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Estimating the pK_a values of basic and acidic side chains in ion channels using electrophysiological recordings: a robust approach to an elusive problem.

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Abstract

As a step toward gaining a better understanding of the physicochemical bases of pK_a -value shifts in ion channels, we have previously proposed a method for estimating the proton affinities of systematically engineered ionizable side chains from the kinetic analysis of single-channel current recordings. We reported that the open-channel current flowing through mutants of the (cation-selective) muscle nicotinic acetylcholine receptor (AChR) engineered to bear single basic residues in the transmembrane portion of the pore domain fluctuates between two levels of conductance. Our observations were consistent with the idea that these fluctuations track directly the alternate protonation–deprotonation of basic side chains: protonation of the introduced basic group would attenuate the single-channel conductance, whereas its deprotonation would restore the wild-type-like level. Thus, analysis of the kinetics of these transitions was interpreted to yield the pK_a values of the substituted side chains. However, other mechanisms can be postulated that would also be consistent with some of our findings but according to which the kinetic analysis of the fluctuations would not yield true pK_a s. Such mechanisms include the pH-dependent interconversion between two conformations of the channel that, while both ion permeable, would support different cation-conduction rates. In this paper, we present experimental evidence for the notion that the fluctuations of the open-channel current observed for the muscle AChR result from the electrostatic interaction between fixed charges and the passing cations rather than from a change in conformation. Hence, we conclude that *bona fide* pK_a values can be obtained from single-channel recordings.

Keywords

electrostatics; proton transfer; pH; patch-clamp technique; single-channel kinetics; nicotinic acetylcholine receptor

INTRODUCTION

Electrostatic interactions are one of the main determinants of structure, solubility, folding stability and function in proteins^{1–4}. These interactions often involve full charges that arise from the protonation of basic side chains or the deprotonation of acidic ones. Establishing whether these protonatable side chains are charged at a given pH using experimental methods, however, has proven difficult, and predictive computational algorithms lose accuracy as the microenvironment around these side chains deviates from bulk water^{5–12}. To some extent, the limitations encountered by these theoretical approaches can be attributed to the incomplete understanding of the factors that cause pK_a s in the interior of proteins to differ from those in bulk water and to the inability to simulate relevant phenomena such as the relaxation of the protein upon proton binding and unbinding. Moreover, in the case of membrane proteins, the paucity of experimentally determined pK_a values that could act as meaningful benchmarks of theoretical calculations adds yet another layer of difficulty.

We have previously studied the effect of engineering basic side chains in the lining of the ion-permeation pathway of the muscle nicotinic acetylcholine receptor (AChR) at the single-channel level^{13–14}. We found that the presence of a single basic group (that is a lysine, an arginine or a histidine) causes the open-channel current through this cation-selective channel to fluctuate between two levels of conductance, with the lower level being favored as the pH is decreased. Interpreting these fluctuations as a direct result of proton binding and unbinding to and from the pore domain led to the notion that the kinetics of protonation and deprotonation of the engineered side chains can be estimated from the kinetics of the oscillations of the open-channel current signal. From here, pK_a values follow trivially from a combination of the ratio of the proton-transfer rates and the pH of the channel-bathing solution.

But other interpretations are possible, too. For example, it has been clearly demonstrated that protonation–deprotonation events of protein side chains can lead to conformational changes that may range from a subtle, local rearrangement of backbone atoms^{15–17} to a global unfolding event¹⁸. Therefore, because the conductance of ion channels is sensitive to the dimensions of the pore domain, it is conceivable that proton-transfer events may lead to current fluctuations as a result of coupled conformational changes rather than as a simple consequence of the electrostatic interaction of charged side chains with the permeating ions. Importantly, if this were indeed the case, the occupancy of either open-channel conductance level would reflect the occupancy of either conformation of the protein, not the side-chain's protonation state, and thus, true pK_a values would not follow directly from the observations.

