Activation Kinetics of Recombinant Mouse Nicotinic Acetylcholine Receptors: Mutations of α-Subunit Tyrosine 190 Affect Both Binding and Gating

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ABSTRACT Affinity labeling and mutagenesis studies have demonstrated that the conserved tyrosine Y190 of the acetylcholine receptor (AChR) α-subunit is a key determinant of the agonist binding site. Here we describe the binding and gating kinetics of embryonic mouse AChRs with mutations at Y190. In Y190F the dissociation constant for ACh binding to closed channels was reduced ~35-fold at the first binding site and only ~2-fold at the second site. At both binding sites the association and dissociation rate constants were decreased by the mutation. Compared with wildtype AChRs, doubly-liganded αY190F receptors open 400 times more slowly but close only 2 times more rapidly. Considering the overall activation reaction (vacant-closed to fully occupied-open), there is an increase of ~6.4 kcal/mol caused by the Y-to-F mutation, of which at least 2.1 and 0.3 kcal/mol comes from altered agonist binding to the first and second binding sites, respectively. The closing rate constant of αY190F receptors was the same with ACh, carbamoylcholine, or tetramethylammonium as the agonist. This rate constant was ~3 times faster in ACh-activated S, W, and T mutants. The equilibrium dissociation constant for channel block by ACh was ~2-fold lower in αY190F receptors compared with in wildtype receptors, suggesting that there are changes in the pore region of the receptor as a consequence of the mutation. The activation reaction is discussed with regard to energy provided by agonist-receptor binding contacts, and by the intrinsic folding energy of the receptor.

INTRODUCTION

Nicotinic acetylcholine receptors (AChR) are pentameric ($\alpha_2\beta\delta\gamma$), ligand-gated channels that can assume three stable structures: closed, open, or desensitized. Closed, unliganded AChRs are induced to open by binding two molecules of ACh (Changeux et al., 1992). To further understand the molecular events that link agonist binding with channel gating, we and others have combined site-directed mutagenesis with measurements of ACh binding and channel activation.

Affinity labeling studies have identified residues in the vicinity of the agonist binding site, including vicinal cysteines of the α-subunit at positions 192 and 193 (Kao and Karlin, 1986) and three regions of the α-subunit rich in aromatic residues: Y190/Y198, Y93, and W149/Y151. Aromatic residues are common at quaternary amine binding sites in other proteins (Sussman et al., 1991; Satow et al., 1986), and both experimental and theoretical studies of model systems support the existence of a specific quaternary amine-aromatic binding interaction (Dougherty and Stauffer, 1990; Gao et al., 1993). In addition to these α-subunit residues, affinity labeling and expression studies have identified residues on the adjacent γ and δ subunits that contribute to ligand binding, including δW55 (Pedersen and Cohen, 1990), γY117 (Sine, 1993), and a segment containing the acidic residues δD180 and δE189 (Czajkowski et al., 1993).

The functional consequences of mutating key aromatic residues in the α-subunit have been examined at the level of equilibrium binding (Sine et al., 1994) and dose response (Tomaselli et al., 1991; O'Leary and White, 1992) properties. These studies demonstrated that residue αY190 is of critical importance to the activation reaction, as even the most conservative substitution, phenylalanine, increased EC_{50}s for agonist up to several hundredfold. However, underlying binding and dose response measurements are contributions from both binding and gating steps, and Y190F has been proposed to affect primarily gating (O'Leary and White, 1992) or primarily binding (Sine et al., 1994). Here, we use single channel kinetic analysis to pinpoint the effects of αY190F at the level of individual rate constants. The results demonstrate changes in both binding and gating rate constants. The relative free energy of the activation reaction is increased by a total of ~6.4 kcal/mol in αY190F, with ~40% of this energy coming from loss of binding affinity for closed receptor states and the remainder from a change in the gating equilibrium constant. We speculate that Y190 both makes direct contact with the ligand and is a key determinant of the transition of the fully occupied closed channel to a high energy conformation that leads to channel opening.

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MATERIALS AND METHODS

The experimental preparation, electrophysiology, and single-channel analysis methods have been described in detail elsewhere (Zhang et al., 1995).

Expression system and recording conditions

Mouse receptor subunits (α, β, δ, and γ) in the vector pRBG4 were expressed via transient calcium-phosphate transfection of human embryonic kidney cells (HEK 293; Sine, 1993). Single-channel currents were recorded (22°C) from cell-attached patches (pipette solution in mM: NaCl 115, HEPES-NaOH 10, $CaCl_2$ 1, KCl 2; pH 7.4). In a few patches the pipette NaCl concentration was 145 mM. Unless stated otherwise, the potential of the patch pipette was held at +70 mV. We assumed the channel reversal potential to be 0 mV, and we estimated the membrane potential from the amplitude of the currents and the current/voltage (I/V) properties of the channel in patches exposed to low concentrations of ACh. In these patches the membrane potential was −80 to −100 mV, indicating that the HEK cells had a resting potential of −10 to −30 mV. Thus, we estimate that in our experiments the membrane potential was −90 ± 10 mV.

Kinetic analysis

Currents were digitized at 94 kHz (fc = 50 kHz; Bessel low pass filter) and were detected after digital filtering (fc = 2 kHz; Gaussian low pass filter) via a half-amplitude threshold-crossing criterion. Clusters of currents, which reflect channels undergoing cycles of desensitization and resensitization, were clearly defined by a critical time that was 5 times longer than the predominant closed interval component in the record. The P_{open} of a cluster was calculated as the sum of open interval durations divided by the sum of closed and open intervals; the last open interval was excluded from this calculation so that an equal number of open and closed intervals were included. Because channel-blocking events were not resolved they did not lower the cluster P_{open}. Rather, channel block by ACh prolonged the apparent open time, thereby decreasing the estimated channel closing rate and increasing the cluster P_{open}. The effective opening rate (β') was calculated as the inverse of the time constant of the predominant component of closed intervals within clusters. In αY190F receptors, at all concentrations of ACh, more than 80% of all closed intervals belong to this component.

The current clusters were idealized a second time using either half-amplitude crossing detection (fc = 2–4 kHz) with a correction for filtering (Colquhoun and Sigworth, 1983) or the Viterbi algorithm (4–30 kHz; Zhang et al., 1995). Rate constants and their standard errors were estimated by an interval-based maximum likelihood method that incorporated first-order corrections for missed events (Horn and Lange, 1993; Roux and Sauve, 1985; Ball and Sansom, 1989). After fitting, probability density functions, dose response, and effective opening rate curves were directly calculated from the rate constants. Equilibrium binding curves for ACh were calculated from the rate constants plus three additional parameters: the dissociation constant for ACh binding to desensitized receptors (3.2 μM in αY190F receptors; Sine et al., 1994), the equilibrium constant for channel block at 0 mV (10 mM), and the fitted desensitization equilibrium constant of vacant receptors (see Sine et al., 1994).

