

Fig. S1- Correlation between open and succeeding closed dwell times. The upper histogram plots each opening against the succeeding closing for the recording from a patch containing AChRs with the L9'S mutation in the β and δ subunits (Fig. 1a of the main text); the total number of events in the histogram is 4315. The lower histogram is constructed from data obtained from simulation¹ using the kinetic model and fitted rate constants in Fig. 1a. The total number of simulated events is 20,000, but for comparison with the experimental data the y-axis is scaled to give equivalent bin heights. Notice each histogram contains two main peaks, a large peak corresponding to brief openings flanked by long closings, and a smaller peak corresponding to long openings flanked by brief

closings. Below the two histograms, the bin heights of the simulated histogram are subtracted from those of the experimental histogram, and the differences are plotted using the color scale to the right. Differences between experimental and simulated bin heights mainly fall between 1 and -1, which corresponds to plus or minus one standard deviation, assuming Poisson-distributed bin heights.²

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expressing AChRs containing Leu to Ser mutations at the 9' or 13' positions of M2 in the indicated subunits. Configuration, cell-attached patch; membrane potential, -70 mV; bandwidth, 10 kHz; channel openings are upward deflections. Prolonged closed periods preceded and followed each trace (not shown).



Fig. S3- Left column, single channel currents elicited by the indicated concentrations of ACh applied to wild type adult human AChRs. Data are from Mukhtasimova, et al.³ Configuration, cell-attached patch; membrane potential, - 70 mV; bandwidth, 10 kHz. Middle and right columns, dwell time histograms with probability density functions (smooth curves) obtained from fitting the Primed (red) or del Castillo-Katz (black) models to the global set of dwell times. Each

pair of closed and open time histograms was constructed from single channel events recorded from a single patch. Each curve resulted from fitting of a kinetic model simultaneously to data from two patches for each of six different ACh concentrations (3-1000 μ M; see Supplementary Methods). Fitted rate constants are given in Table S4.

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Receptor	[ACh]	# of Patches	I ₁₊	۱ ₁₋	β ₁	α ₁	Θ_1	I ₂₊	I ₂₋	β2	α2	Θ_2
	None	3	62 (5)	19 (2.5)	210 (4)	22000 (300)	0.01	10 (0.5)	6700 (450)	84000 (4200)	2300 (200)	37
βL262S	1 nM	2	ND	ND	330 (9)	16000 (400)	0.02	11 (1.3)	1200 (170)	97000 (4600)	2300 (250)	42
δL265S	3 nM	2	ND	ND	340 (11)	15000 (500)	0.02	23 (2.5)	1100 (130)	102000 (4500)	2600 (270)	39
	6 nM	2	610 (100)	1200 (400)	950 (130)	19000 (700)	0.05	70 (12)	550 (65)	88000 (2300)	2200 (130)	38
	10 nM	2	330 (26)	1600 (170)	4000 (300)	20000 (1000)	0.20	1200 (100)	260 (17)	80000 (700)	2300 (40)	35
αY190F +	None	3	120 (14)	46 (9)	400 (14)	32000 (500)	0.01	ND	ND	ND	ND	ND
ρL2023 + δL265S	30 µM	3	ND	ND	ND	ND	ND	7000 (2100)	500 (80)	86000 (1600)	2500 (90)	34
αP272A + βL262S + δL265S	None	3	1000 (120)	2400 (700)	ND	ND	ND	5900 (1200)	1800 (140)	83000 (2200)	2600 (120)	32
εP121L + βL262S + δL265S	None	2	100 (25)	23 (9.9)	140 (8.4)	35000 (1300)	0.004	22 (1.7)	12000 (1300)	65000 (4600)	2000 (180)	33

The model fitted to the tabulated data is shown right. Rate constant definitions: $L_n = I_{n+}/I_n$; $\Theta_n = \beta_n/\alpha_n$. Rate constants are in units of s⁻¹. In the absence of ACh, the I_{n+} , I_n , α_1 and β_1 correspond to true rate constants as they represent reaction steps between states in the left vertical plane of the model in Fig. 4 of the main text. In the presence of ACh, a mixture of un-liganded, mono-liganded and diliganded receptors is expected, as depicted in Fig. 4. Thus although fitting the

model to the data in the presence of ACh described the sequences of open and closed

Θ2

 Θ_1

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±0'

dwell times, each fitted rate constant does not reflect a single reaction step, but instead is a weighted average of all the rate constants;⁴ the resulting rate constants are therefore apparent and not related in a simple way to the microscopic rate constants in the model. ND, parameter not defined. Numbers in parenthesis are error limits computed as described⁵.

The data for the β L262S+ δ L265S mutant receptors (0 through 10 nM ACh) in Table S1 are in principle appropriate for fitting according to the Primed model (Fig. 4). However, additional data obtained over a greater range of ACh concentrations and with more closely spaced intervals of ACh concentrations are likely required to obtain well defined rate constants. Nevertheless the data in Table S1 show that transitions to and from the long open state are the same in the absence of agonist and over a range of ACh concentrations that causes the fractional area of the long component of openings to change from small to large.

