Binding sites contribute unequally to the gating of mouse nicotinic αD200N acetylcholine receptors

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1. Single channel currents were recorded from HEK 293 cells expressing recombinant mouse adult ($\alpha_2\beta\delta\epsilon$) and embryonic ($\alpha_2\beta\delta\gamma$) acetylcholine receptors (AChRs) containing a mutation at residue D200 of the α-subunit. Rate and equilibrium constants for AChR activation were estimated from open and closed times obtained over a range of ACh concentrations.

2. Mutation of αD200 to asparagine (αD200N) dramatically slows the rate constant of channel opening, with adult AChRs slowing 100-fold and embryonic AChRs slowing 400-fold. The rate constant of channel closing increases 3-fold, resulting in a decrease of the gating equilibrium constant of up to 1200-fold. In contrast to channel gating steps, ACh-binding steps are only modestly effected by αD200N.

3. Introduction of a potential glycosylation site in αD200N cannot account for the effect on channel gating because eliminating the consensus for glycosylation with the mutation αD200N + T202V fails to restore efficient gating. Gating is similarly impaired with the substitutions of E, K and Q at position α200.

4. The agonists carbamylcholine and tetramethylammonium also activate the αD200N AChR, but with channel opening rates even slower than with ACh. The agonist dependence of the opening rate constant is similar in αD200N and wild type AChRs.

5. AChRs containing D200N at just one of the two α-subunits show either small or large changes in the gating equilibrium constant, presumably due to the presence of the mutation at either the $\alpha\delta$ or $\alpha\epsilon/\alpha\gamma$ sites. The changes in free energy of channel gating show that the contribution of each binding site is nearly independent. However, the sites do not contribute equally to gating, as an αD200N mutation at the $\alpha\epsilon$ or $\alpha\gamma$ binding site slows channel opening relatively more than at the $\alpha\delta$ site.

Nicotinic acetylcholine receptors (AChRs) mediate synaptic transmission at central and peripheral synapses. AChRs from vertebrate skeletal muscle and homologous tissues have the subunit composition $\alpha_2\beta\gamma\delta$ (embryonic) or $\alpha_2\beta\epsilon\delta$ (adult). Within the AChR pentamer are two binding sites generated by $\alpha\delta$- and either $\alpha\gamma$- or $\alpha\epsilon$-subunit pairs (Blount & Merlie, 1989; Pedersen & Cohen, 1990; Sine & Claudio, 1991). Efficient opening of the AChR channel requires occupancy of both binding sites by agonist, where each site contributes to opening by binding agonist more tightly in the open state. In the resting state, the two sites bind agonist with different affinities in *Torpedo* and embryonic mouse AChRs (Sine, Claudio & Sigworth, 1990; Zhang, Chen & Auerbach, 1995), but in adult mouse AChRs the affinities of the two binding sites are essentially equivalent (Akk & Auerbach, 1996). Jackson (1989) suggested that the binding sites can make different contributions to channel gating depending on the relative affinities for agonist in the resting and open channel states. The experiments described herein combine mutagenesis with single channel recording to examine the contribution of each binding site to the gating reaction.

Affinity labelling and mutagenesis studies have identified residues in three regions of the α-subunit that contribute to stabilization of bound agonist (reviewed in Changeux, Galzi, Devillers-Thierry & Bertrand, 1992). Residues in one of these regions include Y190, C192, C193 and Y198, which are conserved in all α1-subunits. Also in this region is an aspartic acid residue at position 200; aspartic acid is found in an equivalent position in all α1-subunits, as well as in most neuronal α-subunits (Fig. 1). Dose–response measurements of AChRs with an aspartate to asparagine mutation at this position (αD200N) revealed lower apparent affinity, but suggested that D200 contributes to agonist efficacy rather than to agonist affinity (O'Leary & White, 1992). The experiments described here use patch clamp to resolve individual AChR gating events, and show that αD200N affects agonist efficacy by greatly slowing channel opening.

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By introducing D200N into just one of the two α-subunits, we show that occupancy of each binding site contributes independently to the channel opening reaction, but that the contributions are not equal for each site.

METHODS

Construction and expression of AChR

Mouse AChR subunit cDNAs were generously provided by Drs John Merlie and Norman Davidson, and were subcloned into the CMV-based expression vector pRBG4 as described in Sine (1993). Mutations of αD200 were constructed by introducing a unique SnaBI site into α-pRBG4, and bridging from an intrinsic Dra III site to the synthetic SnaBI site using synthetic double-stranded oligonucleotides harbouring the desired mutations. All constructs were confirmed by restriction mapping and dideoxy sequencing.

Human embryonic kidney (HEK) 293 cells were transiently transfected by calcium phosphate precipitation (Ausubel *et al.* 1992). A total of 3·6 μg of DNA per 35 mm culture dish in the ratio 2 : 1 : 1 : 1 ($\alpha:\beta:\delta:\epsilon/\gamma$) was used. The medium was changed 24 h after the start of transfection and 48 h later electrophysiological recordings were started.

Electrophysiology and kinetic analysis

Electrophysiology was performed using the patch clamp technique in the cell-attached configuration (Hamill, Marty, Neher, Sakmann & Sigworth, 1981). The bath solution was Dulbecco's phosphate-buffered saline (PBS; mM): 137 NaCl, 0·9 $CaCl_2$, 2·7 KCl, 1·5 KH_2PO_4, 0·5 $MgCl_2$ and 6·6 Na_2HPO_4, pH 7·3. The pipette solution was buffered to pH 7·4 with 10 mM Hepes and contained (mM): 115 NaCl, 1 $CaCl_2$ and 2 KCl. The interior of the patch pipette was held at +70 mV; assuming a reversal potential of 0 mV and a conductance of either 70 pS (adult AChRs) or 45 pS (embryonic AChRs), we estimate that the membrane potential was ~−100 mV. The temperature was 22 °C. The pipette contained the indicated concentration of acetylcholine, carbamylcholine, or tetramethylammonium (Sigma).

Currents were recorded with an Axopatch-1B amplifier, low-pass filtered at 20 kHz (8-pole Bessel) and stored on videotape in digital format (Instrutech VR-10). For single channel kinetic analysis, the current record was transferred to a PC via a digital interface (Instrutech VR111) at a sampling frequency of 94 kHz. Clusters of channel openings from individual AChRs were elicited by high ACh concentrations (Sakmann, Patlak & Neher, 1980). To identify clusters, the channel traces were digitally filtered (Gaussian filter; half-power frequency, 2 kHz) and channel openings were detected with a half-amplitude criterion using the program IPROC (Sachs, 1983). Clusters were defined as a series of openings separated by closed intervals shorter than some critical duration (τ_{crit}), the value of which depended on the type of AChR and concentration of agonist. τ_{crit} ranged from 5 to 500 ms, but was always at least four times longer than the slowest component of closed intervals within clusters. Once defined, the time constant of the longest closed interval component within clusters was measured and further analyses proceeded only if this time constant was at least four times shorter than τ_{crit}.

