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Detection and Trapping of Intermediate States Priming Nicotinic Receptor Channel Opening

Nuriya Mukhtasimova[1,*], **Won Yong Lee**[1,*], **Hai-Long Wang**[1], and **Steven M. Sine**[1,2]

[1]Receptor Biology Laboratory, Department of Physiology and Biomedical Engineering, Mayo Clinic College of Medicine, Rochester, Minnesota, 55905, USA

[2]Department of Neurology, Mayo Clinic College of Medicine, Rochester, Minnesota, 55905, USA

Abstract

In the course of synaptic transmission in the brain and periphery, acetylcholine receptors (AChRs) rapidly transduce a chemical signal into an electrical impulse. The speed of transduction owes in large part to rapid ACh association and dissociation, implying a binding site relatively non-selective for small cations; selective transduction has been supposed to originate from the ability of ACh, over that of other organic cations, to trigger the subsequent channel opening step. However transitions to and from the open state were shown to be similar for agonists with widely different efficacies.1,2,3 Here, by studying mutant AChRs, we find that the ultimate closed to open transition is agonist-independent and preceded by two primed closed states; the first primed state elicits brief openings, whereas the second elicits long-lived openings. Long-lived openings and the associated primed state are detected in the absence and presence of agonist, and exhibit the same kinetic signatures under both conditions. By covalently locking the agonist binding sites in the bound conformation, we find that each site initiates a priming step. Thus a change in binding site conformation primes the AChR for channel opening in a process that enables selective activation by ACh while maximizing speed and efficiency of the biological response.

Throughout the nervous system, moment-to-moment communication relies on rapid on and off responses of synaptic receptors. Rapid switching is possible through a neurotransmitter binding site freely accessible to solvent, enabling diffusion-limited binding of neurotransmitter, and modest stabilization of the bound complex, enabling quick release. Both adaptations seemingly oppose the binding site's ability to capture the neurotransmitter from a mix of chemically similar molecules within the synapse. However in light of the del Castillo-Katz model proposed fifty years ago4, preferential activation by neurotransmitter over other organic cations could be encoded by a process now recognized as the channel gating reaction in which the fully occupied receptor channel reversibly opens and closes.

Correspondence to: Steven M. Sine (Email: sine@mayo.edu).

*These authors contributed equally to this work.

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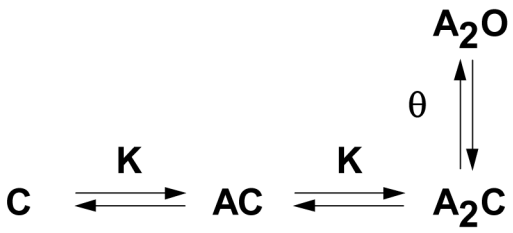

Modified to include two rather than one agonist binding step, this extended del Castillo-Katz model depicts binding of successive agonist molecules A to a receptor in the closed state C, generating an inactive complex A_2C, which isomerizes to the biologically active complex A_2O. Preferential activation by neurotransmitter could thus arise from its structurally-encoded ability to drive the gating reaction in the forward direction, despite a relatively non-selective binding site.

The advent of patch clamp recording enabled registration of current pulses through single ion channels as brief as tens of microseconds5, allowing unprecedented testing of the binding-gating transduction mechanism. Channel openings were found to be interrupted by brief closed periods, and these were proposed to correspond to sojourns in the A_2C state within the extended del Castillo-Katz model.6 By equating brief closed periods with sojourns in the A_2C state, kinetic modeling of current pulse sequences by methods pioneered by Colquhoun and Hawkes7 yielded estimates of the rate at which fully occupied AChR channels opened.8,9 The emerging single channel opening rate approached the rate of rise of macroscopic current elicited by rapid application of agonist to AChR ensembles.10,11 The overall data therefore supported the view that rapid onset of the post-synaptic response arises from low affinity binding of ACh coupled directly to rapid channel opening.

