Detection and trapping of intermediate states priming nicotinic receptor channel opening

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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 is facilitated by rapid ACh association and dissociation, suggesting a binding site relatively non-selective for small cations. Selective transduction has been thought 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 efficacies¹⁻³. By studying mutant AChRs, we show here 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 an 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 the speed and efficiency of the biological response.

Throughout the nervous system, moment-to-moment communication relies on the 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 ability of the binding site 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.



Modified to include two rather than one agonist-binding steps, 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 the registration of current pulses through single ion channels as brief as tens of





¹Receptor Biology Laboratory, Department of Physiology and Biomedical Engineering, ²Department of Neurology, Mayo Clinic College of Medicine, Rochester, Minnesota 55905, USA. †Present address: Department of Biology, University of Utah, Salt Lake City, Utah 84112, USA. *These authors contributed equally to this work. 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 A₂C state within the extended del Castillo-Katz model⁶. By equating brief closed periods with sojourns in the A₂C state, kinetic modelling 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 efficacies¹, contrary to the interpretation that brief closed periods corresponded to sojourns in the A₂C state. Building on kinetic studies of single glycine-activated receptors¹², 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 efficacies³. 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. Furthermore, we 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 centre of the ionconductive 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 that the temporal sequence of singlechannel 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 (Fig. 1a and Supplementary Fig. 1). Notably, although agonist is not present, transitions from the brief-closed to the longopen 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 (Supplementary Fig. 2). A previous study documented an increase of spontaneous brief and long openings after substitution of Thr for a Ser approximately one turn of the pore helix from the Leu substituted here¹³.

Application of ACh to our pore-mutant receptor increases long and decreases brief openings, with a tenfold 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 and Supplementary Table 1). Thus, transition from the major closed to the biologically relevant open state is independent of the presence of agonist.





Figure 2 | Covalent priming of the AChR. a, *Torpedo* AChR (Protein Data Bank accession 2BG9); green, α subunit; orange, δ subunit. Boxed region (magnified to the right) indicates Cys substitutions at positions α 192 and δ 121. b, Upper trace: spontaneous currents through AChR containing Leu9'Ser mutations in the β and δ subunits, and Cys substitutions at both ACh-binding sites. Lower trace: spontaneous currents from the patch above

after the application of H_2O_2 . **c**, Upper trace: spontaneous currents through AChR with Leu9'Ser mutations and Cys substitutions at both ACh-binding sites after application of H_2O_2 . Lower trace: spontaneous currents from the same patch but after application of dithiothreitol (DTT). In panels **b** and **c**, dwell-time histograms are fitted by sums of exponentials. Results are summarized in Supplementary Table 2.

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 ACh-binding protein, with and without bound agonist, shows that a hairpin structure flanking the binding site, called the C-loop, changes from an uncapped to a capped conformation after binding agonist^{14,15}. 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). After oxidation, however, receptor channels activate in episodes of long openings in quick succession (Fig. 2b and Supplementary Table 2), suggesting that 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 and Supplementary Table 2). Application of oxidizing or reducing reagents had minimal effect on pore-mutant AChRs with fewer than four Cys substitutions (Supplementary Table 3).

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 (data not shown), suggesting that the uncapped conformation of the C-loop predominates, rendering the inter-Cys spacing too great for crosslinking. 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 bidirectional manner.

We further reasoned that the ability to prime should depend on the efficiency of bidirectional 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 aY190F suppresses¹⁶ whereas aP272A enhances17 ACh-induced channel opening. When αY190F is engineered into the pore-mutant AChR, channel opening episodes consist solely of brief openings (Fig. 3a), suggesting that 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 aP272A is engineered into the pore-mutant receptor, channelopening episodes consist solely of long openings (Fig. 3b), suggesting that αP272A promotes priming of both binding sites, analogous to the action of agonist. As a control we tested the mutation EP121L, which suppresses AChR activation of the wild-type AChR but is not a component of the principal linkage pathway¹⁸. When EP121L 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 EP121L (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, whereas unprimed 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