An apparently homogeneous population of clusters was selected with regard to the mean amplitude, and open and closed interval lifetime using the program LPROC (Neil, Xiang & Auerbach, 1991). Clusters with overlapping currents were rejected. Approximately 90% of all clusters were selected by these criteria for further analysis when both α-subunits of the AChR were either wild type or mutant. For hybrid AChRs containing one wild type and one mutant α-subunit, sometimes another population of clusters was selected after changing the value of τ_{crit}. All selected clusters were plotted and examined by eye.

The selected clusters (typically, 20 per patch) were extracted at full bandwidth and were again idealized with a half-amplitude method after low-pass filtering (typically, at a half-power frequency of 7 kHz). The intracluster open and closed durations were obtained by fitting sums of exponentials to interval duration histograms. The open and closed durations shown in the figures are the slowest component of each of these distributions. The effective closing rate (α') is the inverse of this open duration, and the effective opening rate (β') is the inverse of this closed duration.

The following kinetic scheme (Magleby & Stevens, 1972; Colquhoun & Hawkes, 1977) was used for single channel kinetic analyses:

$$C \underset{k_{-1}}{\overset{Ak_{+1}}{\rightleftharpoons}} AC \underset{k_{-2}}{\overset{Ak_{+2}}{\rightleftharpoons}} A_2C \underset{\alpha}{\overset{\beta}{\rightleftharpoons}} A_2O,$$

Model 1

Mouse	1	IKEARGWKHW	VFYS**CC**PTTP	YL**D**ITYHFVM	QRLPLYFIVN	VII**P**CLLFSF
Bovine	1	IKESRGWKHW	VFYA**CC**PSTP	YL**D**ITYHFVM	QRLPYLFIVN	VII**P**CLLFSF
Human	1	IKESRGWKHS	VTYS**CC**PDTP	YL**D**ITYHFVM	QRLPLYFIVN	VII**P**CLLFSF
Chicken	1	MKDYRGWKHW	VYYA**CC**PDTP	YL**D**ITYHFLM	QRLPLYFIVN	VII**P**CLLFSF
Torpedo	1	MKDYRGWKHW	VYYT**CC**PDTP	YL**D**ITYHFIM	QRIPLYFVVN	VII**P**CLLFSF
Xenopus	1	MKDYRCWKHW	VYYT**CC**PDKP	YL**D**ITYHFVL	QRLPLYFIVN	VII**P**CLLFSF
Drosophila	1	IMRVPAVRNE	KFYS**CC**_EEP	YL**D**IVFNLTL	RRKTLFYTVN	LII**P**CVGISF
Mongoose	1	KEARGWKHN	VTYA**CC**LTTH	YL**D**ITYHF		
Snake	1	KDYRGFWHS	VNYS**CC**LDTP	YL**D**ITYHFIL	LRLPLYFIVN	VII**P**C
Mouse	7	LMGIPGKRNE	KFYE**CC**_KEP	YP**D**VTYTVTM	RRRTLYYGLN	LLI**P**CVLISA
Rat	2	IINATGTYNS	KKYD**CC**_AEI	YP**D**VTYYFVI	RRLPLFYTIN	LII**P**CLLISC
Rat	3	IIKAPGYKHE	IKYN**CC**_EEI	YQ**D**ITYSLYI	RRLPLFYTIN	LII**P**CLLISF
Rat	4	IVDAVGTYNT	RKYE**CC**_AEI	YP**D**ITYAFII	RRLPLFYTIN	LII**P**CLLISC
Rat	5	IMSAMGSKGN	RTDS**CC**_WYP	Y__ITYSFVI	KRLPLFYTLF	LII**P**CIGLSF
Rat	6	IVDASGYKHD	IKYN**CC**_EEI	YT**D**ITYSFYI	RRLPMFYTIN	LII**P**CLFISF
Rat	7	LMGIPGKRNE	KFYE**CC**_KEP	YP**D**VTYTVTM	RRRTLYYGLN	LLI**P**CVLISA
Rat	9	VHGMPAVKNV	ISYG**CC**_SEP	YP**D**VTFTLLL	KRRSSFYIVN	LLI**P**CVLISF

Figure 1

Sequence alignments of muscle type α-subunits (α1) from different species, and of mouse and rat neuronal type α-subunits. Vicinal cysteines, D200 and a conserved proline in M1 are highlighted.

where A is the concentration of agonist, C is a closed AChR, O is an open AChR, k_{+1} and k_{+2} are the agonist association rate constants, k_{-1} and k_{-2} are the agonist dissociation rate constants, β is the channel opening rate constant, and α is the channel closing rate constant. At agonist concentrations where the binding sites are always occupied, the activation reaction (Model 1) reduces to a single, closed–open step: $A_2C \rightleftharpoons A_2O$. Thus, at sufficiently high concentrations of agonist the predominant closed intervals within clusters approaches the inverse of the opening rate constant, β (closed times corresponding to channel block were rarely detected because of the limited analysis bandwidth). The β' *vs.* concentration (A) curves were fitted by the Hill equation (eqn (1)) in order to estimate β:

$$y = max/(1 + (EC_{50}/A)^n), \quad (1)$$

where y is the effective opening rate, max is β, n is the Hill coefficient and EC_{50} is the concentration yielding $max/2$. Rate constants of the activation reaction were estimated using an interval maximum likelihood method that employed a correction for missed events (Qin, Auerbach & Sachs, 1996). Idealized currents from several patches through a range of agonist concentrations were combined (Auerbach, 1993). An approximately equal number of intervals from each concentration (typically, 2000) was used. After fitting, error limits were estimated as 0·5 likelihood intervals (Colquhoun & Sigworth, 1983). Interval duration histograms and dose–response curves were calculated from the rate constants for comparison with the experimental data.