RESULTS

Characteristics of αY190F current

Fig. 1 shows the basic properties of currents activated by 500 μM ACh in αY190F receptors. On a slow time scale the currents occur in clusters that last 1–20 s, with intercluster intervals of 10–100 s. Earlier studies have noted that αY190F receptors lack the characteristic rapid inactivation response to a step application of ACh (Tomaselli et al., 1991), raising the possibility that this receptor does not desensitize. The clustering behavior clearly demonstrates that αY190F, like wildtype AChR, undergoes cycles of desensitization and resensitization in the continued presence of ACh. The prolonged cluster duration, however, suggests slower onset of desensitization for αY190F. This may arise because of reduced desensitization rate constants, and/or simply because desensitization occurs preferentially from a doubly liganded, open state that is occupied with a low probability in the mutant (see below).

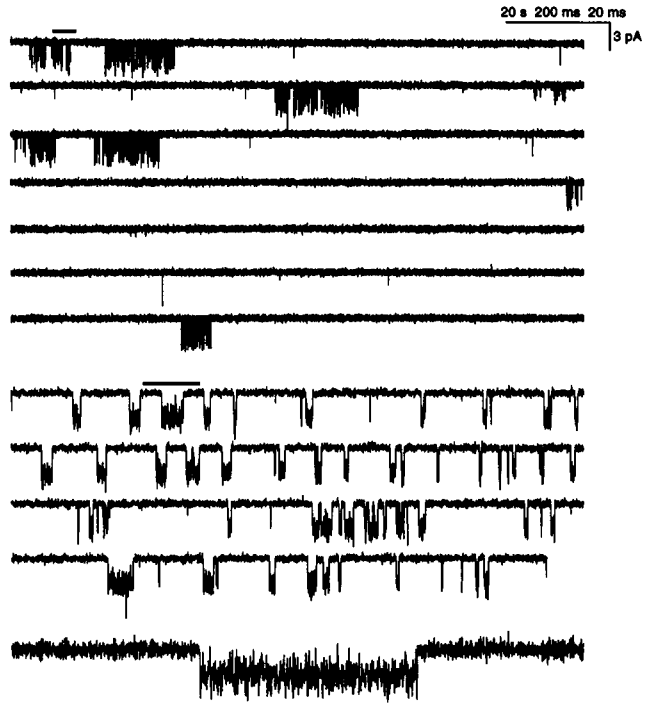

FIGURE 1 Single-channel records from αY190F receptors activated by 500 μM ACh. At low time resolution (*uppermost trace*), the clustering of currents is clear, indicating that the mutant receptors desensitize. The apparently variable amplitude of the currents is an artifact of the low digitization rate. The middle trace shows a cluster (marked by a line in the upper trace) at a higher time resolution and bandwidth (2 kHz). Excess open channel noise is apparent. The lower trace shows an opening event (marked by a line in the middle trace) at still higher resolution and bandwidth (6 kHz). The open channel noise cannot be resolved as discrete events.

Fig. 1 (*middle*) shows one cluster at higher resolution. The closed intervals within clusters are ~1000 times longer than those of wildtype receptor currents (50 ms vs. 50 μs; Zhang et al., 1995) at the same ACh concentration, but the apparent open times are about the same as wildtype receptors (5–10 ms). From these observations we conclude that the Y190 affects the binding and/or opening rate constants to a much greater extent than it does the channel closing rate.

A consistent property of αY190F currents activated by high (>200 μM) ACh concentrations was the presence of excess open channel noise. This can be seen in Fig. 1 (*middle*, *bottom*). Although open channel noise is apparent in wildtype receptors at this ACh concentration, the magnitude of the variance of the noise is greater in the mutant receptors. Below, we show that the properties of this excess noise suggest that it arises from channel block by ACh.

Properties of αY190F as a function of the concentration of ACh

Fig. 2 summarizes the kinetic properties of αY190F receptors over a wide range of ACh concentrations. The probability of being open within a cluster (P_{open}) increases between ~200 μM and 5 mM ACh, and appears to reach a maximum (P_{max}) at ~0.5. When fitted by the Hill equation:

$$P_{open} = P_{max}/(1 + ([A]/EC_{50})^{nH})$$

The dose response data indicate P_{max} = 0.48 ± 0.06, and EC_{50} = 1727 ± 419 μM with nH = 1.8 (8 patches). The corresponding values are P_{max} = 0.996, EC_{50} = 9.4 μM, and nH = 1.6 in wildtype AChR (36 patches; Zhang et al., 1995; Fig. 2, *dashed curve*). The αY190F EC_{50} value is ~3 times larger than that reported by Tomaselli et al. (1991), who reported normalized dose response profiles of macroscopic currents. In the whole-patch experiments channel block reduces the current amplitude more at high ACh concentrations than at low concentrations, producing a leftward shift in the dose response curve. Considering just the channel activation reaction (binding and gating, in the absence of desensitization), the maximum P_{open} can be used to estimate an equilibrium constant for the gating step (θ; see Model 1, below):

$$\theta = (1/P_{max} - 1)^{-1}.$$

This value in wildtype receptors is > 250 (Zhang et al., 1995). From the data shown in Fig. 2, we estimate that in αY190F receptors $\theta < 1$. This estimate of θ is defined as an upper limit because channel block by ACh makes the apparent closing rate slower than the true value. Combined with our observation that the channel closing rate constants are not greatly altered by the mutation, this result indicates that the channel opening rate, β, of αY190F receptors is at least 250 times slower than that of the wildtype.

The effective opening rate (β') is the inverse time constant of the major closed interval within clusters. This measure reflects the time required for an unliganded channel to bind two agonists and open, i.e., β' is a composite rate that reflects the rate constants of association, dissociation, and opening. The effective opening rate of αY190F receptors between 200 μM and 20 mM ACh is shown in Fig. 2 (*middle*). β' increases with increasing ACh concentrations up to ~1 mM, beyond which it plateaus at a value of ~150 s^{-1}. The results of fitting the effective opening rate curve to the Hill equation were β_{max} = 150 ± 15 s^{-1} and EC_{50} = 1051 ± 342 μM, with nH fixed at 1.8 (12 patches). This limit, 150 s^{-1}, is β, the opening rate of fully occupied receptors. Bandwidth limitations prevent the direct observation of this plateau in ACh-activated wildtype receptors (Zhang et al., 1995), but Machonochie and Steinbach (1992) conclude that β~ 60,000 s^{-1} for wildtype embryonic mouse AChR based on the plateau of the risetime of currents elicited by the application of very rapid concentration jumps to outside-out patches. These results regarding the effective opening rate curves indicate that once doubly liganded, αY190F receptors open ~400 times more slowly than do wildtype receptors.

Fig. 2 (*right*) shows the equilibrium binding properties of αY190F receptors (data replotted from Sine et al., 1994).