In this paper, we present compelling evidence for the notion that the pH-dependent current fluctuations recorded from AChRs engineered to bear single ionizable residues in the pore domain indeed track proton-transfer events. Hence, we conclude that *bona fide* pK_a s (as well as the underlying proton association and dissociation rates) can be estimated directly from the kinetics of interconversion between the alternative open-channel conductance levels.

METHODS

DNA clones, mutagenesis and transfection.

HEK-293 cells were transiently transfected with cDNAs encoding the adult muscle-type AChR (mouse $\alpha 1$, $\beta 1$, δ and ϵ subunits, provided by SM Sine, Mayo Clinic College of Medicine, Rochester, MN) using a calcium-phosphate precipitation method. Mutations were engineered using the QuikChange site-directed mutagenesis kit (Stratagene) and were confirmed by dideoxy sequencing. When deemed necessary, a mutation that prolongs the individual activations of the channel (eT264P; ref. 19) was also introduced in the studied AChR constructs to increase the number of proton-transfer events recorded.

Electrophysiological recordings.

Single-channel currents were recorded using the cell-attached configuration of the patch-clamp technique at 22 °C. The solution bathing the transfected cells was (in mM): 142 KCl, 5.4 NaCl, 1.8 CaCl₂, 1.7 MgCl₂ and 10 HEPES/KOH buffer (pH 7.4). The pipette solution used to record currents from mutants having substituted lysine, arginine or histidine residues was identical to that in the bath with the exception of the H⁺ buffer, which changed depending on the desired pH. This buffer was MES (pH 6.0), HEPES (pH 7.4) or TABS (pH 9.0), titrated to a final pH with KOH. The pipette solution used to record currents from mutants having engineered glutamate, aspartate or cysteine residues was identical to that used to obtain recording from basic-residue substituted AChRs with the exception of the divalent cations, Ca²⁺ and Mg²⁺, which were omitted. For all recordings, the pipette solution also contained 1 μ M acetylcholine.

pK_a values.

Single-channel currents were digitized (at 100 kHz), filtered (cascaded cut-off frequency $\approx$ 30 kHz) and idealized (using the SKM algorithm²⁰ in QuB software) to obtain the mean amplitudes of the different current levels and the sequences of dwell times. Protonation and deprotonation rates (as well as all other transition rates) were estimated from maximum-likelihood fits of single-channel dwell-time sequences with kinetic models as described in our previous work^{13,14}. To this end, we used the MIL algorithm²¹ in QuB software with a retrospectively imposed time resolution of 25 μ s. The ratio between the proton-dissociation and proton-association rates thus estimated gives the ratio of the probabilities of the engineered basic or acidic side chain being deprotonated *versus* protonated while the channel is open. pK_a values were calculated from the product of these ratios and the concentration of protons in the pipette solution. These estimates were averaged across a number (*n*) of independent experiments and, unless otherwise indicated, are presented throughout the paper as mean value $\pm$ standard error.

Extent of channel block.

The extent of block was calculated for AChRs bearing engineered basic side chains as the difference between the conductance values of the main level and the sublevel normalized by the conductance of the main level. In turn, these conductances were estimated as the slope of the rectilinear portion of main- and sublevel current–voltage (*I*–*V*) relationships; only

inward currents were analyzed, here. The standard errors of these conductance estimates were used to calculate the standard errors of the extent-of-block values. In some cases, the conductance of the main level could not be estimated with confidence (for example, because the open-channel signal dwelled only briefly and infrequently in the main level). In these cases, the normalization was done relative to the conductance of the background construct (that is, the wild-type AChR with or without the “activation-prolonging” eT264P mutation; see above).

RESULTS AND DISCUSSION

The conductance of ion channels is expected to be affected by the presence of fixed charges in the pore lining. However, single-channel currents through the cation-selective, wild-type muscle AChR do not display any discrete pH-dependent fluctuations that could be ascribed (directly or indirectly) to the alternate binding and unbinding of protons to the many naturally-occurring ionizable residues that decorate the walls of its ion-permeation pathway. At least in part, we attribute this observation to the fact that most native acidic and basic side chains that line the pore segregate to the extracellular and intracellular vestibules where the effect of fixed charges on the passing current is much smaller¹³.