Notice that the estimated β_1 for singly primed $\beta L262S+\delta L265S$ receptors increases as the ACh concentration increases (Table S1), but the Primed model (Fig. 4) suggests β_1 should be independent of ACh concentration. This expectation is likely true if the same site is singly primed in the absence of ACh and in its presence. However, agonist-bound, singly-primed closed states have multiple escape pathways with different rate constants, potentially altering the apparent β_1 obtained from fitting the simplified model in this table legend. Furthermore, it is possible that the α - ε site primes in the absence of agonist, but the α - δ site is the first to prime in its presence; if β_1 is not the same for each binding site, it may show an apparent dependence on ACh concentration.

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Receptor	Treatment	Patch #	Mode #	# of events	P _{open}	τ _o (msec)	τ _c (msec)	
αC193S +εl 119C	Before H ₂ O ₂	1	1	2998	0.026	0.13	4.5	
+δL121C		·	1	406	0.015	0.093	6.0	
	H ₂ O ₂		2	345	0.52	1.74	1.6	
			3	1229	0.87	2.30	0.36	
αC193S	Before H ₂ O ₂	2	1	3678	0.018	0.11	6.6	
+8L119C			1	1885	0.027	0.15	5.4	
TULIZIC	H_2O_2		2	112	0.38	2.1	3.3	
			3**	384	0.90	2.8	0.32	
αC193S +εL119C +δL121C	Before H ₂ O ₂	3	1	4006	0.011	0.084	9.0	
			1	335	0.015	0.092	6.1	
	H_2O_2		2	623	0.64	2.6	1.5	
			3	1176	0.90	3.6	0.27	
			1	150	0.025	0.12	4.7	
αC193S	H_2O_2		2	15	0.36	2.1	3.7	
+ɛL 119C			3	878	0.88	3.3	0.44	
+δL121C		1	1	1659	0.034	0.15	4.2	
	DTT		2	470	0.27	0.69	1.8	
			1	1/35	0.040	0.10	37	
	H ₂ O ₂		2	74	0.040	19	3.0	
αC193S	11202		3	817	0.00	22	0.24	
+εL119C		2	0	017	0.00	2.2	0.24	
+0L121C	DTT	-	1	2657	0.043	0.19	4.2	
			2	68	0.24	0.49	1.6	
			1	220	0.016	0.091	5.5	
aC103S	H_2O_2		2	114	0.47	2.3	2.6	
+εL119C		3	3	628	0.87	2.9	0.46	
+δL121C	DTT		1	2897	0.018	0.12	6.2	

Table S2- Properties of episodes of currents through single AChR channels.

For each patch and treatment condition, episodes of spontaneous unitary currents belonging to the same channel were identified and separated into the indicated number of kinetic modes as described⁶. After separation into modes, open probability, mean open time and mean closed time were calculated for each episode and averaged for each mode. ** The number of events in mode 3 is underestimated because, for this patch, the frequency of events was so high following addition of H_2O_2 that many brief events superimposed upon the episodes of long events, requiring exclusion from analysis.

Receptor	Treatment	Patch #	Mode #	# of events	P_{open}	τ _ο (msec)	τ _c (msec)
αC193S	Before H ₂ O ₂	1	1	2554	0.004	0.054	14.0
+821190	H_2O_2		1	1840	0.005	0.052	11.0
αC193S +εL119C	Before H ₂ O ₂	2	1	4778	0.012	0.080	9.4
	Defense III O		1	2444	0.007	0.094	14.0
αC193S	Before H ₂ O ₂	3	1	3204	0.019	0.090	5.0
+£L119C	H_2O_2		1	1702	0.021	0.097	6.8
0.4000	Before H ₂ O ₂		1	2810	0.029	0.094	3.4
401935	HaOa	1	1	1003	0.025	0.28	11.0
+0L121C	11202		2	330	0.025	0.20	14.0
	Before H ₂ O ₂		1	3830	0.014	0.087	6.7
αC193S		2					
+δL121C	H_2O_2		1	300	0.034	0.11	3.1
	<u> </u>		2	4680	0.22	0.28	1.0
αC193S	Before H ₂ O ₂	з	1	4598	0.010	0.084	9.6
+δL121C	H_2O_2	0	1	4730	0.071	0.35	6.2
d 110C	Before H ₂ O ₂		1	3264	0.005	0.060	14.0
+δL121C	H.O.	1	1	2219	0.006	0.062	12.0
			1	2564	0.000	0.002	12.0
εL119C		2	1	2004	0.000	0.005	11.0
+0L121C	H_2O_2		1	2746	0.006	0.063	12.0
εL119C	Before H ₂ O ₂	•	1	2004	0.053	0.19	4.0
+δL121C	H_2O_2	3	1	4596	0.053	0 19	56
	Before DTT		1	3222	0.009	0.089	14.0
εL119C	Boloro BTT	1	•	0222	0.000	0.000	11.0
	DTT		1	2810	0.008	0.064	7.8
εL119C	Before DTT	2	1	1418	0.004	0.070	15.0
+δL121C	DTT	2	1	3664	0.006	0.080	12.0
d 110C	Before DTT		1	2776	0.011	0.097	9.2
+δL121C	DTT	3	4	4004	0.010	0.000	7.0
			1	4224	0.010	0.063	7.0
βL262S	Belore H ₂ O ₂	1	I	3434	0.40	0.75	0.2
+0L265S	H_2O_2		1	2364	0.081	0.84	5.0
BL262S	Before H ₂ O ₂	0	1	1228	0.047	0.44	9.1
+δL265S	H_2O_2	2	1	1988	0.048	0.57	10.0
	Before H ₂ O ₂		1	2876	0.052	0.45	7.4
βL262S +δL265S	<u>L</u> - L	3					
-012030	H ₂ O ₂		1	2856	0.031	0.37	6.2
βL262S	Before DTT	1	1	2334	0.082	0.53	5.0
+δL265S	DTT	I	1	4684	0.16	0.6118	2.5
RIDEDE	Before DTT		1	3392	0.076	0.55	4.9
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			1	2874	0.10	0.55	3.7
βL262S		3	I	5142	0.041	0.444	0.754
+0L2655	DTT		1	2876	0.052	0.451	7.378