The probability of being open within a cluster (P_{open}) *vs.* concentration can be described by eqn (1), with $y = P_{open}$. Also, according to Model 1:

$$P_{open} = ((K_{d1}K_{d2}/A^2\Theta) + (K_{d2}/A\Theta) + (1/\Theta) + 1)^{-1}, \quad (2)$$

where K_{d1} and K_{d2} are the equilibrium dissociation constants at the two binding sites, and Θ is the gating equilibrium constant (β/α). If the two binding sites have similar association and dissociation rate constants for ACh (Akk & Auerbach, 1996), Model 1 simplifies to:

$$C \underset{k_-}{\overset{2Ak_+}{\rightleftharpoons}} AC \underset{2k_-}{\overset{Ak_+}{\rightleftharpoons}} A_2C \underset{\alpha}{\overset{\beta}{\rightleftharpoons}} A_2O,$$

Model 2

where k_+ is the association rate constant to each binding site, and k_- is the dissociation rate constant from each binding site, and the apparent equilibrium dissociation constant at each site is K_d (equal to k_-/k_+). Accordingly, the equilibrium dissociation constant K_d can be estimated by fitting P_{open} *vs.* concentration curves to give:

$$P_{open} = ((K_d^2/\Theta A^2) + (2K_d/\Theta A) + (1/\Theta) + 1)^{-1}. \quad (3)$$

RESULTS

αD200N adult AChRs activated by ACh

To determine the kinetic steps affected by αD200N, we recorded single channel currents in the presence of a range of concentrations of ACh from HEK 293 cells transiently expressing αD200N adult AChRs. At intermediate and high ACh concentrations, channel events appeared in clusters, or a series of closely spaced openings due to a single channel, separated by prolonged closed periods due to desensitization. At all concentrations of ACh, the presence of αD200N dramatically decreased the probability that a channel is open within a cluster (P_{open}; Fig. 2). The EC_{50} of the open probability was increased 3-fold by the mutation, but the major effect was to decrease the maximum open probability from 0·98 for wild type to 0·13 for αD200N. Analysis of open and closed duration histograms showed that the decrease in maximum P_{open} was due primarily to a 100-fold decrease in the channel opening rate constant (Table 1). The

Table 1. Dose–response parameters of wild type and αD200N ACh receptors

	Adult		Embryonic	
	Wild type	αD200N	Wild type	αD200N
P_{open} (eqn (1))				
$P_{open,max}$	0·98	0·13	0·99	0·12
EC_{50} (μM)	16	73	9·4	18
n	1·7	2·8	1·6	2·9
P_{open} (eqn (3))				
K_d (μM)	102	33	—	11
Θ	47	0·13	—	0·17
β' (eqn (1))				
β'_{max} (s^{-1})	55000	535	60000	153
EC_{50} (μM)	377	116	—	33
n	1·6	1·5	—	1·6

Adult receptors were expressed by transfecting with $\alpha\beta\delta$ and ϵ subunits. Embryonic receptors were expressed by transfecting with $\alpha\beta\delta$ and γ subunits. Receptors were activated by ACh. The values of maximum P_{open} ($P_{open,max}$), EC_{50} and the Hill coefficient (n) were obtained by fitting eqn (1), and those of K and Θ were obtained by fitting to eqn (3). The maximal β' values (β'_{max}) for wild type receptors are from previous results (see Table 2).

channel closing rate constant increased 3-fold, so the overall gating equilibrium constant, β/α, decreased 350-fold due to the presence of αD200N.

Rate constants governing agonist association and dissociation were estimated by fitting Model 2 to single channel open and closed durations obtained over a range of ACh concentrations (10–200 μM; Table 2). The presence of αD200N decreased the rate of ACh dissociation 3-fold, with no change in the rate of association. Thus, the affinity of the αD200N AChR for ACh actually increased 3-fold. The

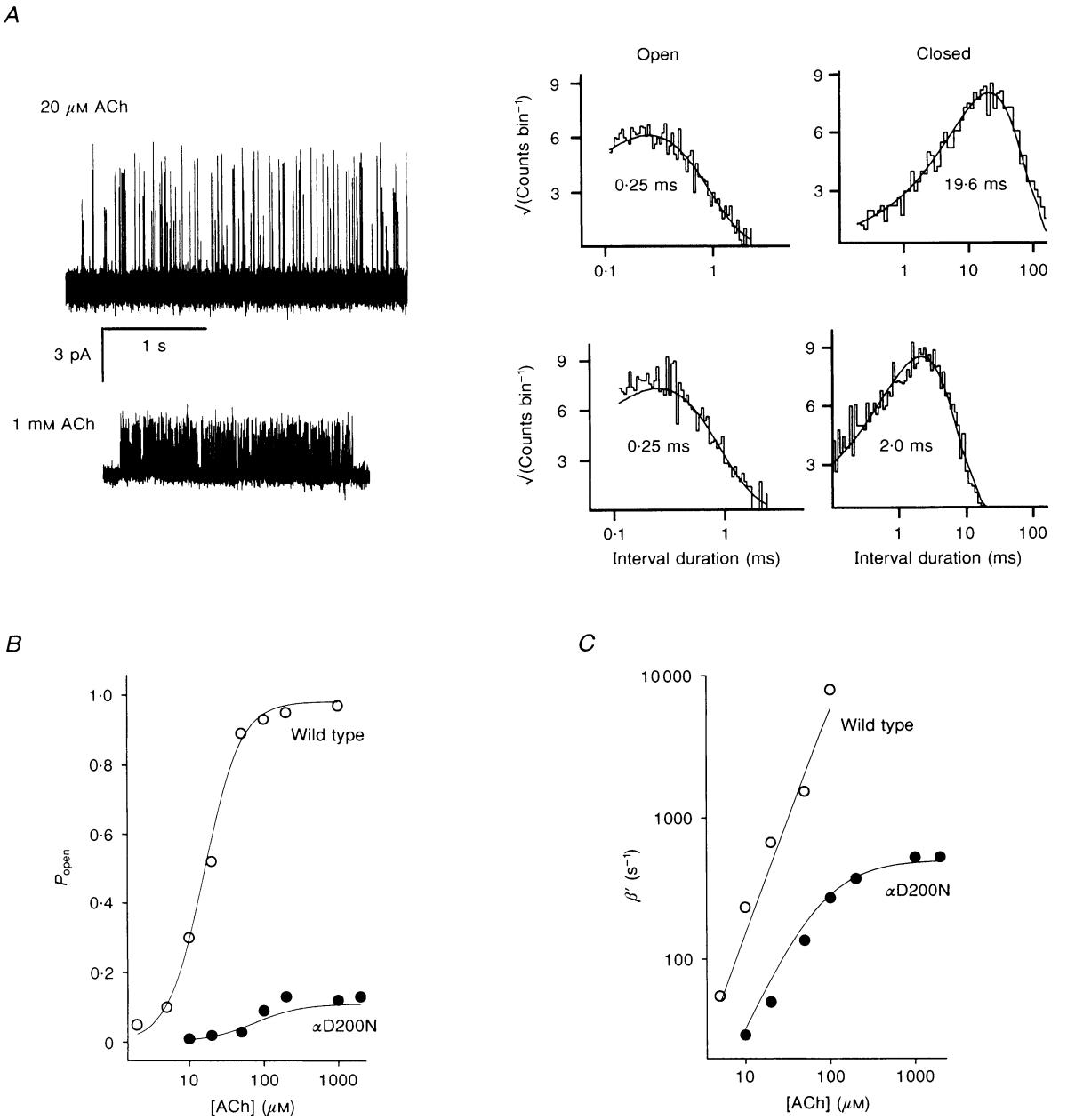

Figure 2. Single channel currents and the activation properties of adult αD200N receptors