However, a concurrent study found that brief closed periods that interrupted openings of single AChR channels were similar for agonists with widely different efficacies,1 contrary to the interpretation that brief closed periods corresponded to sojourns in the A_2C state. Building upon kinetic studies of single glycine-activated receptors,12 a recent study of full and partial agonists provided evidence for a closed state called flipped, that followed agonist binding and preceded channel opening, but whose mean lifetime was similar for agonists with diverging efficacies.3 Here, by studying mutant AChRs, we detect two distinct closed states, called primed, that follow agonist binding, couple tightly to channel opening and exhibit agonist-independent properties. We further show that priming results from conformational changes at the two ACh binding sites.

To look for agonist-independent transitions between closed and open states, we recorded single channel currents through AChRs from adult skeletal muscle in the absence of agonist. Because wild type AChRs rarely open spontaneously, we increased spontaneous opening by substituting Ser for a conserved Leu in the center of the ion-conductive pore. In the absence of agonist, single AChR channels containing the Ser substitution in the β and δ subunits activate in long episodes of brief and long openings flanked by prolonged quiescent periods (Fig. 1a). Within such episodes, three closed and two open states are detected, indicating the temporal sequence of single channel current pulses arises from a minimum of five distinct states. Fitting a five-state model to the sequences of open and closed dwell times reveals that

brief openings arise from a closed state with intermediate duration, whereas long openings arise from a closed state with brief duration (Figs. 1a, S1). Remarkably, although agonist is not present, transitions from the brief closed to the long open state occur rapidly and with high probability.

Substituting Ser for the central Leu in other pairs of AChR subunits also increases spontaneous channel opening, which again appears as episodes of brief and long openings from a single receptor channel (Fig. S2). A previous study documented an increase of spontaneous brief and long openings following substitution of Thr for a Ser approximately one turn of the pore helix from the Leu substituted here.19

Application of ACh to our pore-mutant receptor increases long and decreases brief openings, with a 10-fold change in ACh concentration increasing the fraction of long openings from 0.05 to greater than 0.90 (Fig. 1b). Fitting a five-state model to the sequences of ACh-evoked current pulses yields the surprising result that rates for entering and leaving the long-lived open state mimic those observed in the absence of agonist (Fig. 1c; Table S1). Thus transition from the major closed to the biologically-relevant open state is independent of the presence of agonist.

To account for these observations, we propose that closed states that immediately precede channel opening correspond to AChRs with “primed” binding sites; priming of one site triggers brief openings whereas priming of two sites triggers long openings. Comparison of crystal structures of the related acetylcholine binding protein, without and with bound agonist, shows that a hair-pin structure flanking the binding site, called the C-loop, changes from an uncapped to a capped conformation upon binding agonist.13,14 Thus we further propose that each priming step results from transition of a C-loop from the uncapped to the capped conformation.

To test this idea, we engineered a Cys residue at the tip of the C-loop of each binding site of our pore-mutant receptor (Fig. 2a), and engineered another Cys in each of the two juxtaposed subunits. We then monitored channel opening, in the absence of agonist, before and after applying an oxidizing reagent. Before oxidation, single receptor channels activate in episodes of predominantly brief openings (Fig. 2b). Following oxidation, however, receptor channels activate in episodes of long openings in quick succession (Fig. 2b; Table S2), suggesting covalent reaction arrests the C-loops in the capped conformation, generating the doubly-primed state that triggers long-lived channel openings. The functional consequences of oxidation are reversible; after applying the oxidizing reagent and generating long channel openings, application of a reducing reagent restored brief openings (Fig. 2c; Table S2). Application of oxidizing or reducing reagents had minimal effect on pore-mutant AChRs with fewer than four Cys substitutions (Table S3).

Receptors with the Cys pair at both binding sites, but without Ser substitutions in the pore, showed only rare spontaneous channel opening and no change after applying the oxidizing reagent (not shown), suggesting the uncapped conformation of the C-loop predominates, rendering the inter-Cys spacing too great for cross-linking. However with Ser substitutions in the pore, the capped conformation predominates, enabling covalent reaction. This

retrograde communication between binding site and pore confirms the expectation that the two distal locations communicate in a bi-directional manner.