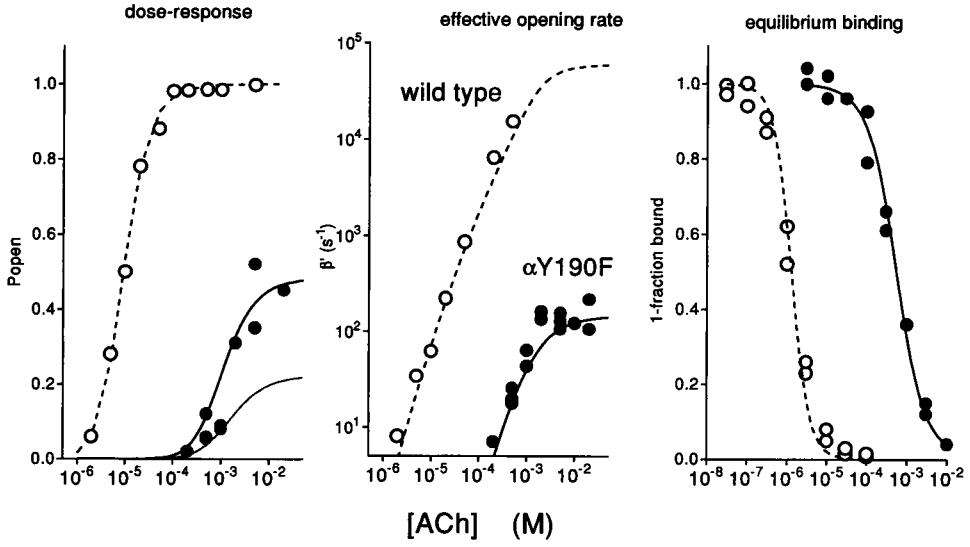

FIGURE 2 Properties of αY190F receptors as a function of the concentration of ACh. Each symbol in the left and center panels are each from a different patch, while those in the right panel are from the binding experiments of Sine et al., 1994. All lines were calculated directly from the rate constants estimated by kinetic modeling with out further adjustments. (-----, ○) Wildtype receptors (rates given in text from Zhang et al., 1995); (———, ●) αY190F receptors (rates shown in Fig. 4); (· · · · ·) αY190F receptors with the effects of channel block by ACh removed. The equilibrium binding curves had one free parameter to fit ($M = 1.4 \times 10^{-5}$ for αY190F receptors and 1.8×10^{-4} for wildtype receptors). Compared with wildtype receptors αY190F receptors show a right-shifted dose response curve, a lower maximum probability of being open (P_{open}), a slower maximum effective opening rate (β'), and a higher apparent equilibrium dissociation constant.

The curve for the mutant shows an apparent affinity that is ~400 times lower than that of the wildtype receptor. The position and shape of the binding curve is determined by occupancy of closed, open, blocked and desensitized receptors. We therefore combined our kinetic parameters (described below) with parameters of desensitization and channel block to obtain the smooth curve that overlays the data in Fig. 2.

αY190F receptors activated by low concentrations of ACh, carbamylcholine (CCh), and tetramethylammonium (TMA)

The kinetic properties of αY190F receptors activated by low concentrations of ACh, CCh, or TMA were examined in 16 patches. Example results are shown in Fig. 3. At low concentrations of agonist (ACh = 20 μM; CCh = 500 μM; TMA = 1 or 2 mM), brief closures were rare, the distribution of open interval durations >0.1 ms was that of a single exponential, and excess open channel noise was not substantial (Fig. 3, *top*). The open channel lifetimes (τ; analysis bandwidth = 2 kHz, membrane potential ~−90 mV) of αY190F receptors activated by these three agonists were: $\tau_{ACh} = 2.0 \pm 0.4$ ($n = 4$), $\tau_{CCh} = 2.1 \pm 0.4$ ($n = 6$), and $\tau_{TMA} = 2.0 \pm 0.6$ ($n = 6$). Because we have established that in the mutant the opening rate is slow in ACh-activated receptors, it is safe to assume that this is also true for CCh- and TMA-activated receptors. Therefore, the channel closing rate for each agonist can be directly estimated as the inverse of the open channel lifetime. These results indicate that the channel closing rate of αY190F receptors, α, is 500 s^{-1} and is independent of the nature of the agonist. Compared with wildtype receptors (Zhang et al., 1995) for the three agonists we have examined, αY190F receptors close 1.5-fold (CCh), >2.1-fold (ACh), and 3.3-fold faster (TMA).

Using the estimates $\alpha = 500\ s^{-1}$ and $\beta = 150\ s^{-1}$, we calculate that for ACh-activated receptors, the equilibrium constant for the opening of doubly liganded channels, θ, equals 0.3, and that P_{max} is 0.23. The gating rate constants predict that a macroscopic current from Y190F receptors elicited by an instantaneous step to a saturating concentration of ACh should rise with a time constant equal to $(\alpha + \beta)^{-1}$, or 1.53 ms (ignoring channel block).

Activation rate constants of αY190F receptors

We estimated the rate constants of association, dissociation, and channel closing using the following standard kinetic scheme:

$$C \underset{k_{-1}}{\overset{[A]k_{+1}}{\rightleftharpoons}} AC \underset{k_{-2}}{\overset{[A]k_{+2}}{\rightleftharpoons}} A_2C \underset{\alpha}{\overset{\beta}{\rightleftharpoons}} A_2O \quad \text{(Model 1)}$$

In embryonic, wildtype receptors, the activation rate constants were estimated by Zhang et al. (1995) to be (in s^{-1} or $\mu M^{-1}\ s^{-1}$):

$$C \underset{650}{\overset{21[A]}{\rightleftharpoons}} AC \underset{>25{,}000}{\overset{40[A]}{\rightleftharpoons}} A_2C \underset{<240}{\overset{60{,}000}{\rightleftharpoons}} A_2O$$

(activation rate constants, wildtype)

In the fit of αY190F currents, the channel opening rate constant was constrained to be 150 s^{-1}, as determined from the saturation of the effective opening rate curve (Fig. 2). Rate constants were estimated separately for three patches at 500 μM ACh (Table 1). Fig. 4 shows the optimal rates, as well as the loglikelihood surface in the vicinity of the