A, example clusters and the corresponding open and closed interval duration histograms at different ACh concentrations. The membrane potential was −100 mV; inward current is up. Channel block by agonist reduced the amplitude at higher concentrations. Times are the characteristic time constants of the slowest component of the intracluster interval duration histograms. *B*, dose–response curves for wild type and αD200N mutant receptors. The probability of a channel being open within a cluster (P_{open}) is plotted as a function of the ACh concentration. αD200N receptors cannot open as efficiently as wild type receptors. *C*, effective opening rate of wild type and αD200N receptors. Each symbol is calculated as an inverse of the slowest component in the closed duration histograms. αD200N receptors opened ~100 times more slowly than wild type receptors. In both *B* and *C*, each symbol is one patch (3–69 clusters), and the continuous lines are calculated from the rate constants shown in Table 2. The optimal dose–response parameters are given in Table 1.

overall results show that the dominant effect of mutating αD200 was to impair channel gating.

αD200N embryonic AChRs activated by ACh

To characterize further the contribution of residue D200 to channel gating, single channel curents were recorded from embryonic AChRs containing αD200N. As observed for adult AChRs, the primary effect of αD200N was to decrease the open probability by slowing the rate of channel opening (Fig. 3, Table 2). The opening rate constant decreased 400-fold, an effect even more pronounced than for adult AChRs. The closing rate constant again increased about 3-fold in the presence of αD200N, giving an overall decrease in the gating equilibrium constant of 1260-fold.

Rate constants governing ACh association and dissociation were again examined by kinetic analysis of open and closed durations obtained over a range of ACh concentrations. For wild type mouse embryonic AChRs, ACh bound to the two sites with distinct affinities of 21 and 675 μM (Zhang *et al.* 1995). When αD200N was present together with the embryonic complement of subunits, ACh binding affinities

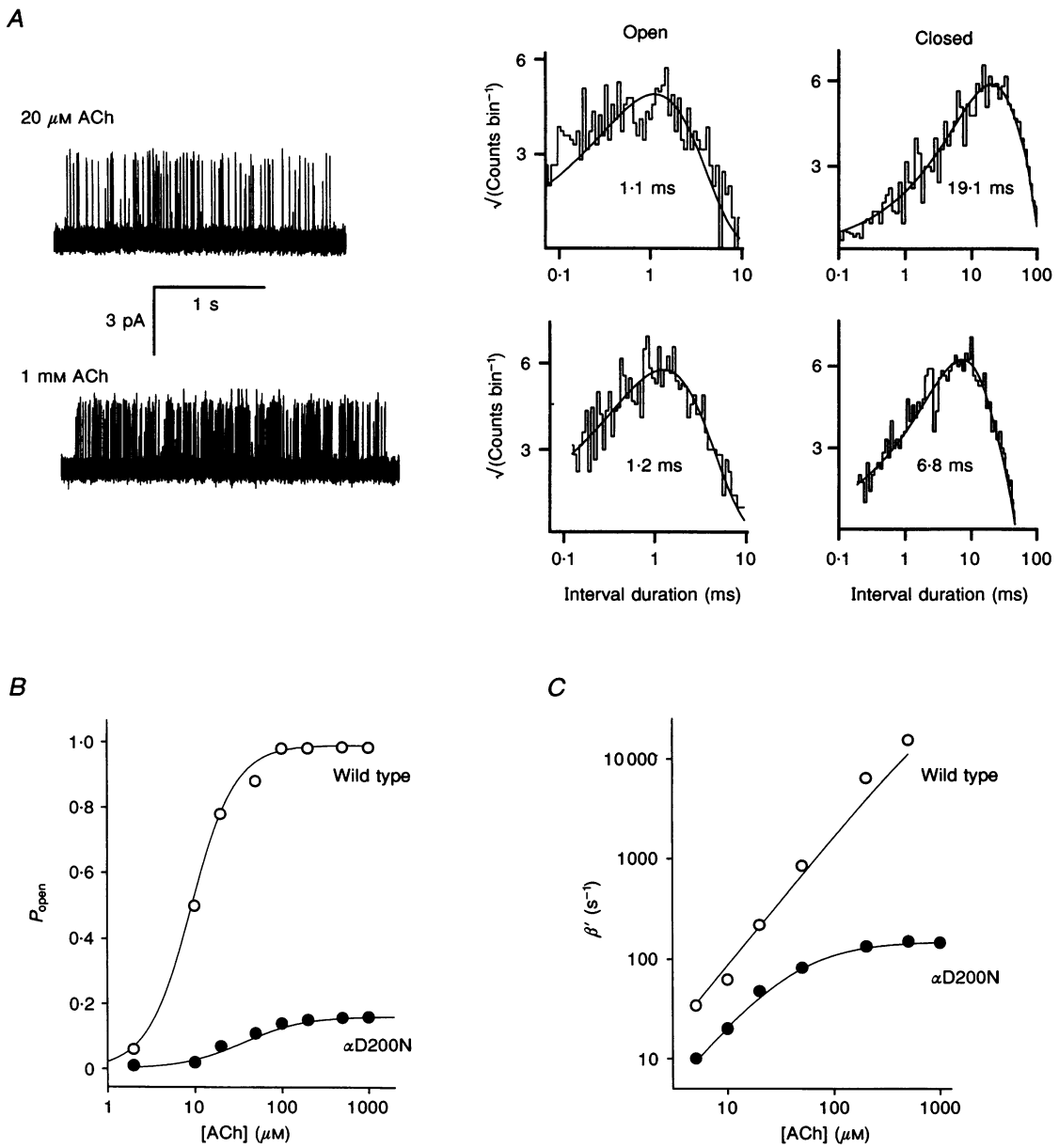

Figure 3. Single channel currents and the activation properties of embryonic αD200N receptors

A, example clusters and the corresponding open and closed interval duration histograms at different ACh concentrations. The membrane potential was −100 mV; inward current is up. *B*, the probability of being open within a cluster (P_{open}) is plotted as a function of agonist concentration. *C*, effective opening rate of wild type and αD200N mutant receptors. Mutant receptors opened ~400 times more slowly than wild type receptors. Each symbol is one patch (6–43 clusters per patch), and the continuous lines are calculated from the rate constants shown in Table 2.