We further reasoned that the ability to prime should depend on the efficiency of bi-directional communication. To test this idea, we mutated key residues within the binding-pore linkage pathway in our pore-mutant AChR and recorded single channel currents in the absence of agonist. In the wild type AChR, the mutation αY190F suppresses15 whereas αP272A enhances16 ACh-induced channel opening. When αY190F is engineered into the pore-mutant AChR, channel opening episodes consist solely of brief openings (Fig. 3a), suggesting αY190F allows only a single priming step; application of ACh, however, triggers episodes of long openings in quick succession, suggesting agonist overcomes the attenuated priming caused by the mutation. When αP272A is engineered into the pore-mutant receptor, channel opening episodes consist solely of long openings (Fig. 3b), suggesting αP272A promotes priming of both binding sites, analogous to the action of agonist. As a control we tested the mutation εP121L, which suppresses AChR activation of the wild type AChR but is not a component of the principal linkage pathway.17 When εP121L is engineered into the pore-mutant receptor, channel opening episodes contain both brief and long openings (Fig. 3c), similar to the pore-mutant receptor without εP121L (Fig. 1a). Thus mutations that alter the linkage between binding and pore domains suppress or enhance priming and consequently alter channel opening.

To explain our collective findings, we propose a Primed model to describe activation of the AChR (Fig. 4). The model consists of closed states arrayed in three columns, one for each degree of agonist occupancy (0-2), and three rows, one for each degree of priming (0-2). Primed states, **C**’ and **C**”, give rise to channel opening, while un-primed states do not. Each element of the array represents a theoretically possible state, although only a subset of the states may be experimentally detectable. The left vertical plane of states depicts priming and channel opening in the absence of agonist, and in the wild type AChR these transitions are rare. However substituting hydrophilic residues in the pore unmasks states and reaction steps underlying unliganded channel gating; the singly-primed state **C**’ elicits brief openings, whereas the doubly-primed state **C**” elicits long openings. The right vertical plane depicts priming and channel opening with agonist bound to both binding sites, and in our pore-mutant AChR, transitions from the doubly-primed to long open state predominate and mimic those that occur in the absence of agonist.

As a further test, we fitted the Primed model to sequences of single channel closed and open dwell times obtained from the wild type AChR activated by a wide range of ACh concentrations. Because all states in the Primed model are not expected to occur with high probability, we fitted the most likely subset of the model to the data (Fig. 4). For comparison we fitted the extended del Castillo-Katz model to the data, as described previously.15,18 Both models provide good descriptions of the dwell time distributions (Table S4; Fig. S3). However the computed log likelihood for the Primed model is significantly greater than that for the del Castillo/Katz model. The Primed model yields greater ACh association and dissociation rate constants than the del Castillo-Katz model, suggesting the binding site is more accessible to small molecules than previously recognized. In the Primed model, the rate constant for generating the doubly-primed closed state is similar to the rate constant for

channel opening in the del Castillo-Katz model, which explains why the true channel opening step was obscured before.

More than two decades ago, biologically-relevant channel openings of the AChR were found to be interrupted by agonist-independent brief closings.1,2 Yet the functional significance of the interruptions remained elusive until a recent study of channel opening by partial agonists; the doubly-occupied AChR was envisioned to flip to a transient closed state, similar for all agonists, before the channel could open.3 Here by studying mutant AChRs, we detect two transient closed states, called primed, tightly coupled to channel opening; the singly-primed state has an intermediate duration and triggers brief openings, whereas the doubly-primed state has a brief duration and triggers long-lived openings. Using disulfide trapping, we show that capping of each binding site C-loop initiates priming, and that capping of both C-loops evokes long-lived openings. Our ability to unmask and modulate primed states is possible through the bi-directional nature of communication between binding and pore domains. Priming of the AChR is thus a major determinant of its biological activity; reduced priming by partial agonists would explain why they elicit a low maximal response but at the same time generate a stable open state interrupted by brief closings.1,3 Priming appears to be an adaptation to endow the AChR with a rapid response, while maintaining preferential activation by agonist, thus preventing spurious responses to organic cations such as choline, a product of ACh hydrolysis. Furthermore, priming may represent a general adaptation by which chemically-mediated processes achieve both high speed and ligand specificity. Disruptions of a neurotransmitter's ability to prime receptor channels for opening may underlie neurological diseases associated with the AChR and relatives in the Cys-loop superfamily.