Table S3- Properties of episodes of currents through single AChR channels.

Note that when Cys was engineered at both faces of one binding site, or at one face of both binding sites, brief openings observed before oxidation, persisted following oxidation. Also, receptors without Cys substitutions at the binding sites, but with Ser substitutions in the pore, showed no changes in the kinetics of spontaneous channel gating following application of oxidizing or reducing reagents.

del Castillo-Katz Model

Primed Model



Table S4- Fitted rate constants underlying activation of wild type human adult AChRs

Model	LL	k ₊₁	k .1	p +1	p ₋₁	β1	α1	k ₊₂	k . ₂	p ₊₂	p.2	β₂	α2	k₊₀	k. _b
del	304306	350	3500			65	3500	92	19000			43000	2100	33	162000
Castillo- Katz		(40)	(440)			(6)	(300)	(2.1)	(300)			(1100)	(43)	(2.9)	(3800)
Primed	304480	840	48000	27000	11000	180	3200	420	72000	43000	32000	82000	3400	19	144000
		(60)	(7000)	(3400)	(1400)	(21)	(250)	(30)	(5800)	(2300)	(1400)	(1500)	(54)	(2.5)	(15000)

Rate constant definitions: K=k/k₊; P= p₊/p₋; $\theta = \beta/\alpha$. Units are μ M⁻¹s⁻¹ for k₊₁, k₊₂ and k_{+b} and s⁻¹ for all others. Rate constants were estimated by fitting each model to sequences of dwell times obtained for wild type AChRs from previous work (a total of six ACh concentrations, ranging from 3 to1000 μ M at half log unit intervals of the concentration, with two patches per concentration)³ using MIL software (<u>www.qub.buffalo.edu</u>). LL is the log likelihood obtained from fitting each model to the same data. Numbers in parenthesis are error limits computed by MIL. To fit the Primed model, the constraint k₊₁=2k₊₂ was imposed. The subset of the Primed model chosen for fitting is indicated in Fig. 4.

The presence of primed intermediates is expected to slow the rate at which current increases following a step increase of ACh concentration. Experimental measurements of the limiting rate of current increase have been obtained by sudden exposure of out-side-out patches containing AChRs to saturating concentrations of ACh.^{7,8} The resulting current time courses were sigmoid-shaped, presumably due to finite solution exchange times, so rise times were quantified as the time for the current to change from 20 to 80% or 10 to 90 % of the maximum plateau current. Liu and Dilger observed limiting 20-80% rise times of 45 to 80 μ s,⁷ while Maconochie and Steinbach observed

limiting 10-90% rise times of 25 to 40 µs.⁸ Using the fitted rate constants for the Primed model and a concentration of 10 mM ACh, simulations using QUB software yielded a 20-80% rise time of 89 µs, and a 10-90% rise time of 130 µs. Thus our simulated limiting rise time is at the upper end of the range observed by Liu and Dilger and longer than observed by Maconochie and Steinbach. Simulation using the fitted rate constants for the extended del Castillio-Katz model yielded a 10-90 % rise time of 47 µs. Limiting rise times have not been measured for the adult human AChR studied here, so differences between simulated and experimental rise times may result from use of the fetal mouse AChR by Liu and Dilger and adult mouse AChR by Maconochie and Steinbach. Another possibility is the open channel state may also be reached through the bind-bind-prime-prime or bindprime-prime-bind pathways within the Primed model (Fig. 4). Fitting either alternative pathway to single channel dwell time sequences did not yield well defined rate constants, but this could have arisen because the single channel measurements were made in the presence of a range of ACh concentrations for which multiple pathways within the Primed model are possible. Application of a step pulse of a saturating concentration of agonist may enforce the bind-bind-prime-prime pathway at the expense of the bind-prime-bind-prime pathway that described the single channel data. Thus it is possible that only a subset of the fitted rate constants from single channel measurements contribute to the rise time of macroscopic current following a step pulse of ACh.

Supplementary References-

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