On the other hand, when single basic side chains are engineered in the transmembrane portion of the pore (say, in one of the five M2 α -helices; Fig. 1), the open-channel current alternates between two conductance levels the higher of which is very close to, and in some cases even indistinguishable from, the single level of the wild-type channel. The pH dependence of these fluctuations (Fig. 2; channel openings are downward deflections) indicates that the channel with a “protonated pore” conducts cations at a slower rate than does the channel with a “deprotonated pore”. This raises the possibility that it is the extra positive charge that lowers the channel’s conductance, and therefore, that the fluctuations are a direct result of proton binding and unbinding to and from the engineered basic side chain. In this framework, the phenomenon is described by the kinetic scheme in Figure 3(A). The kinetics of proton transfer are given by:

$$\begin{aligned} &\text{Deprotonation rate} \\ &= \\ &k_{-\text{H}_2\text{O}}[\text{H}_2\text{O}] \\ &+ \\ &k_{-\text{OH}^-}[\text{OH}^-] \\ &+ \\ &k_{-\text{B}^-}[\text{B}^-] \end{aligned} \quad (1)$$

and

$$\begin{aligned}
 &\text{Protonation rate} \\
 &= \\
 &k_{+H_3O^+}[H_3O^+] \\
 &+ \\
 &k_{+H_2O}[H_2O] \\
 &+ \\
 &k_{+BH}[BH]
 \end{aligned} \tag{2}$$

where H_2O , OH^- and B^- (the latter denoting the deprotonated form of the pH buffer) are the three proton acceptors, and H_2O , H_3O^+ and BH are the three proton donors typically present in solutions used for biological assays. [Note from equations 1 and 2 that (unlike the kinetics of binding and unbinding of all other ligands) the dissociation rate of protons is not independent of, and the association rate is not directly proportional to, the concentration of protons]. Furthermore, the ratio between these two rates gives the ratio of deprotonation *versus* protonation probabilities:

$$\begin{aligned}
 &\frac{\text{Deprotonation rate}}{\text{Protonation rate}} \\
 &= \\
 &\frac{\text{Probability of the engineered side chain being deprotonated}}{\text{Probability of the engineered side chain being protonated}}
 \end{aligned} \tag{3}$$

and hence,

$$\begin{aligned}
 &K_a \\
 &= \\
 &\frac{\text{Deprotonation rate}}{\text{Protonation rate}}[H_3O^+]
 \end{aligned} \tag{4}$$

where K_a (10^{-pK_a}) is the acid-dissociation equilibrium constant of the side chain in question. Following this line of reasoning, we estimated the protonation and deprotonation rates of engineered basic side chains from single-channel recordings by fitting sequences of dwell times in the shut, open-protonated and open-deprotonated current levels (denoted in the plotted single-channel current traces as “shut”, “sublevel” and “main level”, respectively) with kinetic models [Fig. 3(A)] using maximum-likelihood methods.

Using the methodology outlined above, we analyzed the kinetics of the current fluctuations caused by basic side chains engineered, one at a time, at 89 different positions along transmembrane segments M1, M2 and M3, and the two intervening linkers of the muscle AChR^{13,14} (Fig. 1). As expected if these fluctuations reflected proton-transfer events, we found that the probability of substituted side chains being protonated at any given pH depends on their position along the channel’s sequence and on the bulk-water pK_a of the introduced side chain. For example, at certain positions of M2, the open-channel current through histidine mutants remained in the high-conductance (wild-type-like) level even at pH 6.0, whereas the open-channel current through mutants bearing lysine substitutions at these same positions oscillated between two conductance levels, and that through arginine mutants appeared to remain fixed in the sublevel even at pH 9.0 [Fig. 2(B)]. This behavior is consistent with a microenvironment that lowers the pK_a of the three side chains (which, in