Methods Summary

Construction of mutant AChR subunits, their expression in BOSC 23 cells, recordings of single channel currents and kinetic analyses of the currents are described in Online-only Methods.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Appendix

Methods

Construction of wild type and mutant AChRs

Human α, β, δ, and ε subunit cDNAs, subcloned in the CMV-based mammalian expression vector pRBG420, were described previously.21 Site-directed mutations were made using the

QuickChange mutagenesis kit (Stratagene, Cedar Creek, TX) and confirmed by sequencing the entire subunit cDNA. To generate a single free Cys at the tip of the C-loop of the α-subunit, Cys 193 was mutated to Ser, generating a free Cys at position 192.

Mammalian cell expression

All experiments used the BOSC 23 cell line22, a variant of the 293 HEK cell line. Cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) containing fetal bovine serum (10% vol/vol) at 37°C until they reached 50-70% confluence. Wild type or mutant AChR cDNAs were transfected by calcium-phosphate precipitation using cDNA concentrations of 0.68 μg/ml for non-α-subunits and 1.36 μg/ml for the α subunit. Patch-clamp measurements were performed 1 or 2 days after transfection.

Patch-clamp recordings

To record single channel currents, transfected cells were maintained in (mM): KCl 142, NaCl 5.4, $CaCl_2$ 1.8, $MgCl_2$ 1.7, and HEPES 10 (pH was adjusted to 7.4). The same solution was used to fill patch pipettes. Acetylcholine (Sigma Chemical Co., St. Louis, MO) was kept as a 100 mM stock dissolved in bath solution and stored at -80°C until use. Glass micropipettes (type 7052, Garner Glass Co., Claremont, CA), coated with Sylgard 184 (Dow Corning Co., Midland, MI), were heat polished to yield resistances of 5-8 M Ω. Single channel currents were recorded in the cell-attached configuration at 21°C using an Axopatch 200B (Axon Instruments, Inc., Union City, CA) at a membrane potential of -70 mV. Data were collected from 2 to 4 different patches for each experimental condition; recordings were accepted for further analysis only when channel activity was low enough to clearly identify activation episodes from a single channel. The current signal was low-pass filtered at 50 kHz and recorded to hard disk at 200 kHz using the program Acquire (Bruxton Co., Seattle, WA).

Single channel kinetic analysis

The digitized current signal was filtered using a 10 kHz digital Gaussian filter,23 and channel events were detected by the half-amplitude threshold criterion using the program TAC (Bruxton Co.), using an imposed dead time of 10 μs. Precise determination of the dwell time at threshold was achieved by interpolating the digital signal and correcting the measured dwell time for the effects of the Gaussian filter.23 Open and closed time histograms were fitted by the sum of exponentials using the program TACFit (Bruxton Co.). Openings corresponding to a single receptor channel were identified by assigning a critical closed time defined as the point of intersection of the longest closed time component, corresponding to closings between independent episodes of single channel openings, with the preceding briefer component, corresponding to closings between openings from a single channel. Kinetic analysis was performed using MIL software (QuB suite, State University of New York, NY), which employs a maximum likelihood method, corrects for missed events and gives error estimates of the fitted rate constants.24 An instrument dead time of 22 μs was uniformly applied to all recordings prior to fitting.