Table 2. Activation rate constants for wild type and αD200N receptors

	k_{+1} (μM s^{-1})	k_{-1} (s^{-1})	k_{+2} (μM s^{-1})	k_{-2} (s^{-1})	β (s^{-1})	α (s^{-1})
Adult						
Wild type	—	16540	169	—	55000*	1150
αD200N	—	5225	160	—	535	4025
Embryonic						
Wild type†	29	616	40	27000	60000	240
αD200N	—	1496	88	—	152	780

Adult receptors are those expressed with $\alpha\beta\delta$ and ϵ subunits. Embryonic receptors are those expressed with $\alpha\beta\delta$ and γ subunits. All rates are from a kinetic analysis of ACh-activated single channel currents, using Model 2 or Model 1 (embryonic wild type receptors). Some values are from previous results (*Sine *et al.* 1995; †Zhang, Chen & Auerbach, 1995).

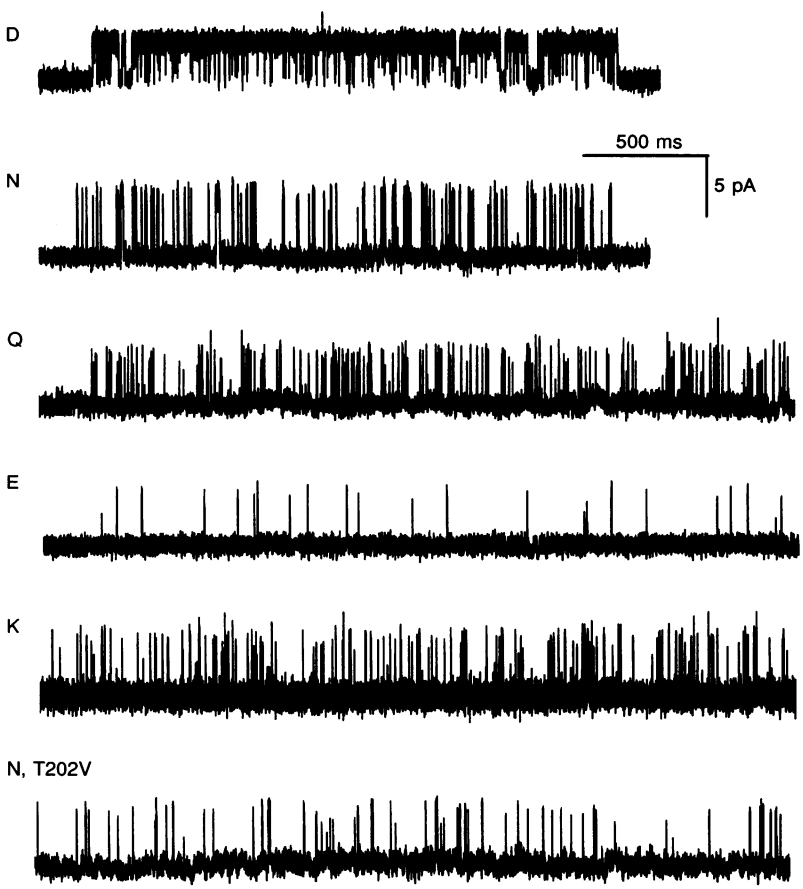

Figure 4. Example clusters of receptors with different side-chains at positition α200

The receptors are embryonic (co-expressed with γ subunit). Replacement of D (wild type) by N, Q, E, or K caused a substantial decrease in the probability of the channel being open. The closed intervals in the mutant traces were longer than in the wild type because these channels had a smaller opening rate constant. Introduction of a potential glycosylation site in αD200N cannot account for the effect on channel gating because eliminating the consensus for glycosylation with the mutation αD200N + T202V failed to restore efficient gating. All clusters were elicited by 100 μM ACh.

were no longer distinct at the two sites (Table 2); ACh bound to each site with a dissociation constant of 17 μM. Thus, αD200N appeared to alter the structure of the low affinity binding site so that it became similar to the high affinity site. Although unchanged in overall affinity, the high affinity site was also altered by αD200N, as both the association and dissociation rate constants increased about 3-fold.

The effect of D200N is not due to glycosylation

Because residue α202 is a threonine, introducing N at position 200 creates a potential glycosylation site (NxT). To determine whether glycosylation can account for the observed changes in channel gating, we made the double mutant αD200N + T202V and recorded single channel currents. Figure 4 shows that the effective opening rate at high ACh concentrations was still decreased for the mutant lacking the consensus signal for glycosylation. Thus the presence of a potential glycosylation site in αD200N cannot account for the decreased efficiency of channel gating.

Other αD200 mutations

We examined the side-chain dependence of residue 200 by substituting glutamate (E), glutamine (Q) and lysine (K). The three mutant α-subunits were expressed at high levels similar to αD200N. In all three of these mutants, both the open probability and opening rate were low in the presence of 100 μM to 1 mM ACh (Fig. 4). Thus channel gating was