bulk water, are ~6.4 for histidine ~10.4 for lysine and ~12.5 for arginine) enough to completely deprotonate the imidazol group of histidines but not enough to abstract the strongly bound proton from the guanidinium group of arginines. When mapped onto a structural model of an α -helix, the positions where this type of behavior was observed form two narrow stripes that would correspond to the sides of M2 that are in contact with the adjacent M2 segments. Yet, at another subset of M2 positions, lysine or arginine substitutions lowered the open-channel current amplitude, and no excursions to the main level could be recorded even at pH 9.0, consistent with bulk-water-like pK_a s for these two side chains. Also consistent with an aqueous microenvironment, the open-channel current through AChRs bearing single histidines at these positions did display current fluctuations, and the corresponding pK_a values were only slightly upshifted: on average, the pK_a value at pH 7.4 was estimated to be 6.86 ± 0.32 (mean $\pm$ standard deviation; $n = 7$ different positions). When mapped onto a model of an α -helix, these positions cluster to a single stripe that, based on the behavior of the pK_a s of the three basic side chains, would face the water-filled pore directly.

An alternative explanation

It may be argued that mechanisms other than a direct electrostatic blockade of the currents by the extra proton bound to the channel's pore could also underlie the observed pH-dependent current fluctuations. For example, suppose that ionizable residues engineered along the transmembrane lining of the pore caused the protein to adopt two alternative conformations supporting different ion-conduction rates, say, a wild-type-like value and a lower one. Furthermore, suppose that the pK_a value of the engineered side chain were higher in the lower-conductance conformation, and thus, that this conformation were favored upon proton binding. If this were the case, then single-channel currents through this hypothetical channel would display many of the pH-dependent properties we observed for the engineered AChRs. For example, the probability of the open-channel current signal dwelling in the low conductance level would increase as the pH is decreased and, at any given pH and mutated position, the probability of the current signal dwelling in the low conductance level would be highest for an arginine-substituted channel and lowest for a histidine-substituted one. In this context, the phenomenon would be described by the kinetic scheme in Figure 3(B). Note that, in this case, the two levels of conductance would correspond to two different conformations of the protein rather than to the different protonation states of the substituted side chain. As a result, the ratio of the rates that would be obtained from the kinetic analysis of the open-channel current fluctuations would give the ratio of the probabilities of the channel adopting either conformation regardless of the protonation state. This ratio is given by:

$$\frac{P_{\text{high-conductance conformation}}}{P_{\text{low-conductance conformation}}} = \frac{(K_{a,H} + [H_3O^+])K_{a,L}}{(K_{a,L} + [H_3O^+])K_{a,H}K_{H \rightarrow L}} \quad (5)$$

where $K_{a,H}$ and $K_{a,L}$ are the acid-dissociation equilibrium constants of the engineered ionizable side chain in the conformations supporting a high single-channel conductance and a low single-channel conductance, respectively, and $K_{H \rightarrow L}$ is the equilibrium constant corresponding to the interconversion between these two conformations when the mutated side chain is deprotonated (note that, owing to the detailed-balance constraint, the “protonated counterpart” of this equilibrium constant can be expressed in terms of the other three constants as $K_{H \rightarrow L} \times K_{a,H} / K_{a,L}$). As can be clearly seen, equation 5 differs markedly from equation 4, which (to allow for a direct comparison) can be combined with equation 3 and be rearranged as:

$$\frac{P_{\text{deprotonated}}}{P_{\text{protonated}}} = \frac{K_a}{[H_3O^+]} \quad (6)$$

It follows that, if the observed pH-dependent current oscillations reflected the ability of the channel to adopt alternative conformations, rather than the different protonation states of a side chain, then the product of the proton concentration and the ratio of rates we calculate from the electrophysiological recordings would not give the K_a of a side chain but a more complicated (and less useful) quantity, instead. Certainly, compare equation 4 with the product of the right-hand side of equation 5 and the concentration of protons.