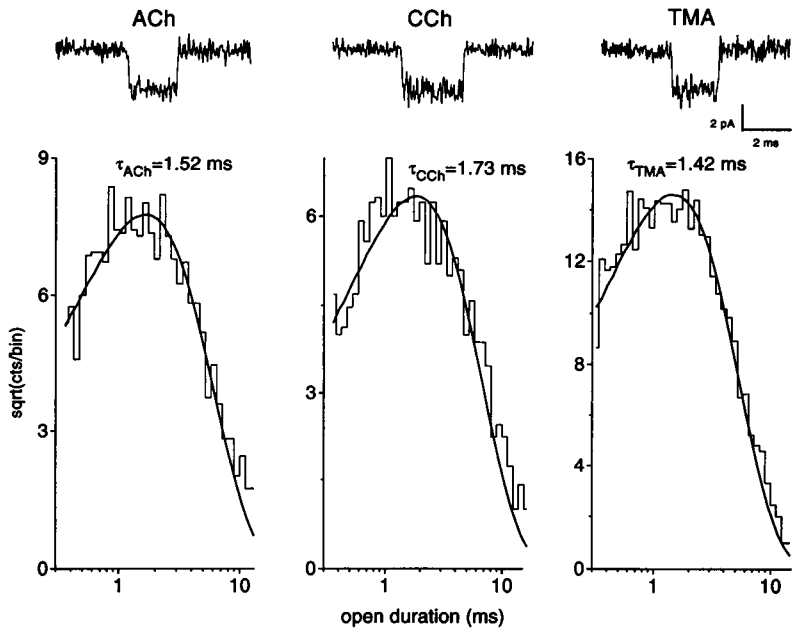

FIGURE 3 Examples of αY190F receptor currents activated by concentrations of agonists that do not produce significant channel block. ACh, CCh, and TMA elicit openings that have similar lifetimes.

TABLE 1 Optimal activation rate constants for αY190F receptors activated by 500 μM ACh

File	No. clusters	No. events	No. open/ms	k_{+1}	k_{-1}	k_{+2}	k_{-2}	α
232700	15	3180	19.1	0.33	199	2.66	3673	159
724784	56	1686	23.8	0.36	163	0.92	1302	315
737221	48	2708	19.6	0.97	361	2.60	3856	247

The rate constants are in s^{-1} or $mM^{-1}\ s^{-1}$ (k_{+1} and k_{+2}). The error limits are the 0.5 likelihood intervals. File 232700 was in 115 mM NaCl in the pipette, and files 724784 and 737221 were in 145 mM NaCl; channel block is reduced at the higher salt concentration; thus, the decrease in the apparent closing rate is less. The rates (with error limits) obtained by fitting these three files together are shown in Fig. 4.

optimal values, obtained by combining the intervals from these three patches. The error limits (0.5 likelihood intervals) for the association and dissociation rate constants were within ~40% of the best estimates; the error limits on the closing rate constant estimate was within 2% of the optimal value. The open and closed interval duration histograms for all three files are well described by the optimal rate constants. These values predict that at 500 μM ACh closed interval durations should be comprised of three exponential components with time constants (and relative areas) of 53.8 ms (94%), 1.94 ms (5%), and 0.27 ms (1%).

The dose response and effective opening rate curves calculated from the mean rate constants summarized in Table 1 are superimposed (with no additional curve fitting) on the experimental data over all tested ACh concentrations as solid lines in Fig. 2. For the equilibrium binding data, the activation rate constants are combined with the measured dissociation constant for binding to desensitized receptors (K = 3.2 μM; see Materials and Methods and Sine et al., 1994), the dissociation constant for channel block (assumed to be 20 mM at 0 mV membrane potential; see below), and the allosteric constant describing the equilibrium between vacant-closed and desensitized receptors. The allosteric constant, which was fitted to the binding data as a free parameter, is 13-fold smaller in αY190F ($M = 1.4 \times 10^{-5}$) than in wildtype ($M = 1.84 \times 10^{-4}$ from the rate constants of Zhang et al., 1995) indicating that, like channel activation, desensitization is less efficient in the mutant. Thus for all three sets of results there is agreement between the calculated curves and the experimental data over a 1000-fold range of ACh concentration.

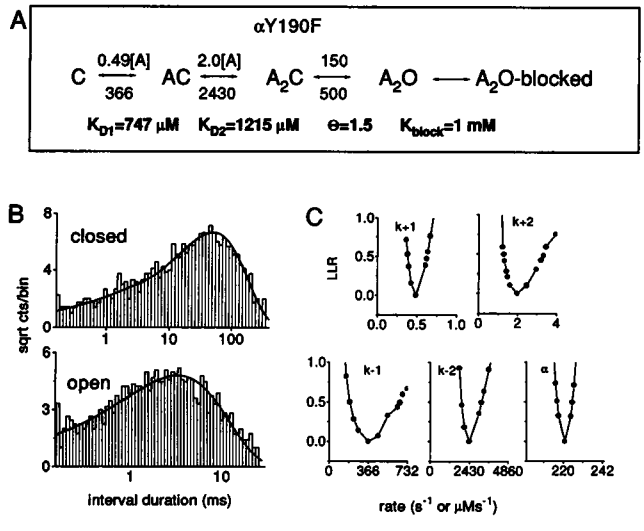

FIGURE 4 Kinetic modeling of αY190F single-channel currents. (*A*) Optimal rate constants (s^{-1} or $\mu M^{-1}\ s^{-1}$); [*A*] is the concentration of ACh in μM) obtained by combining intervals from three different patches exposed to 500 μM ACh. Rate constants obtained separately for each patch are given in Table 1. (*B*) Open and closed interval duration histograms for one patch. The solid curves were calculated directly from the global optimal rate constants without fitting to the histogram. (*C*) Loglikelihood ratio (LLR) surfaces in the vicinities of the global optima. A standard error is defined by the 0.5 LLR interval. The limits of the abscissa represent ±100% of the optimal value, except for α, where it represents ±10% of the optimum.

The results indicate that both binding and gating rate constants are altered by the mutation. The equilibrium dissociation constant at the first site (K_{D1}) is 747 μM, more than 35 times greater than that of the wildtype. The equilibrium dissociation at the second site (K_{D2}) is also greater than that of the wildtype, but only by a factor of ~2. Thus, in the mutant receptor there is only about a 1.6-fold difference in the K_D values at the two sites, compared with a 32-fold difference in wildtype receptors. In apparent contrast to this greater difference in affinity, the equilibrium binding curve for wildtype receptors is steeper (nH = 1.8) than for αY190F (nH = 1.5) (Fig. 2 and Sine et al., 1994). The shallow slope in αY190F results from decreases in both the opening equilibrium constant and the allosteric constant for desensitization, so that equilibrium binding mostly reflects binding to activatable states.

The modeling results suggest that the decrease in the agonist binding affinity for closed channels in the mutants results largely from slower rate constants of ACh association, particularly at the first binding site. These association rate constants are ~60 and 20 times slower in αY190F receptors compared with the wildtype at the first and second binding sites, respectively. The results indicate that the dissociation rate constants also slow with the mutation, but only by factors of ~2 and 11 at the two sites.