For kinetic analysis of wild type AChRs, episodes of single channel currents containing at least 5 openings were analyzed for open probability, mean open time and mean closed time,

and episodes within two standard deviations of the mean were accepted for further analysis.25 Kinetic analysis was performed by fitting models to data obtained across a range of ACh concentrations using MIL software. ACh concentrations ranged from 3 μM through 1 mM, with the concentrations spaced at half log unit intervals. Recordings from two patches for each ACh concentration were subjected to analyses, with an average number of events per patch of 4208 (range 2754-5628); each kinetic model was fitted to data from all patches simultaneously. Data from different patches at the same ACh concentration were not pooled prior to fitting.

Disulfide trapping

After establishing a cell-attached gigaohm seal to BOSC 23 cells expressing Cys-substituted AChRs, spontaneous single channel currents were recorded under control conditions. Freshly prepared H_2O_2 was then added to the bath solution, as described previously,26 to establish a final concentration of 8.8 mM, and a second recording was obtained after the change in current kinetics appeared complete (2-4 minutes).

To demonstrate reversal of the change in single channel kinetics following oxidation, cells were treated with 8.8 mM H_2O_2 for 5 minutes, rinsed with normal bath solution, and a cell-attached gigaohm seal was established. After recording control spontaneous single channel currents, DTT was added to the bath solution to establish a final concentration of 0.02 mM, and a second recording was obtained after reversal of the current kinetics appeared complete (4-5 minutes).26

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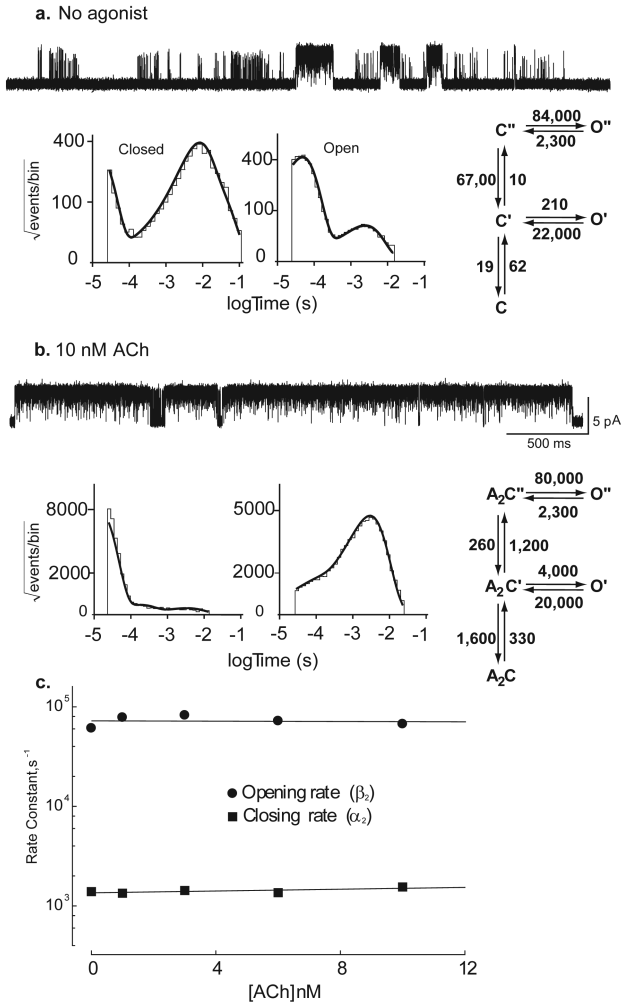

Fig. 1.

Agonist-independent channel gating. **a**, Spontaneous single channel currents through AChR containing Leu-to-Ser mutations at position 9′ of the second transmembrane domain (L9'S) of the β and δ subunits. Cell-attached configuration; membrane potential, -70 mV; bandwidth, 10 kHz; channel openings are upward deflections. Dwell time histograms are shown with probability density functions obtained by fitting the scheme, right, to the dwell times. Rate constants are from Table S1. **b**, Same as **panel a** but with 10 nM ACh. **c**, Plot of fitted channel opening and closing rate constants against ACh concentration (Table S1).