Figure 4 shows the predictions of the two mechanisms discussed above by plotting equations 5 and 6 as a function of pH. For these two plots, we assumed that neither the pK_a values nor the conformational-change equilibrium constant, $K_{H \rightarrow L}$, change with proton concentration. In the case of the “electrostatic-block mechanism” (blue line), the plotted ratio changes as a function of pH as a straight line (slope = -1) that intercepts both the y and x axes at the pK_a value of the side chain in question ($pK_a = 6.0$, in this particular example). In the case of the “conformational-change mechanism” (red line), three types of behavior are predicted. At high and low pH values, the plotted ratio becomes independent of pH as the engineered ionizable side chain approaches full (de)protonation. At intermediate pH values, on the other hand (and provided that proton binding favors the low-conductance conformation), the plotted ratio decreases from one asymptote to the other one as the pH is increased. Interestingly, it can be seen that a range of pH values exists (indicated in the figure with a gray background) within which the plotted ratios are expected to vary as a function of proton concentration with similar pH dependence regardless of the underlying mechanism. We conclude that, depending on the end values of this pH range relative to the range of pH values that is experimentally accessible, it may be impossible to discriminate between these two models solely on the basis of plots like those in Figure 4. Importantly, mistaking one mechanism for the other one would lead to meaningless pK_a -value estimates. For example, applying equation 4, which pertains to the “electrostatic-block mechanism”, to the red-red-line plot in Figure 4 at pH 7.4 yields a (single) pK_a of ~ 9.0 when, actually, the model consists of two pK_a s (6.0 and 10.0) and two conformational-change equilibrium constants (0.1 and 1,000).

Different protonation states or different conformations?

Below, we present three separate lines of experimental evidence that support the idea that the open-channel current fluctuations recorded from AChRs engineered to bear single ionizable side chains in the transmembrane-pore lining reflect protonation–deprotonation events and that their kinetic analysis leads, using equation 4, to true pK_a s.

On the one hand, we found that the extent to which the single-channel current is attenuated in the low-conductance level relative to the (wild-type-like) high-conductance level is a function of the distance between the substituted basic group and the long axis of the pore. Indeed, a plot of the degree to which the currents are attenuated (referred here to as the “extent of channel block”) as a function of position along the M2 α -helix (Fig. 1) results in a nearly ideal α -helical pattern [Fig. 5(A); gray symbols]. This finding is entirely consistent with the expectation that the electrostatic interaction between the positively charged side chains and the permeating cations is greatest for substitutions along the pore lumen-facing stripe of the helix, smallest for substitutions along the helix’s backside, and intermediate at all other positions. Furthermore, when single basic side chains were systematically substituted, one position at a time, along the M1 and M3 transmembrane segments (Fig. 1), current attenuation was observed only for a subset of positions that map to a narrow stripe in each helix, and the extent of block was similar to that exerted by basic side chains engineered along the sides or backside of M2 [Fig. 5(A); red and black symbols, respectively]. This result is consistent with the known location of the M1 and M3 segments relative to M2 and the long axis of the pore²², and suggests that these stripes of M1 and M3 point toward the channel’s lumen in the open-channel conformation¹⁴. Needless to say, a conformational change (however graded) is extremely unlikely to underlie the observed α -helical periodicities.

Another strong evidence for the notion that the attenuation of the single-channel currents results from the electrostatic interaction between the positively charged basic side chains and the passing cations is the effect of substituted acidic residues. As indicated above, the effect of systematically substituted basic side chains along M2 on the single-channel currents led to the identification of the rotational angle of this α -helix in the open-channel conformation. When glutamates or aspartates were engineered at positions of M2 expected to face the aqueous lumen of the pore, the open-channel current also displayed pH-dependent interconversions between two conductance levels, but this time, the current fluctuated between a wild-type-like level and a level of even higher conductance [Fig. 5(B,C)]. This is exactly what is expected from the fluctuating charge on acidic side chains and from the electrostatic effect of negative charges on the permeation rate of cations. Again, it seems very unlikely that conformational changes would selectively lead to a decreased conductance upon the introduction of basic residues and to an increased conductance upon the introduction of acidic residues.