Open channel noise

Excess open channel noise in αY190F receptors compared with wildtype receptors was consistently observed, but only at high concentrations of ACh, suggesting that channel block properties were altered by the mutation. To test this hypothesis, we quantified the current-voltage properties of receptors activated by different concentrations of ACh. If the duration of the blocked state is too brief to be resolved as discrete gaps, then the apparent open channel current amplitude will decrease with increasing concentrations of ACh. Moreover, if the site of block by ACh is within the field of the membrane, the extent of this reduction in current amplitude will increase with the membrane potential, lead-

ing to outward rectification of the single-channel current-voltage relationship.

Current-voltage (I/V) relationships of the open channel amplitude for wildtype and αY190F receptors at different ACh concentrations (analyzed at 1 kHz) are shown in Fig. 5 *A*. The outward rectification that is apparent at high, but not low, ACh concentrations is consistent with fast, voltage-dependent channel block by ACh. That rectification is more pronounced for αY190F receptors compared with wildtype receptors suggests that equilibrium channel block is enhanced by the mutation. The absence of rectification at very hyperpolarized potentials further suggests that ACh can permeate the channel.

We can obtain a first order estimate of the equilibrium dissociation constant for channel block (K_b) by assuming a simple two-state kinetic model of channel block that predicts:

$$K_b = [A]/(i_o/i - 1) \quad (1)$$

where i is the observed current amplitude, i_o is the current amplitude in the absence of channel block (taken from the 20 μM ACh I/V curve), and $[A]$ is the concentration of ACh. Using this equation, the I/V curves at 2 mM ACh indicate that at −80 mV, for αY190F receptors K_b = 1.2 mM and for wildtype receptors K_b = 2.0 mM. Thus, an analysis of the I/V properties indicates that the mutation decreases the equilibrium dissociation constant for block about twofold.

As a second test of the channel block hypothesis, we examined the apparent open channel lifetime as a function of the concentration of ACh (Fig. 5 *B*). Unresolved channel block should result in a decrease in the apparent closing rate with increasing concentrations of ACh. This was the trend we observed in Y190F receptors, where the apparent closing rate decreased from 500 s^{-1} at 20 μM ACh to 220 s^{-1} at 500 μM ACh. For a two-state blocking model, the equation that describe this decrease is identical to Eq. 1, with the apparent closing rate replacing the observed current amplitude (i) and the true closing rate replacing the true channel amplitude (i_o). Fig. 5 *B* shows the apparent closing rate (at an analysis bandwidth of 2 kHz) as a function of the ACh concentration. When fitted by Eq. 1, these data indicate a true closing rate of 565 ± 60 s^{-1} and an equilibrium dissociation constant for channel block (at −90 mV) of 1.02 ± 0.38 mM for αY190F receptors. This value agrees with that obtained from the analysis of the I/V relationship, and supports the conclusion that the mutation increases equilibrium block by ACh by about a factor of 2.

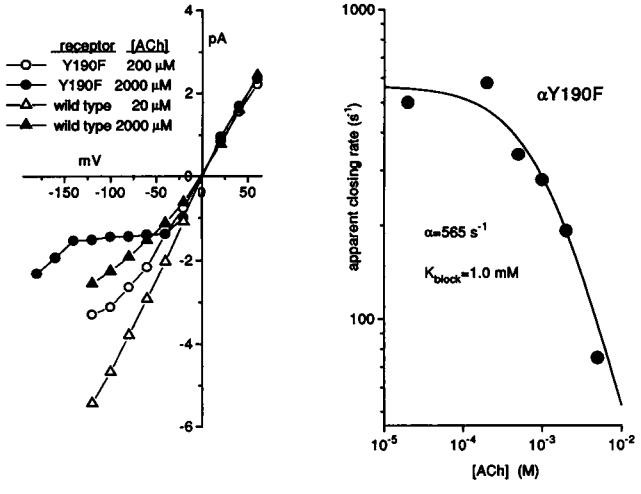

FIGURE 5 (*A*) I/V profiles for wildtype and αY190F receptors. The outward rectification at high concentrations of ACh as consistent with fast, unresolved channel block by ACh. The equilibrium dissociation constant for block obtained by fitting Eq. 1 (given in the text) 2 mM for wildtype receptors, and 1 mM for mutant receptors. (*B*) The apparent closing rate decreases with increasing concentrations of ACh in αY190F receptors because of unresolved channel. The symbols are the means of three to five patches (except at 5 mM, which is only one patch); the solid line is drawn according to the best fit to Eq. 1, with the indicated parameters.

This agreement of the blocking dissociation constant obtained by two independent measures supports the conclusion that the shortening of the open channel lifetime with increasing ACh concentration arises from channel block rather than, e.g., the increased probability of monoliganded channel openings. Very short-lived openings (<1 ms) were not observed in αY190F receptors at any ACh concentration, suggesting that the opening rate constant for monoliganded receptors is extremely low.

A change in the equilibrium dissociation constant for channel block by ACh in the mutant receptors was unexpected because the mutated residue and the site of blockade are likely to be separated by a distance of ~30–40 Å (Unwin, 1993, 1995; Valenzuela et al., 1994). The alteration in block properties in αY190F therefore suggests that there are long distance effects caused by the mutation. This result also emphasizes the fact that binding site and the pore domains are tightly linked. However, the structural changes in the pore region that are induced by the Y-to-F substitution at the binding site may be small because the ionic permeation properties of the open channel are similar in the wildtype and mutant, and because the twofold increase in equilibrium block by ACh represents a change in relative free energy of only 0.4 kcal/mol.

Other mutations at position αY190

We further examined the influence of side chain chemistry of position 190 by recording currents from aromatic (Y190W) and alkyl hydroxyl (Y190S/T) mutations. These results are summarized in Fig. 6.

For Y190W, concentrations of ACh above ~200 μM elicited clustering of currents, with the cluster P_{open} consistently less than those of αY190F currents. This difference arises from both a slower effective opening rate and a faster channel closing rate in αY190W. In five patches (0.2–2 mM ACh), the open channel lifetime of these receptors was 0.68 ± 0.15 ms; thus, the closing rate for this mutant (1464 s^{-1}) is almost 3 times faster than that of the αY190F mutant