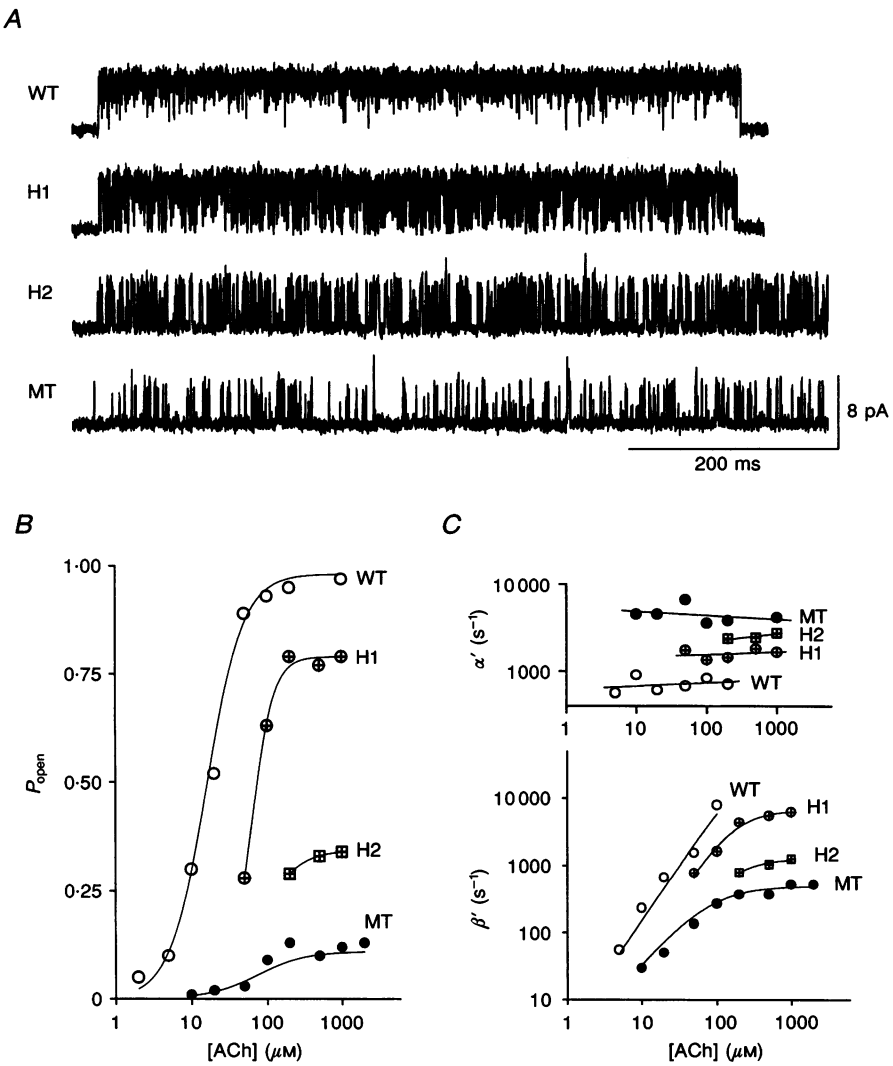

Figure 5. Activation properties of hybrid adult receptors

A, single channel clusters elicited by 500 μM ACh. WT, wild type receptors (no mutant α-subunits); MT, αD200N mutant receptors (two mutant α-subunits); and H1 and H2 are hybrid receptors (exactly one mutant α-subunit). Inward current is up. *B*, the dose–response curves of wild type, mutant and hybrid receptors. For the hybrids each symbol represents one patch (having 6–28 clusters). *C*, effective closing (α′) and opening (β′) rates of wild type, hybrid and mutant receptors. Only the effective opening rate depended on agonist concentration. In *B* and *C* the lines are the fits by eqn (1). The opening and closing rate constants for the hybrid receptors are given in Table 3.

impaired regardless of whether αD200 was replaced with a negatively charged, neutral, or positively charged side-chain. Efficient opening thus required aspartic acid at α200.

Different agonists

In the wild type embryonic AChR, the channel opening rate depends on the nature of the agonist, whereas the channel closing rate is nearly agonist independent (Zhang *et al.* 1995). Also, the agonist with lowest efficacy, tetramethylammonium (TMA), failed to elicit detectable currents in oocytes expressing *Torpedo* $\alpha_2\beta\delta\gamma$ αD200N AChRs (O'Leary & White, 1992). To examine further the effect of αD200N on the agonist dependence of gating, we examined the ability of both TMA and carbamylcholine (CCh) to activate embryonic and adult αD200N AChRs.

For adult AChRs, high concentrations of CCh elicited clear clustering of openings, whereas no clustering was apparent even at the highest concentration of TMA (2 mM). For CCh, the effective opening rates remained constant between 500 μM (50 s^{-1}) and 1 mM CCh (55 s^{-1}), indicating about a 10-fold decrease in opening rate compared with ACh. Thus agonist dependence of the channel opening rate was maintained in adult AChRs containing αD200N. For embryonic AChRs, clustering of openings was not apparent for either CCh or TMA, which is most likely to be because the opening rates for these agonists are too low to measure.

The mean open times for ACh, CCh and TMA were found to be within a factor of two for embryonic AChRs containing αD200N. For ACh $\tau_0 = 1{\cdot}17 \pm 0{\cdot}15$ ($n = 5$ patches), for TMA

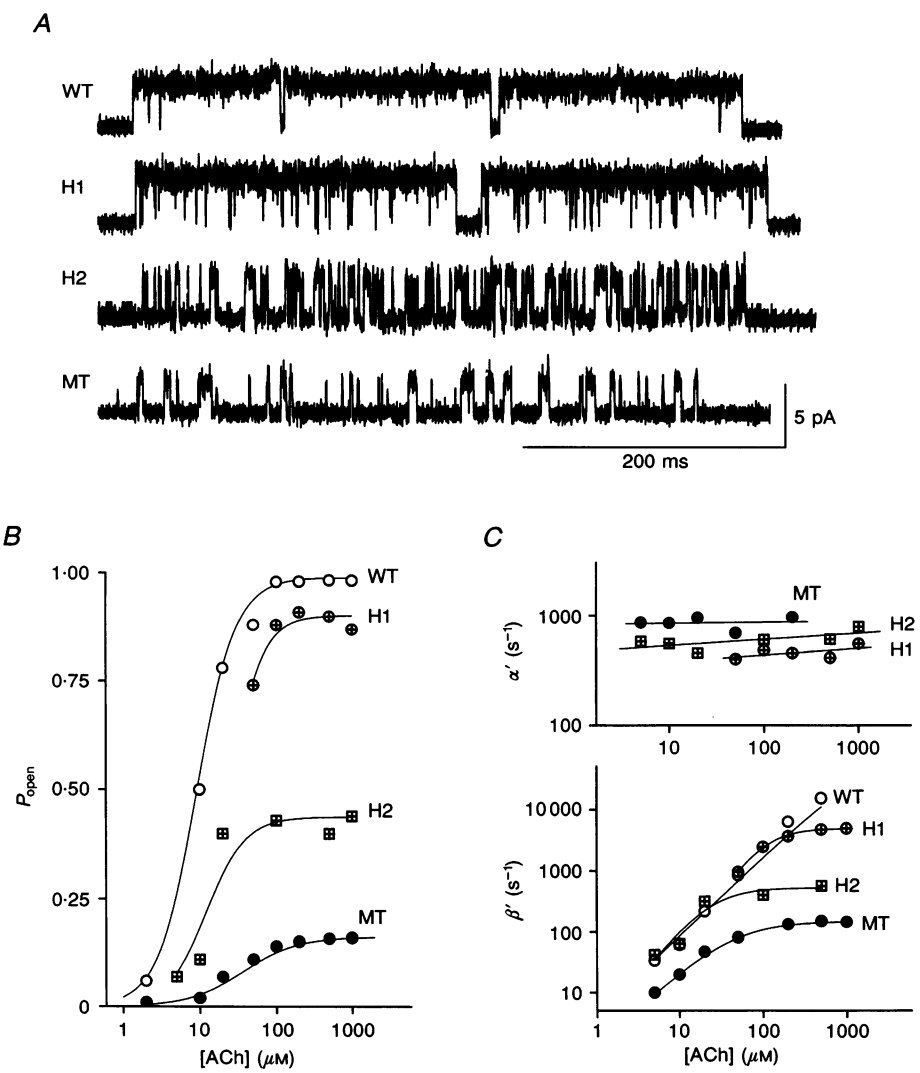

Figure 6. Activation properties of hybrid embryonic receptors

A, single channel clusters elicited by 500 μM ACh. WT, wild type receptors (no mutant α-subunits); MT, αD200N mutant receptors (two mutant α-subunits), and H1 and H2 are hybrid receptors (exactly one mutant α-subunit). Inward current is up. *B*, the dose–response curves of wild type, mutant, and hybrid receptors. Each symbol represents one patch (for hybrids, having 6–49 clusters). *C*, effective closing and opening rates of wild type, hybrid, and mutant receptors. Only the effective opening rate depends on agonist concentration. In *B* and *C* the lines are the fits by eqn (1). The opening and closing rate constants for the hybrid receptors are given in Table 3.