With this in mind, even a cursory inspection of the current traces in Figure 5(B,C) reveals that glutamates engineered at pore-facing positions of the M2 segment of the δ subunit are mostly protonated at pH 7.4 (and thus, mostly neutral) despite the expected water-filled microenvironment around these side chains but consistent with the anion-rejecting properties of a cation-selective pore. Kinetic analysis of the current fluctuations indicated that the pK_a s

of these glutamate side chains [8.02 ± 0.02 ($n = 4$) at position $\delta 262$ and 7.97 ± 0.06 ($n = 5$) at position $\delta 269$] are upshifted by >3.5 units relative to the bulk-water value of ~ 4.4 . We also measured the proton affinity of an aspartate (pK_a in bulk water $\cong 4.0$) engineered at position $\delta 269$ and found that the pK_a upshift is comparable to that of a glutamate at this position; indeed, this pK_a turned out to be 7.81 ± 0.01 ($n = 4$). Consistent with the pK_a shifts of these acidic side chains, when cysteines (pK_a in bulk water $\cong 8.3$) were substituted at these same pore-facing positions along $\delta M2$, the open-channel current appeared to remain fixed at the wild-type-like level even at pH 9.0. This indicates that the deprotonation events of the (poorly acidic) thiol group of cysteine were so brief and/or infrequent that they could not be detected.

Yet a third line of experimental evidence for the electrostatic origin of the alternative conductance levels is the effect of multiple basic-residue substitutions. For example, when pairs of basic residues were engineered along the M2 segments, three alternative open-channel conductance levels could now be observed. It could be argued, however, that if ionizable side chains were able to swing into and out of the pore lumen in such a way as to partially occlude and de-occlude the pore, then the rearrangement of several such side chains could give rise to several conductance levels. To address this point, we engineered a lysine in the M1 segment (at position 225) of the two $\alpha 1$ muscle AChR subunits (Fig. 1). At this position, a lysine side chain is expected to be too far from the channel's long axis so as to hinder ion conduction sterically. Recordings from this double mutant, however, exhibited three well-defined and pH-dependent open-channel conductance levels [Fig. 5(D)]. This leads us to propose that the multiplicity of conductance levels stems, simply, from the multiplicity of engineered proton-binding sites. Most likely, the three open-channel levels reflect the protonation of both, one, or none of the substituted basic side chains rather than associated conformational changes.

Concluding remarks

One of the clear advantages of the electrophysiological approach discussed here is that it allows the estimation of side-chain pK_a values of physiologically-relevant ion channels embedded in their native membrane environment, in the context of living cells, and in an unequivocally-defined functional state (namely, the open-channel conformation). Furthermore, our method generates estimates of the individual rates of side-chain protonation and deprotonation, thus adding an additional layer of information that is not provided by methodologies that only deal with equilibrium quantities.

An evident limitation of our approach, though, is that its use is restricted to the transmembrane portion of the pore. Indeed, pK_a measurements can only be performed at the subset of positions along the ion-permeation pathway where single fixed charges attenuate or enhance the passing current to a measurable degree. However, to the extent that it is the properties of the transmembrane region of the protein that are most elusive, our experimental data and future applications of this methodology are expected to play a fundamental role in the elucidation of the physicochemical bases of pK_a -value shifts in this important class of membrane protein.

Lastly, we realize that reproducing the highly shifted pK_a s in our dataset^{13,14} using theoretical calculations remains a formidable challenge. However, inasmuch as high-resolution structural models of the AChR are forthcoming and a large set of meaningful benchmarks is now available, it seems that such an endeavor should no longer be intractable.

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Figure 1. The AChR channel.