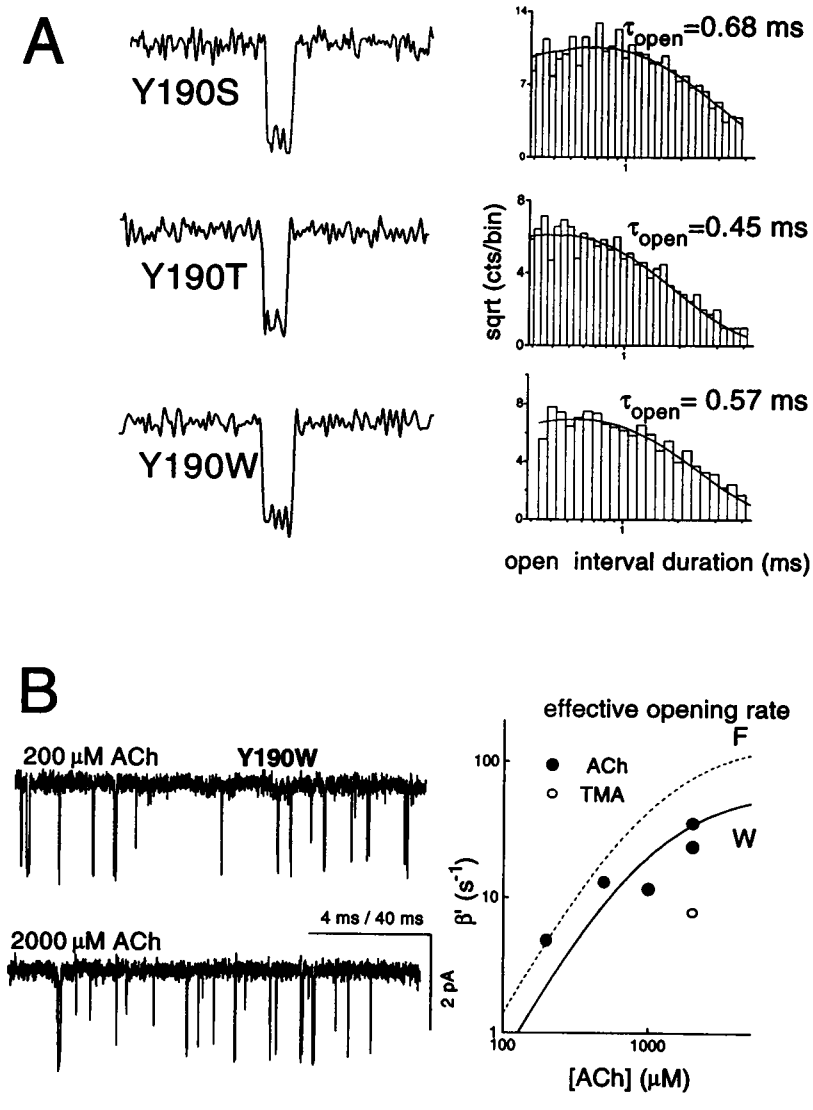

FIGURE 6 Activation properties of receptors with serine, threonine, or tryptophan substitutions at position αY190. (*A*) Examples of opening and open interval duration histograms for three mutant receptors. The closing rate constants were similar to each other, but ~3 times faster than that of the αY190F mutant. (*B*) Clusters from αY190W receptors. Closed intervals within clusters decrease in duration with increasing ACh concentration. The effective opening rate of this mutant receptor activated by ACh (●, ——) is slightly less than that of the aY190F mutant (-----; see Fig. 2).

(500 s^{-1}), and more than 6 times faster than that of the wildtype (<240 s^{-1}).

The effective opening rate of αY190W receptors increased with increasing concentration of ACh (Fig. 6, *bottom right*), but in no single patch were we able to obtain a set of rate constants that fitted the kinetics of the idealized currents and that were well defined (i.e., had parabolic likelihood surfaces). If we assume that the association and dissociation rate constants at both agonist binding sites are the same as in αY190F, then the kinetic data indicate that the receptors bearing the tryptophan mutation open at a rate of 96 s^{-1}, or about ⅔ the rate of αY190F receptors.

In one patch αY190W receptors were activated by 2 mM TMA. The currents were clustered with the effective opening rate being somewhat less than ACh-activated receptors (Fig. 6). In this patch the open channel lifetime was 0.68 ms, i.e., the same as that of ACh-activated αY190W receptors.

Fig. 6 also shows the properties of receptors having an alkylhydroxyl sidechain at position Y190. No clear clustering behavior was apparent at ACh concentrations up to 2 mM in both αY190S and αY190T receptors; this could result from a lower effective opening rate and/or lower affinity of these mutants compared with the aromatic mutations (Sine et al., 1994). The open channel lifetimes of these mutant receptors was 0.68 ms (S) and 0.47 ms (T). Thus, the closing rate with alkyl hydroxyl substitutions is about the same as that of the tryptophan substitution. The very low efficacy of activation of the alkyl hydroxyl mutations may explain failure of previous attempts to detect their activation (O'Leary and White, 1992).

DISCUSSION

Summary of results

The kinetic modeling results (Fig. 4) indicate that Y190F alters binding of ACh to closed channels. Both binding sites show reduced affinity for ACh, mostly because of a lower association rate constant. The effect is larger at the first (high affinity) site, where the association rate of the mutant

is almost 60 times slower than that of the wildtype. The opening rate of doubly liganded αY190F receptors is ~400 times lower than that of the wildtype, but the closing rate of this mutant is only twice that of the wildtype, and is the same for ACh-, CCh-, and TMA-activated receptors. The opening rate is also greatly reduced in receptors bearing other substitutions at position αY190. With regard to channel closing, the rank order was T, S,W > F> Y, with less than a 10-fold difference separating the variants. Like wildtype AChR, αY190F and W receptors undergo cycles of desensitization and resensitization. In addition, the I/V properties of Y190F receptors suggest that equilibrium channel block is enhanced ~2-fold by this mutation.

Together these results indicate that a relatively minor change in the residue at position αY190–the removal of two oxygen atoms from a molecular complex that is more than 250,000 Da in atomic mass–profoundly influences the binding, gating, and channel block properties of the receptor channel.

Comparison with previous studies

Previous studies have examined dose response (Tomaselli et al., 1991; O'Leary and White, 1992) and equilibrium binding (Sine et al., 1994) properties of αY190F. Because both binding and gating steps underlie these measurements, it was not possible to definitively ascribe effects of the mutation to altered binding or gating. In support of an effect on gating, O'Leary and White (1992) found that TMA failed to activate Y190F AChRs at concentrations up to 10 mM, but retained its potency in competitively inhibiting activation by ACh. Our measurements, however, show clear activation of αY190F by TMA, and Sine et al. (1994) found that Y190F produced a large decrease in TMA binding affinity. Sine et al. (1994) measured equilibrium ACh binding and, by combining their observations with dose response results (Tomaselli et al., 1991), concluded that αY190F receptors had a 100-fold decreased binding affinity to activatable states. This value is greater than our estimate because, in the absence of single-channel kinetic data, they assumed that there were no changes in gating as a consequence of the mutation.

Our results show that the mutation causes both affinity decreases (2–35-fold) for activatable states and a slower opening rate constant (400-fold). When these changes in both binding and gating are taken into account, the dose response measurements of Tomaselli et al. (1991) and the equilibrium binding measurements of Sine et al. (1994) are well described (Fig. 2).

Relative free energies

The differences in the activation kinetics of wildtype and αY190F receptors may be interpreted in terms of the differences in free energies ($\Delta\Delta G$) for each step of the activation sequence (Fig. 7):

$$C \underset{0.3}{\overset{2.4}{\rightleftharpoons}} AC \underset{1.4}{\overset{1.8}{\rightleftharpoons}} A_2C \underset{-0.4}{\overset{3.5}{\rightleftharpoons}} A_2O$$

where the numbers are $\Delta\Delta G^{\ddagger}$ values for each reaction step, in kcal/mol.

