavior observed for all mutant AChRs clearly demonstrates that, like wild-type, they can undergo cycles of desensitization and resensitization in the continued presence of ACh.

Based on gating kinetics, one can distinguish the polar serine and asparagine from the nonpolar or aromatic amino acid mutations at position 422 as follows. (a) Kinetic changes due to replacement of T422 by serine or asparagine: recordings obtained from these mutant AChRs are similar to those from wild-type AChRs (Fig. 1). Slight changes in the open- and closed-time histograms are observed (Fig. 2). Data can be well fitted by the classical description of activation for wild-type AChR. The only detectable change is a twofold in-

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**TABLE IV**

Equilibrium Constants for Mouse Wild-Type and \( \alpha\)T422 Mutant AChRs

|           | \( K_1 \)  | \( K_2 \) |
|-----------|------------|------------|
| Wild type | 70 \( \mu M \) | 320 \( \mu M \) |
| T422S     | 140 \( \mu M \) | 560 \( \mu M \) |
| T422N     | 70 \( \mu M \) | 260 \( \mu M \) |
| T422V     | 190 \( \mu M \) | 780 \( \mu M \) |
| T422A     | 110 \( \mu M \) | 460 \( \mu M \) |
| T422W     | 120 \( \mu M \) | 500 \( \mu M \) |
| T422Y     | 100 \( \mu M \) | 410 \( \mu M \) |
| T422C     | 62 \( \mu M \) | 260 \( \mu M \) |

Equilibrium constants were obtained with data from Table II. \( K_1 = k_{-1}/k_{+1}, K_2 = k_{-2}/k_{+2} \).
crease in the channel closing rate. (b) Kinetic changes due to replacement of αT422 by nonpolar or aromatic residues: recordings show very brief openings and prolonged closings. The classical activation scheme does not fit the experimental data. However, expanding the classical scheme to include two sequentially connected open states (Scheme II) satisfactorily describes the data. Kinetic analysis based on Scheme II reveals that the rate of opening to the first open state is slower than the analogous step in wild-type AChRs, but the closing rate from this state ($\alpha_3$) is much faster. Because $\alpha_3$ is faster than the competing rate $\beta_3$, the open channel ($A_2R^*$) has a much greater probability of closing than of reaching the second, more stable open state ($A_2R^{**}$). Thus, nonpolar or aromatic mutations dramatically impair the initial opening step in the gating pathway. Interestingly, αT422C shows a different behavior within this group of mutants; it is the only mutant in which the opening rate is much more affected than the closing rate. Comparison of rate constants of wild-type and M4 mutant AChRs, calculated on the basis of both Schemes I and II, confirms that αT422 contributes to channel opening and closing regardless of the kinetic scheme used for analysis. Scheme II was recently used to explain the activation of an AChR mutated in the M3 domain (Wang et al., 1999). This mutation, V285I, also causes abnormally slow opening and rapid closing rates. Thus, Scheme II may represent a general mechanism that is not distinguished in wild-type AChR because the intermediate open state may be too short-lived to be detected, and the channel rapidly reaches the final open state. Thus, certain mutations that disrupt gating may have the ability to unmask intermediate open states.

The results also reveal the structural basis of the contribution of T422 to channel gating. Threonine and serine both contain polar hydroxy groups, but differ by the presence of a methyl group attached to the beta carbon. Although the lack of a methyl group in serine slightly affects gating, αT422S AChRs are kinetically similar to wild-type AChRs. However, if only the hydroxy group of threonine is replaced by a methyl group, as in valine, gating is substantially disrupted. Moreover, αT422V AChRs behave similarly to those containing structurally unrelated amino acids, such as tryptophan. The asparagine side chain provides similar electronegative atoms to the original threonine side chain, and the polar amide group is similar to the hydroxyl group in its propensity for donating hydrogen bonds; T422N AChRs are almost kinetically identical to wild-type AChRs. Although cysteine can form hydrogen bonds, the kinetics of T422C AChRs differ from those of wild type. This finding can be explained by the fact that the thiol group is a poor hydrogen bond donor and forms weaker hydrogen bonds compared with hydroxyl or amide groups. Because hydroxyl and amide groups can equally well accept hydrogen bonds, and because, in contrast, cysteine is a poor hydrogen bond acceptor (Pal and Chakrabarti, 1998), an alternative interpretation would be that the side chain at position 422 acts as a hydrogen bond acceptor. In addition, the lower pKa of cysteine compared with threonine, or the greater hydrophobicity of cysteine compared with threonine, serine, or asparagine may contribute to the impaired channel gating. The observed gating kinetics of T422Y AChRs indicate that tyrosine does not provide the correct hydrogen bonding capability, perhaps owing to delocalization of electron density from the hydroxyl oxygen to the aromatic ring, or to the bulk of the aromatic ring. Interestingly, the tyrosine hydroxyl group is a poorer hydrogen-bond acceptor than an aliphatic or water hydroxyl group (Fersht et al., 1985). In summary, our results show that correct gating requires a hydrogen bond involving the side chain at position 422 in the M4 domain of the α subunit.

T422 is located in the last third of the M4 domain, closest to the outer leaflet of the bilayer (Fig. 4). Whether the M4 domain is an α helix (Blanton and Cohen, 1994) or a β-sheets (Unwin, 1993) remains controversial. In addition, current models of the AChR place M4 with a tilt of ~30° with respect to the membrane (Ortells et al., 1997). Thus, atomic structure resolution of the AChR will be needed to unequivocally identify the hydrogen-bonding partner for αT422. Although T422 is labeled by the hydrophobic probe TID, and therefore is probably exposed to the lipid, its hydroxyl group could interact with a hydrogen bond acceptor or donor within a transmembrane domain of the same or another subunit. The more energetic hydrogen bonding expected in a hydrophobic environment may account for the substantial functional importance of the hydrogen bond with which T422 is involved. Thus, the present work demonstrates that subtle interactions originating far away from the two main functional domains of the AChR, the ion pore and the binding site, significantly affect channel gating.

![Figure 4. Sequence alignments of the M4 domains. The conserved T422 is shown as a shaded column.](Image)
Calculation of free energy changes for channel opening and closing disclosed only slight decreases in free energy for closing between serine and asparagine mutant AChRs, but revealed significant changes in free energy for both opening and closing steps for all other mutant AChRs. The free energy differences are on the order of those calculated for hydrogen bonds between uncharged residues in an aqueous solution (0.5–1.5 kcal/mol; Fersht et al., 1985), or for other weak interactions such as dipole-dipole interactions. Our observation that a hydrogen bond is required for proper gating agrees with the calculated free energy changes. However, the correlation between the free energy of a hydrogen bond and free energy of activation for a state transition is not necessarily strict, given that disruption of a chemical bond can have global consequences.

The significant role of a hydrogen bond at position 422 in channel gating demonstrated in the current work explains the high degree of conservation of T422 among subunits and species (Fig. 4). Abnormal activation of AChR has been shown to underlie congenital myasthenic syndromes (CMS) (Engel et al., 1998). Although mutations in the M4 domain have not been identified in CMS, our findings indicate that if they naturally occurred they could lead to a CMS.

Given the present understanding of the topology of the M4 domain, our results demonstrate that a lipid-exposed residue involved in hydrogen bonding is necessary for proper gating of the AChR.

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