(A) Membrane-threading pattern common to all Cys-loop receptors, a superfamily of pentameric ligand-gated ion channels that, in vertebrates, includes receptors to acetylcholine, serotonin, γ -aminobutyric acid and glycine. (B) Ribbon representation of the transmembrane portion of the PDB file 2BG9 (4-Å resolution; ref. 22), a model of the AChR from the electric organ of *Torpedo marmorata* (a muscle-type AChR) in the absence of activating ligands as viewed from the extracellular side. The color code is the same as in (A). In adult muscle, the γ subunit is replaced by the ϵ subunit; thus, the stoichiometry of the adult-type AChR is $(\alpha 1)_2\beta 1\delta\epsilon$. The molecular image was made with VMD (ref. 23).

Figure 2. pH-dependent fluctuations of the open-channel current.

Upon engineering single basic residues in the pore lining of the (cation-selective) AChR, the open-channel current fluctuates between two levels of different conductance: a wild-type-like level (the “main level”) and a level of lower conductance (the “sublevel”). (A) Effect of pH. Single-channel inward currents recorded from the $\delta A266K$ AChR mutant using pipette solutions of different pH, at ~ -100 mV (negative inside the cell). One representative burst of channel openings is shown at each pH. Residue $\delta A266$ is in the M2 segment of the δ subunit, approximately halfway across the membrane width. As the concentration of protons increases so does the probability of the open-channel current signal dwelling in the sublevel. The pK_a of a lysine substituted at this position (which, using the prime-numbering system, is referred to as position $10'$) was estimated to be 7.53 ± 0.01 ($n = 8$), at pH 7.4 (ref. 13). (B) Effect of type of basic side chain. Inward currents recorded at ~ -100 mV; one representative burst of openings is shown for each mutant. The probability of the open-channel current signal dwelling in the sublevel is highest for the arginine-substituted, and lowest for the histidine-substituted, $\delta S268$ mutant AChR. Residue 268 of the δ subunit corresponds to position $12'$ of M2. At pH 7.4, fluctuations of the open-channel current signal between the main level and a sublevel could only be detected (and hence, a pK_a could only be estimated) for the lysine mutant; the pK_a was found to be 8.87 ± 0.01 ($n = 4$) (ref. 13). All current traces are displayed at a cut-off frequency of ~ 6 kHz.

Figure 3. Two possible mechanisms.

Different mechanisms can account for pH-dependent fluctuations of the open-channel current. (A) The channel can adopt ion-conductive (“open”) and non-conductive (“shut”) conformations, which, in turn, can have the substituted ionizable residue in a protonated or deprotonated state. The alternate protonation and deprotonation of the engineered titratable group while the channel is open (see gray-shaded boxes) may manifest as fluctuations of the open-channel current; proton-transfer events occurring while the channel is shut would go undetected. The kinetic scheme in (A) illustrates this mechanism for the particular case of substituted basic side chains (hence, the positive charge on the protonated forms) but the same type of mechanism applies to acidic side chains. (B) While open, the channel can adopt a high-conductance or a low-conductance conformation (denoted as “Open_H” and “Open_L”, respectively), which, in turn, can have the substituted ionizable residue in a protonated or deprotonated state. The alternate occupancy of either conformation, irrespective of the protonation state of the substituted side chain (see gray-shaded boxes), would give rise to fluctuations in the open-channel current. If the pK_a values of the side chain in question differed in these two conformations, then the probability of the channel occupying either conformation would be a function of pH. For example, if the pK_a were higher in the low-conductance conformation, then the probability of the channel adopting this conformation would increase as the pH decreases. The equilibrium constant corresponding to the interconversion between the two conformations of different conductance when the substituted side chain is protonated can be expressed in terms of the other three equilibrium constants around the cycle. For clarity, neither the proton donors/acceptors nor the shut states are shown.

Figure 4. Different mechanisms, similar pH dependences.