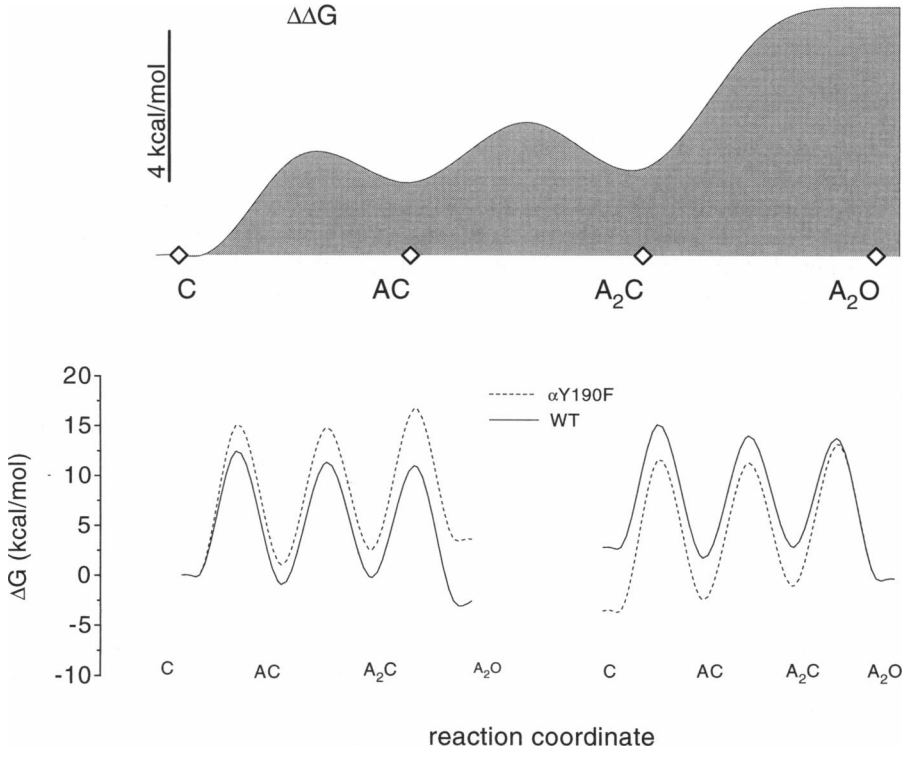

FIGURE 7 Relative free energy profiles of wildtype and αY190F receptors activated by ACh. (*Top*) The differences in relative free energy ($\Delta\Delta G^{\ddagger}$, in kcal/mol) are shown for each step and state of the activation reaction. In mutant receptors there is a ΔG_0 of 2.1 kcal/mol after the first binding step, with only an additional 0.4 kcal/mol difference after the second. In both binding steps the barriers to entry and exit of the agonist are increased by the mutation. The largest energy difference occurs upon opening, as doubly liganded mutant receptors must surmount an excess barrier of 3.5 kcal/mol to reach the transition state. In the open conformation, mutant receptors are only slightly (0.4 kcal/mol) less stable than the mutant. Overall, there is a 6.4 kcal/mol difference between the mutant and the wildtype receptor. (*Bottom*) Alternative alignments of ΔG profiles. (*Left*) The ground states (C) of the wildtype and the mutant receptors are assumed to be equal. (*Right*) The A_2O states of the wildtype and the mutant receptors are assumed to be equal. Both interpretations are consistent with the dose response, kinetic, and equilibrium binding results.

First we consider the effects of the Y-to-F mutation on the binding steps. At the first (high affinity) binding site, which may be the $\alpha(\delta)$ site (Zhang et al., 1995), the association rate slows to a much greater extent than does the dissociation rate, leading to a relative destabilization of the AC state by 2.1 kcal/mol. At the second (low affinity) binding site the mutation again slows both association and dissociation, and here the barriers increase rather symmetrically so that the relative destabilization of the A_2C state is minimal, only 0.4 kcal/mol. In terms of equilibrium binding constants, the mutation results in a $\Delta\Delta G_0$ of ~2.5 kcal/mol from binding to the closed receptor.

The effects of the mutation are most significant at the gating step. Compared with wildtype receptors, the energy barrier to opening is ~3.5 kcal/mol higher in αY190F receptors. However, the extra energy required for closing is only ~0.4 kcal/mol (assuming a closing rate of 500 s^{-1} in the mutant; Figs. 3 and 5). Thus, $\Delta\Delta G_o$ of the gating step increases by ~3.9 kcal/mol in the mutant. Considering the equilibrium of the full reaction (C to A_2O), the $\Delta\Delta G_0$ is ~6.4 kcal/mol.

Several aspects of the $\Delta\Delta G^{\ddagger}$ profile can be emphasized. First, the consequences of the mutation are significantly greater at the high affinity binding site than at the low affinity site. Second, the barriers to association and dissociation are larger in the mutant than in the wildtype. In αY190F receptors binding and unbinding each incur an extra energy penalty of ~1.5 kcal/mol. Third, more than half of the total energy difference induced by the mutation occurs at the channel opening transition, with rather little extra energy penalty accompanying full residence in the open state.

Nature of the mutation

Although we were able to obtain estimates of the $\Delta\Delta G_0$ for the entire reaction only for the phenylalanine substitution, some information is available about receptors with a tryptophan, threonine, or serine sidechain at position α190. With regard to the closing rate constant, the rank order is S, T, W > F > Y in the approximate ratio 6:2:1. In energetic terms, the $\Delta\Delta G^{\ddagger}$ for closing is −1.2 kcal/mol for the S, T, and W mutants compared with the wildtype, and that of the F mutant is −0.4 kcal/mol. With regard to the opening rate constant, we can conclude that the rank order is the inverse of that of the closing rate constant, i.e., Y ≫ F = W > S, T. There may be relatively little energy difference among the mutants; while the Y-to-F substitution results in a $\Delta\Delta G^{\ddagger}$ of opening of 3.5 kcal/mol, the F-to-W substitution may only result in an additional loss of 0.2 kcal/mol to this process.

The results indicate that with regard to closing, the nature of the sidechain is relatively unimportant. A modicum of stability, ~1 kcal/mol, is provided by the benzene ring of the Y and F sidechains. With regard to opening, we conclude that only a Y can support a fast opening rate, i.e., neither the aromatic nor the hydroxyl moieties of the sidechain alone can be thought of as the key elements for this reaction.

The αY190F mutant closing rate was the same for all three agonists we tested (ACh, CCh, and TMA), and the αY190W receptor closing rate was the same for ACh- and TMA-activated receptors. This result indicates that not only is the nature of the sidechain at position 190 relatively unimportant with regard to channel closing, but the ester portion of the agonist molecule is also relatively unimportant. A similar conclusion was reached for these three agonists for the wildtype receptors (Zhang et al., 1995).