Table 3. Gating rate and equilibrium constants for wild type and αD200N receptors

Receptor type	β (s^{-1})	α (s^{-1})	β/α
Embryonic			
WT*	60000	240	250·0
H1	4984 ± 191	473 ± 65	10·5
H2	533 ± 188	640 ± 151	0·8
MT	153 ± 43	780 ± 11	0·2
Adult			
WT	55000†	1132 ± 22	48·6
H1	6676 ± 1142	1640 ± 291	4·1
H2	1362 ± 182	2420 ± 284	0·6
MT	535 ± 36	4027 ± 45	0·1

For each receptor type, WT is wild type, H1 and H2 are the two hybrid populations (i.e. receptors with one mutant and one wild type α-subunit), and MT is a receptor with two mutant α-subunits. The mutation was a D-to-N substitution at position α200. We hypothesize that H1 receptors have a mutated α_δ site and that H2 receptors have a mutated $\alpha_{\gamma,\epsilon}$ site. Adult receptors have an ε-subunit, and embryonic receptors have a γ-subunit. Errors are S.D. The receptors were activated by ACh. For the mutant and hybrid receptors β was estimated using eqn (1) (see Figs 5 and 6), and α was calculated as the inverse of the open channel lifetime ($n = 3$–7 patches). Some values are from previous results (* Zhang, Chen & Auerbach, 1995, † Sine *et al.* 1995).

$\tau_0 = 0{\cdot}94$ ms ($n = 2$), and for CCh $\tau_0 = 2{\cdot}18 \pm 0{\cdot}09$ ms ($n = 3$). Similar observations were made for adult AChRs containing αD200N: for ACh $\tau_0 = 0{\cdot}22 \pm 0{\cdot}06$ ms ($n = 4$), for TMA $\tau_0 = 0{\cdot}29$ ms ($n = 2$), and for CCh $\tau_0 = 0{\cdot}37 \pm 0{\cdot}04$ ($n = 3$). The closing rate constant for these agonists is simply the inverse of the mean open time because αD200N receptors rarely reopen before losing an agonist. The results indicate that in αD200N AChRs the channel closing rate constant is within a factor of two for these three agonists.

Hybrid AChRs

To gain insight into the contribution of each binding site to the channel gating reaction, we examined AChRs containing one mutant and one wild type α-subunit. To form such hybrid AChRs, we co-transfected HEK cells with both wild type and D200N α-subunits (plus wild type β-, δ- and either γ- or ε-subunits). This procedure should give rise to four populations of AChRs. Two populations should be homogeneous with regard to the α-subunits (AChRs with either two wild type or two mutant subunits), and the other two populations should be hybrids (AChRs with one mutant and one wild type subunit). Because the two binding sites are formed by αδ- and either αγ- or αε-subunit pairs, the two hybrid populations are potentially different in terms of gating properties.

When both wild type and D200N α-subunits were co-expressed, high concentrations of ACh elicited four distinct types of clusters. Qualitatively similar observations were made for both adult and embryonic mutant AChRs (Figs 5 and 6, respectively). Clusters with the highest and lowest effective opening rates correspond to AChRs containing either two wild type or two D200N α-subunits, respectively. The two intermediate populations of clusters are assumed to arise from hybrid AChRs containing the D-to-N mutation at only one of the two binding sites. Channel opening rate constants for these hybrid AChRs were estimated from the limiting effective opening rate obtained at high ACh concentrations, while the closing rate constants were estimated from the distribution of open durations (Table 3). The hybrid AChR that opens more quickly (designated H1) had a similar opening rate constant in both embryonic and adult AChRs, ~6000 s^{-1}. The hybrid AChR that opens more slowly (designated H2) had an opening rate constant significantly slower in embryonic (~500 s^{-1}) than in adult (~1400 s^{-1}) AChRs. In both embryonic and adult AChRs, the channel closing rate constants of the two hybrid populations were intermediate to the wild type and mutant AChRs.

The effects of αD200N on free energy of channel gating is presented for the two hybrid and double mutant AChRs in Table 4. For both embryonic and adult AChRs, the $\Delta\Delta G$ values (the change in the free energy of channel closing caused by the mutation) clearly differed for the two types of hybrid AChRs, indicating different contributions of each binding site to channel gating. The sum of $\Delta\Delta G$ values for the two hybrids was slightly greater than that for the double mutant AChR, indicating nearly independent contributions of the two sites to the overall gating reaction.

Table 4. Energetics of gating of hybrid and αD200N receptors activated by ACh

	ΔΔG (kcal mol^{-1})		
Receptor type	β	α	β/α
Embryonic			
H1	1·46	−0·40	1·86
H2	2·79	−0·58	3·34
MT	3·52	−0·70	4·22
H1 + H2	4·25	−1·28	5·20
Adult			
H1	1·30	−0·22	1·52
H2	2·23	−0·45	2·68
MT	2·78	−0·75	3·53
H1 + H2	3·53	−0·67	4·20

Values are ΔΔG in kcal mol^{-1}, and are computed as $0{\cdot}59 \ln(k_{WT}/k_{mutant})$, from the rate constants in Table 3. β is the channel opening rate constant, α is the channel closing rate constant, and β/α is the gating equilibrium constant (Model 2). H1 and H2 refer to hybrid receptors that have a D200N mutation in only one α-subunit; MT refers to receptors with this mutation at both binding sites. The effect of the mutation was significantly greater in the H2 hybrid, and the effect in all-mutant receptors was slightly less than the sum of the effects in the hybrids.

DISCUSSION

Effects of the mutation on AChR activation

By far the most significant effect of mutations to residue D200 of the α-subunit was to slow the rate constant for AChR channel opening. When both binding sites were occupied by ACh, both embryonic and adult wild type AChRs opened at ~60 000 s^{-1}. In contrast, diliganded αD200N mutant AChRs (with the mutation in both α-subunits) opened at least 100 times more slowly. Other rate constants in the activation pathway were altered by the mutation, but to a lesser extent than channel opening. In adult AChRs, the channel closing rate constant was only ~4-fold faster in the mutant than the wild type, while ACh bound 3-fold more tightly to the mutant AChR than to wild type. These results support the conclusion of O'Leary & White (1992) that the D-to-N mutation at α-residue 200 impairs channel gating to a greater extent than ACh binding.