Whether reflecting the fluctuating protonation state of a pore's ionizable side chain or the alternative conformations of the channel's pore domain, the interconversion between different levels of conductance may be sensitive to pH. In blue, the plot shows the ratio of deprotonated *versus* protonated forms of a hypothetical side chain whose alternate charging and uncharging leads to two different conductance levels according to the “electrostatic-block mechanism” shown in Figure 3(A); in this example, $pK_a = 6.0$. In red, the plot shows the ratio of high-conductance *versus* low-conductance conformations of a hypothetical channel that can switch between these forms in a pH-dependent manner according to the “conformational-change mechanism” in Figure 3(B); in this example, $pK_{a,H} = 6.0$, $pK_{a,L} = 10.0$ and $K_{H \rightarrow L} = 0.1$. Note that, regardless of the underlying mechanism, a pH range exists within which the computed ratio is predicted to vary with pH in a remarkably similar fashion.

Figure 5. Experimental evidence for the electrostatic-block mechanism.

(A) Extent of channel block. The extent to which the single-channel conductance is attenuated in the sublevel relative to the main level as a result of engineering single lysines in the AChR pore domain is plotted as a function of the mutated position along M1 (open red circles), M2 (including the M1–M2 linker and part of the M2–M3 linker; filled gray circles) and M3 (including the rest of the M2–M3 linker; filled black circles). Most of the plotted extent-of-block values correspond to the effect of substitutions in the δ subunit. Positions in and flanking M2 are denoted using the prime-numbering system according to which, in the δ subunit, proline 250 corresponds to position $-7'$ whereas threonine 281 corresponds to $25'$ (we omit the $-3'$ label in the δ subunit). Positions in and flanking M1 or M3 are denoted using the δ -subunit residue numbers. The unbroken line joining the gray symbols is a cubic-spline interpolation. Proposed membrane boundaries and the relative depths of the three helices in the membrane are tentative. For most positions, the horizontal error bars (standard errors) are smaller than the symbols. The lumen of the pore would be to the right of the plot; see also ref. 14. (B) Single-channel inward currents recorded from the δ S262E AChR mutant using pipette solutions of different pH, at ~ 100 mV. This construct also included the T264P mutation in the ϵ subunit (see Methods). As the concentration of protons increases so does the probability of the open-channel current signal dwelling in the (wild-type-like) main level. The high-conductance level (154 ± 2 pS; main-level conductance = 114 ± 1 pS) is denoted as a “superlevel”. Residue 262 of the δ subunit corresponds to position $6'$ of M2. The pK_a of a lysine substituted at this position could not be estimated because the open-channel current signal appeared to remain fixed at the low-

conductance level even at pH 9.0. Thus, in order to estimate the pK_a of a basic side chain at this position, we engineered a histidine; its pK_a was estimated to be 7.30 ± 0.01 ($n = 3$), at pH 7.4 (ref. 13). (C) Single-channel inward currents recorded from the $\delta V269E$ AChR mutant using pipette solutions of different pH, at ~ -100 mV. The conductance of the superlevel was found to be 166 ± 1 pS (main-level conductance = 127 ± 1 pS). Residue 269 of the δ subunit corresponds to position 13' of M2. As was the case for position 6', the pK_a of a lysine substituted at position 13' could not be estimated even at pH 9.0. The pK_a of a histidine at this position was estimated to be 6.68 ± 0.03 ($n = 3$), at pH 7.4 (ref. 13). (D) Single-channel inward currents recorded from the $\alpha 1F225K$ AChR mutant using pipette solutions of different pH, at ~ -100 mV. The two $\alpha 1$ subunits of the channel contained the substituted lysine. Note that the presence of two lysines (in this case, in M1) gives rise to two conductance sublevels. For panels (B), (C) and (D), one representative burst of channel openings is shown at each pH (display cut-off frequency ≈ 6 kHz). The larger amplitude of the main-level current through acidic residue-substituted AChRs [this figure, panels (B) and (C)] compared to that through the main level of basic residue-substituted channels [this figure, panel (D), and Fig. 2] is simply due to the different pipette solutions employed in each case. Currents through acidic residue-substituted AChRs were recorded in the nominal absence of Ca^{2+} or Mg^{2+} in the pipette solution (see Methods). Even at low millimolar concentrations, these divalent cations reduce the amplitude of single-channel channel currents through the AChR²⁴.