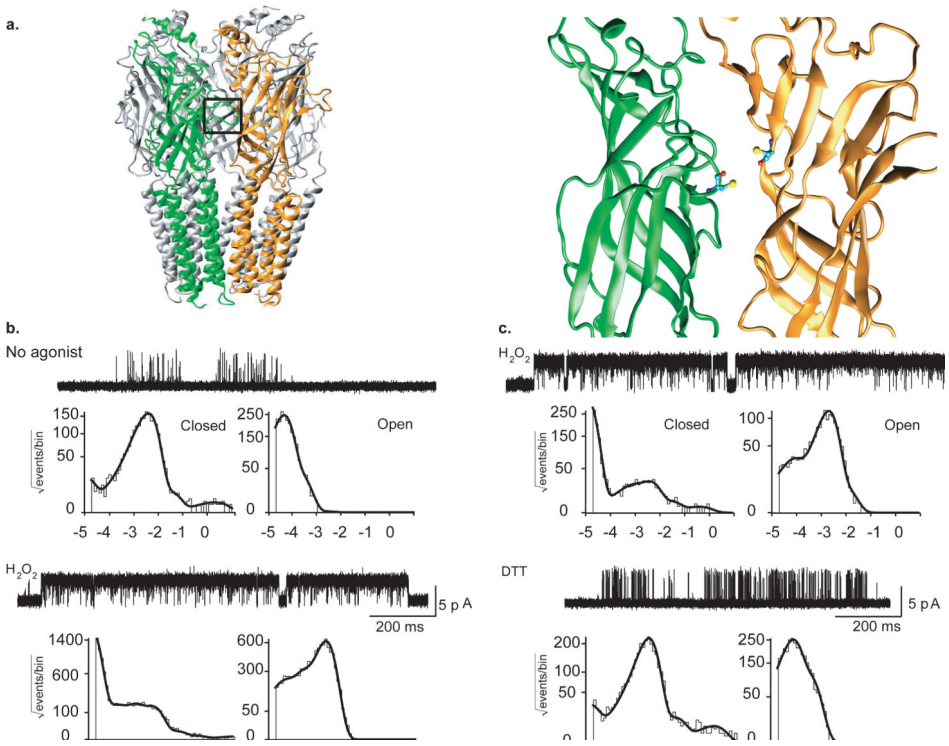

Fig. 2.

Covalent priming of the AChR. **a**, *Torpedo* AChR (PDB code: 2bg9); α-subunit green, δ-subunit orange. Boxed region, magnified right, indicates Cys substitutions at positions α192 and δ121. **b**, upper trace: spontaneous currents through AChR containing L9'S mutations in the β and δ subunits and Cys substitutions at both ACh binding sites. Lower trace: Spontaneous currents from the patch above following application of H_2O_2. **c**, upper trace: spontaneous currents through AChR with L9'S mutations and Cys substitutions at both ACh binding sites treated with H_2O_2. Lower trace: Spontaneous currents from the patch above following application of DTT. In panels **b** and **c**, dwell time histograms are fitted by sums of exponentials. Results are summarized in Table S2.