We observed a qualitatively similar effect on gating with E, Q and K mutations at αD200, as well as in the double mutant αD200N + T202V. We conclude that introduction of a new glycosylation site (N*x*T) by the αD200N mutant is not the basis for the effects on channel gating. The similarly impaired gating of the αD200E mutant further suggests an absolute requirement for D at this position in order to maintain rapid opening of the channel. The high specificity for an aspartate suggests that it is the side-chain rather than the backbone atoms that form the structure that enables rapid opening. We speculate that in wild type receptors, the αD200 side-chain participates in an intra- or intersubunit bond (e.g. a salt bridge) that is required for fast opening. The similar behaviour of mutant receptors with positively charged, negatively charged, or neutral side-chains, and the uniformly high expression levels of these receptors, leads us to speculate that in mutant AChRs, the critical bond is broken and the α200 side-chain is exposed to solvent.

Agonist dependence

The channel opening rate constant of diliganded, wild type AChR depends on the nature of the agonist (Zhang *et al.* 1995), as AChRs occupied by ACh open faster than those occupied by CCh (~5-fold) or TMA (~20-fold). We found a similar pattern of agonist dependency of the opening rate constant in αD200N AChRs, indicating that the mutation does not disrupt the structures that determine the agonist selectivity of the AChR.

Effect on agonist binding

Although the major effect of the mutation was to slow the channel opening rate constant, binding of ACh was also affected by the αD200N mutation. In adult AChRs, where the two agonist binding sites appear to have a similar affinity for ACh, the D-to-N mutation lowered the K_d about 3-fold. Kinetic analysis showed that this reduction was entirely due to a slower ACh dissociation rate constant. The D-to-N mutation resulted in loss of a negative charge; if the αD200 side-chain interacted directly with the cationic group of the agonist, we would expect destabilization rather than stabilization of bound ACh$^+$. Because ACh actually bound more tightly to the D200N AChR, we hypothesize that the side-chain of residue α200 does not come into close contact with the quaternary amine moeity of the agonist. That the mutants αD200E and αD200K had qualitatively similar behaviours to αD200N supports this hypothesis. We suspect that the changes in the agonist equilibrium dissociation

constant resulting from the mutations arose from delocalized changes in the structure of the transmitter binding pocket.

A second effect on transmitter binding was apparent in embryonic AChRs. In AChRs expressed with a γ-subunit (for example, *Torpedo* and embryonic mouse AChRs), the two binding sites (αδ and αγ) have different affinities for agonists. In mouse embryonic AChRs containing αD200N, however, we could detect no difference in K_d between the two binding sites. This result suggests that αD200N not only increases the affinity of αδ and αγ sites, but it also eliminates the asymmetry in affinity of the two sites.

Hybrid AChRs

In an unoccupied, resting AChR the energy barriers that define the closed-to-open transition are sufficiently high to prevent frequent spontaneous opening. Channels open somewhat more rapidly after binding one agonist, and very rapidly after binding two. By co-transfecting HEK cells with both wild type and D200N α-subunits, we expressed and measured the gating rate constants of hybrid AChRs containing one mutant and one wild type α-subunit (Figs 5 and 6). In this section, we discuss three topics regarding these AChRs: association of each hybrid with a mutation in αδ or αγ/αε, the degree of independence of the free energy changes at the binding sites, and the degree of asymmetry of the effect of the mutation at the two binding sites.

We observed a fast- and a slow-opening hybrid AChR, called H1 and H2, respectively. We can tentatively identify the site of the mutation in each hybrid by comparing the change in free energy of channel opening in embryonic and adult AChRs. Presumably, in mutant receptors the opening rate constant is determined by the binding site containing the αD200N mutation. Embryonic and adult AChRs have a common αδ site, but different αγ or αε sites. We hypothesize that the hybrid with D200N at the common αδ site is the one that shows similar change in free energy for channel opening in adult and embryonic AChR, namely H1 (Table 3). The adult and embryonic H2 AChRs show greater differences in free energy for channel opening, suggesting these contain D200N at the αγ/αε site. The same conclusion follows by comparing the free energy of channel closing.

Whether αD200N affects each binding site independently can be determined from the energetics of gating in hybrid and mutant AChRs (Table 4). For both adult and embryonic AChRs, the sum of $\Delta\Delta G$ values for the opening rate constant for each hybrid was slightly greater (by ~0·7 kcal mol^{-1}) than for the double-mutant AChR. That is, AChRs with two αD200N mutations open about 3–4 times faster than would be expected from a simple additive contribution of each mutation. We can compute a coupling coefficient (Ω) between α-subunit mutations (see Hidalgo & MacKinnon, 1995):

$$\Omega = (\Theta_{WT} \times \Theta_{MT})/(\Theta_{H1} \times \Theta_{H2}),$$

where Θ is the gating equilibrium constant (β/α in Table 3) and the subscripts refer to wild type (WT), αD200N (MT), hybrid 1 (H1) or hybrid 2 (H2) AChRs. For adult AChRs Ω = 2·1, and for embryonic AChRs Ω = 5·9. In terms of free energies, there is ~0·5 kcal mol^{-1} (adult) and ~1 kcal mol^{-1} (embryonic) more energy available for gating in αD200N AChRs than would be expected from completely independent effects of each hybrid (Table 4). However, because these free energies are relatively small, we conclude that the changes in the structures of the binding sites caused by the D-to-N mutation are largely independent.

The effects of the mutation on the channel opening rate constant are not equal at the two binding sites. The free energies shown in Table 4 indicate that the mutation has nearly twice the effect on gating in H2 AChRs (i.e. at the putative αγ/αε site) compared with H1 AChRs (i.e. at the putative αδ site). This asymmetry may reflect intrinsic differences in the transmitter binding sites with respect to their influence on gating. If so, the results indicate that the αε/αγ site contributes to channel gating to a greater extent than does the αδ site. However, our analysis is limited to receptors having αD200N mutations, and it is also possible that the asymmetry arises as a consequence of the mutation.

The effect of the mutation on the channel closing rate constant was relatively small, with destabilization of ~0·4 kcal mol^{-1} occurring at either binding site. Thus, in addition to slowing opening and agonist dissociation, αD200N also leads to modestly less favourable agonist–protein and protein–protein interactions in the open channel conformation.

The experiments described here show that in AChR αD200 is an important transduction element that links receptor occupancy with channel gating. A homologous aspartate is present in several other channels, located twenty-one residues upstream from a conserved proline in M1 (ligand-gated subunits) or M2 (connexins). It remains to be determined whether the aspartate is as important in the gating of these channels as it is in AChRs.

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