Figure 3 | Mutating residues linking binding and pore domains increases or decreases priming. a, Upper trace: spontaneous single channel currents through AChR containing Leu9'Ser mutations in the β and δ subunits and α Y190F. Lower trace: ACh-evoked single-channel currents from a different patch containing the same mutant AChR. b, Spontaneous currents through AChR containing Leu9'Ser mutations in the β and δ subunits and α P272A. c, Spontaneous currents through AChR containing Leu9'Ser mutations in the β and δ subunits and α P272A. through a subunits and ϵ P121L. Dwell-time histograms are shown with fitted probability density functions (Supplementary Table 1).

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 the long-open state predominate and mimic those that occur in the absence of an agonist.

As a further test, we fitted the primed model to sequences of singlechannel closed and open dwell times obtained from the wild-type AChR activated by a wide range of ACh concentrations. Because not all states in the primed model are 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^{16,19}. Both models provide good descriptions of the dwell-time distributions (Supplementary Table 4 and Supplementary Fig. 3). However the computed log likelihood for the primed



Figure 4 | **Primed model of AChR activation.** Agonist binding, priming and channel gating steps are indicated (inset). C, C' and C'' symbolize closed states, whereas O' and O'' symbolize open states. For the wild-type AChR in the absence of ACh, the C' and C'' states are negligible, indicating that the first step in the activation process generates AC, from which there are three possible paths towards A_2C' . Fitting the path bind–bind–prime–prime did not give well-defined rate constants, possibly owing to an inability to distinguish directly interconnected A_2C' and 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 Supplementary Table 4.

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 that 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 proposed to flip to a transient closed state, similar for all agonists, before the channel could open³. 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 disulphide trapping, we show that capping of each bindingsite 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 because of the bidirectional nature of communication between binding and pore domains. Priming of the AChR is thus a fundamental 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 closings1.3. Priming seems 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 the ability of a neurotransmitter 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 Methods.

Full Methods and any associated references are available in the online version of the paper at www.nature.com/nature.

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

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METHODS

Construction of wild-type and mutant AChRs. Human α , β , δ and ϵ subunit complementary DNAs, subcloned in the CMV-based mammalian expression vector pRBG4 (ref. 20), were described previously²¹. Site-directed mutations were made using the QuickChange mutagenesis kit (Stratagene) 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 DMEM containing fetal bovine serum (10% v/v) 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 μ g ml⁻¹ for non- α -subunits and 1.36 μ g ml⁻¹ 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, CaCl₂ 1.8, MgCl₂ 1.7 and HEPES 10 (pH adjusted to 7.4). The same solution was used to fill patch pipettes. Acetylcholine (Sigma Chemical Co.) 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.), coated with Sylgard 184 (Dow Corning Co.), 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.) at a membrane potential of -70 mV. Data were collected from two to four 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.).

Single-channel kinetic analysis. The digitized current signal was filtered using a 10-kHz digital Gaussian filter²³, 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 filter²³. 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 closing to channel. Kinetic analysis was performed using MIL software (QuB suite, State University of New York), which uses a maximum likelihood method, corrects for missed events and gives error estimates

of the fitted rate constants²⁴. An instrument dead time of $22\,\mu s$ was uniformly applied to all recordings before fitting.

For kinetic analysis of wild-type AChRs, episodes of single channel currents containing at least five openings were analysed for open probability, mean open time and mean closed time, and episodes within two standard deviations of the mean were accepted for further analysis²⁵. Kinetic analysis was performed by fitting models to data obtained across a range of ACh concentrations using MIL software. ACh concentrations ranged from 3 μ M to 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 4,208 (range 2,754–5,628); each kinetic model was fitted to data from all patches simultaneously. Data from different patches at the same ACh concentration were not pooled before fitting.

Disulphide 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 previously²⁶, 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 min).

To demonstrate reversal of the change in single-channel kinetics after oxidation, cells were treated with $8.8 \text{ mM H}_2\text{O}_2$ for 5 min, 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 min)²⁶.

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