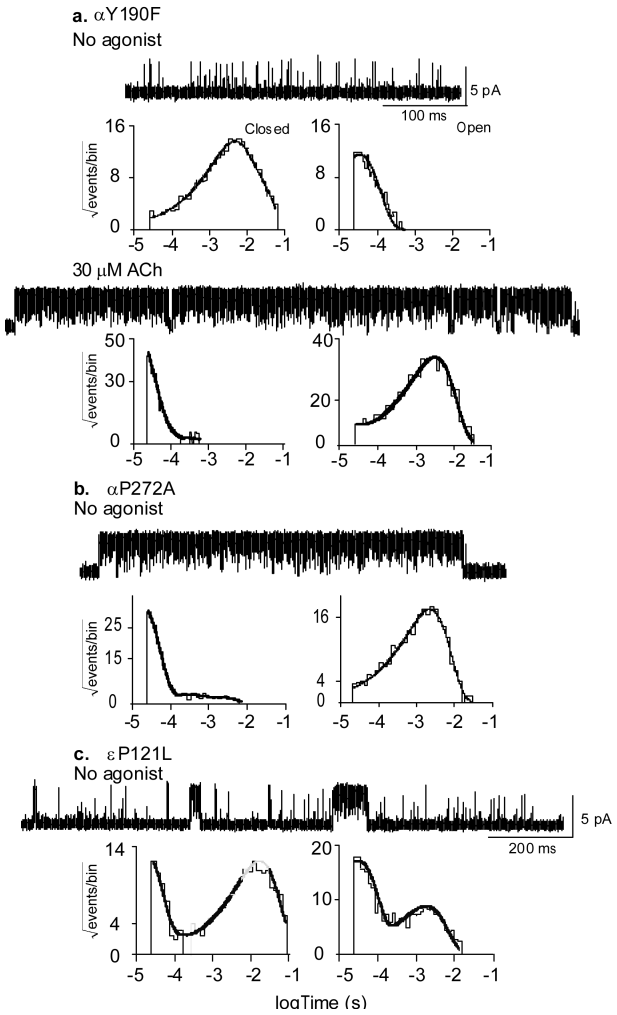

Fig. 3.

Mutating residues linking binding and pore domains increases or decreases priming. **a**, Upper trace: spontaneous single channel currents through AChR containing L9'S mutations in the β and δ subunits and αY190F. Lower trace: ACh-evoked single channel currents from a different patch containing the same mutant AChR. **b**, Spontaneous currents through AChR containing L9'S mutations in the β and δ subunits and αP272A. **c**, Spontaneous currents through AChR containing L9'S mutations in the β and δ subunits and εP121L. Dwell time histograms are shown with fitted probability density functions (Table S1).

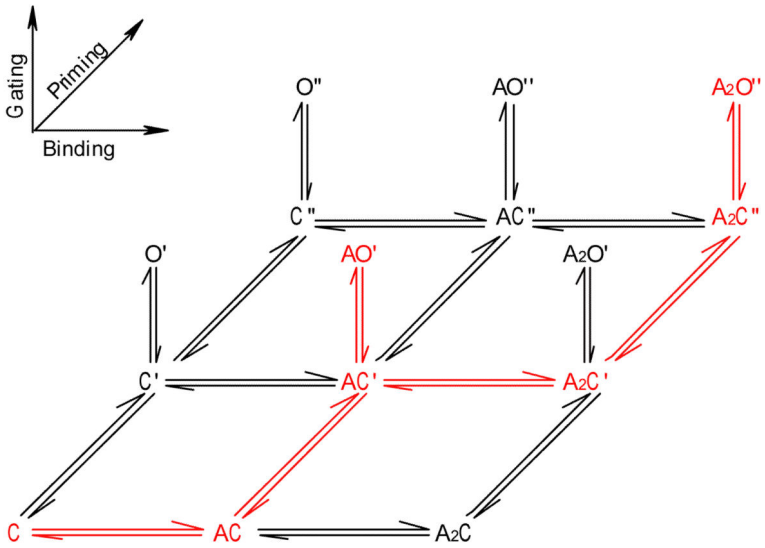

Fig. 4.

Primed model of AChR activation. Agonist binding, priming and channel gating steps are indicated (inset). **C, C**’ and **C**” symbolize closed states, while **O**’ and **O**” symbolize open states. For the wild type AChR in the absence of ACh, the **C**’ and **C**” states are negligible, indicating the first step in the activation process generates **AC**, from which there are three possible paths toward $\mathbf{A_2C}$”. Fitting the path, bind-bind-prime-prime, did not give well defined rate constants, possibly due to an inability to distinguish interconnected $\mathbf{A_2C}$’ and $\mathbf{A_2C}$” states. Fitting the path, bind-prime-prime-bind, also did not give well defined rate constants, possibly because the second binding step would be to a primed site presumed to have reduced accessibility to small molecules. The remaining path in red was fitted to agonist-dependent dwell times from the wild type AChR, yielding the rate constants in Table S4.