Interpretation of ΔΔG

Kinetic experiments only concern relative free energy differences, i.e., we have no absolute reference by which to align the wildtype and mutant ΔG profiles. Thus, considering the entire reaction, the kinetic and binding results cannot distinguish whether a mutation raises the relative free energy of the A_2O state, or lowers the relative free energy of the C state, or both. This distinction is illustrated in Fig. 7 (*bottom*), where the solid and dashed lines pertain to the activation kinetics of wildtype and αY190F receptors, respectively. The curves at the left have been aligned at the C state, i.e., the ground states of the wildtype and mutant systems are assumed to be of equal energy. In this example, the mutation raises the free energy of the A_2O state by 6.4 kcal/mol. In Fig. 7 (*bottom right*) the free energy curves have been aligned at the A_2O state, i.e., the liganded-open forms of the wildtype and mutant systems are assumed to be of equal energy. Here, the mutation lowers the free energy of the A_2C state by 3.9 kcal/mol (and that of the ground state, C, by 6.4 kcal/mol). Our experimental results do not allow us to distinguish between these interpretations. Both views are completely consistent with all of the available observations, i.e., they predict a 2.5 kcal/mol loss of binding energy at the two sites and the observed single-channel kinetic, dose response, equilibrium binding, and $\Delta\Delta G$ profiles.

Gating mechanisms

The ambiguity in the absolute positioning of the $\Delta\Delta G$ profile leads us to propose two different mechanisms by which αY190F influences channel gating.

It is possible that mutant receptors open with a lower probability than the wildtype because of altered agonist-receptor contacts. ACh binds more tightly to the open channel conformation than to the closed conformation, and it has been proposed that gating is driven by differences in the ligand-protein interaction free energy between the closed and open receptor structures (Jackson, 1989). In equilibrium terms, for the Y190F receptor the $\Delta\Delta G_0$ of ~4 kcal/mol at the gating step may be considered to arise from less favorable ligand-protein interactions in the higher affinity, open

conformation. Quantitatively, the total loss of relative free energy (at two sites, to both open and closed structures) of 6.4 kcal/mol from binding contacts alone is reasonable; a tyrosine-phenylalanine substitution in tyrosyl tRNA synthetase, an enzyme-substrate system that has evolved specifically to distinguish between these two sidechains, results in a loss of ~6.3 kcal/mol at one site (Fersht, 1985). The following interpretation of the Y190F mutation is logical: ~2.5 kcal/mol is lost in binding the closed receptor (2.1 kcal/mol to the high affinity site and 0.4 kcal/mol to the low affinity site), with an additional 3.9 kcal/mol lost from altered ligand-protein contacts in the transition to the open channel configuration, perhaps at both sites. As we observed for the closed receptor, the Y190F mutation may alter the ligand-receptor contacts in the open receptor to different extents at each of the two binding sites. This ligand-driven view of the gating process is appealing in its simplicity: all of the relative free energy changes induced by the mutation can be attributed to a disruption in binding interactions at the receptor site.

It is also possible that mutant receptors open with a lower probability than the wildtype because of changes in the overall structure of the binding site. As discussed above, we can equivalently explain our kinetic results by assuming that the mutation at position 190 lowers the free energy of the A_2C state (with little effect on the height of the barrier that separates the A_2C and the A_2O structures). According to this view, free energy for gating is normally provided by the intrinsic fold of the protein, and some of this energy is lost as a consequence of the Y-to-F substitution. For example, "tension" in the resting receptor's binding site could be coupled to a "relaxation" somewhere else in the protein (perhaps the pore region) so that the unliganded channel remains closed. Binding an agonist could shift the equilibrium between the coupled structures toward one where the binding site is "relaxed" and the coupled region "tense," thus increasing the probability of an open channel conformation. In this scenario energy from both the ligand-protein interaction and that stored in the protein fold drives gating. The following interpretation of αY190F is entirely consistent with our results: the mutation alters the folding of the protein so as to stabilize the ground state of the receptor (by up to 3.9 kcal/mol), and an additional ~2.5 kcal/mol are lost in binding the closed receptor (2.1 kcal/mol to the high affinity site and 0.4 kcal/mol to the low affinity site). Reduced association and dissociation rate constants in αY190F receptors would be expected if such a stabilization corresponded to a more sterically restricted binding pocket, i.e., a structure that imposes increased entry and exit barriers to the agonist.

These two hypotheses of the mechanism by which residue αY190F influences gating are not mutually exclusive. The activation reaction may be powered by both the free energy of ligand binding and by the intrinsic free energy contained in the protein fold. It is likely that both agonist-receptor contacts and intra-protein interactions at the binding site and elsewhere combine to determine the magnitude of the gating rate constants.

Structural interpretations

Our result indicate that both binding and gating are altered in αY190F. Of the total $\Delta\Delta G_0$ of the reaction, a little more than one-third is lost in the binding interaction of ACh with closed receptors. This observation is consistent with the idea that αY190 is in close contact with ACh, and that the interaction with the phenolic hydroxyl of the sidechain contributes to this binding. The loss in binding energy, however, is unequally distributed between the two binding sites, with more than 80% lost from the high affinity (possibly $\alpha(\delta)$) site as a consequence of the mutation. This suggests that the nature of the sidechain-agonist contact at position α190 is different at the two binding sites. Perhaps the high affinity site is more structurally constrained and therefore more sensitive to the effects of mutation.

If ACh indeed binds to residue 190, and if contact with the ligand is weaker in αY190F receptors, then the escape of ACh from this docking site does not determine the rate of dissociation from the receptor. If this were the case we would expect an asymmetric effect on binding, with a slower association rate constant and a faster dissociation rate constant in the mutant. That both association and dissociation are slower in αY190F receptors is most simply accounted for by proposing that there is an increase in the height of a barrier to both the entry and exit of the agonist, which in turn suggests that there is a change in the overall structure of the binding pocket as a consequence of the mutation.

The kinetic results demonstrate that it is the rate constant of channel opening, and not the rate constant of channel closing, that is greatly diminished in the mutant. The asymmetry in the effects of the mutation on gating kinetics reflects an asymmetry in the role played by residue 190 in the transition of the receptor between closed and open structures. It is possible that agonist- or protein-protein interactions with the hydroxyl of residue 190 serve to increase the opening rate constant when the binding site is in the closed-channel (low affinity) conformation, and that these interactions are relatively unimportant to channel closing when the binding site is in its open-channel (high affinity) conformation. Once open, other interactions become more important determinants of the closing rate constant. These interactions may be between the agonist and the benzene ring of 190, between the agonist and other residues at the binding site, and/or between sidechains both within and distant from the binding sites.

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