Mutations that stabilize the open state of the Erwinia chrisanthemi ligand-gated ion channel fail to change the conformation of the pore domain in crystals

Giovanni Gonzalez-Gutierrez, Tiit Lukk, Vinayak Agarwal, David Papke, Satish K. Nair, and Claudio Grosman

The determination of structural models of the various stable states of an ion channel is a key step toward the characterization of its conformational dynamics. In the case of nicotinic-type receptors, despite different structures have been solved but, thus far, these different models have been obtained from different members of the superfamily. In the case of the bacterial member ELIC, a cysteamine-gated channel from Erwinia chrisanthemi, a structural model of the protein in the absence of activating ligand (and thus, conceivably corresponding to the closed state of this channel) has been previously generated. In this article, electrophysiological characterization of ELIC mutants allowed us to identify pore mutations that slow down the time course of desensitization to the extent that the channel seems not to desensitize at all for the duration of the agonist applications (>20 min). Thus, it seems reasonable to conclude that the probability of ELIC occupying the closed state is much lower for the ligand-bound mutants than for the unliganded wild-type channel.

To gain insight into the conformation adopted by ELIC under these conditions, we solved the crystal structures of two of these mutants in the presence of a concentration of cysteamine that elicits an intra-cluster open probability of ∼0.9. Curiously, the obtained structural models turned out to be nearly indistinguishable from the model of the wild-type channel in the absence of bound agonist. Overall, our findings bring to light the limited power of functional studies in intact membranes when it comes to inferring the functional state of a channel in a crystal, at least in the case of the nicotinic-receptor superfamily.
of the ligand-bound channel to the extent that no net desensitization could be observed for the duration of our electrophysiological recordings. However, aside from the mutated side chains, no difference could be appreciated between the structural models of these open-state stabilizing mutants and that of the wild-type ELIC channel in the absence of activating ligand (9). Several scenarios that may account for this puzzling finding are discussed.

Results

Two main structural models of bacterial nicotinic-type receptors have been inferred from X-ray crystallographic data, thus far: that of the unliganded ELIC channel (PDB ID code: 2VL0), which was deemed to represent the closed-channel conformation (9), and that of the proton-gated GLIC channel in the presence of a desensitizing concentration of protons (PDB ID codes: 3EAM and 3EHZ), which was deemed to represent the open state (5, 6). These models have been interpreted to represent the end states of the closed ⇌ open conformational transition (5, 6), and thus, form the basis of recently proposed channel-gating mechanisms. However, it may be argued that it would be desirable to obtain crystal structures of the same channel in the different functional states. Because favoring the crystallization of specific functional states of these channels using ligands has so far proved ineffective, we set out to test the effect of mutations. Here, we present data on ELIC.

Because the 2VL0 model of ELIC was obtained from crystals grown in the absence of activating ligands, it seems reasonable to assume that the crystallized conformation represents the closed state, at least, of this particular channel. Hence, we decided to engineer mutations to ELIC that would tilt the conformational free-energy landscape away from the closed state. In the absence of much data on the effect of mutations on ELIC function, it also seemed reasonable to take advantage of the wealth of information that is available for the eukaryotic members of the superfamily. In these channels (to our knowledge, without exception; e.g., see refs. 11–24), mutation of the aliphatic residues that line the extracellular half of the transmembrane pore (between positions 9′ and 20′ of M2) to residues bearing shorter or more polar side chains has been found to cause a dramatic increase in the closed ⇌ open equilibrium constant; the effect on channel desensitization has been less well documented. It is this increase in gating equilibrium constant that leads to the well-known lower EC50 values, the much higher frequency of unliganded openings, and the prolongation of the mean duration of bursts of single-channel openings (or, in macroscopic terms, the slowing of the deactivation time course) that are characteristic of these pore mutants.

Remarkably, when engineered in all five subunits of ELIC, the leucine-to-alanine mutation at position 9′ of the M2 α-helix, leads to a faster—not slower—deactivation time course upon cysteamine (that is, the agonist) washout (Fig. 1 A and B). This finding is highly surprising because even the deactivation time course of the bacterial GLIC channel is prolonged by an isoform of cysteamine for 1 min. (Fig. 1 C and D). This effect of the leucine-to-alanine mutation at position 9′ on the kinetics of desensitization is unsurprising inasmuch as similar findings have been reported for other members of the nicotinic-receptor superfamily; here, we show that this is also the case for GLIC (Fig. S2B).

Incidentally, we noticed that both wild-type and L9′A mutant ELIC channels, heterologously expressed in Xenopus oocytes, activate extremely slowly in response to cysteamine-concentration jumps from 0 to 10 mM (Fig. 2 A and B): the time constants of monoeponential fits to the rising phase of the macroscopic currents are 84.6 ± 7.0 ms (n = 15) and 58.6 ± 13.1 (n = 9), respectively. These values are much slower than those of the

![Fig. 1. Effect of canonical gain-of-function mutations on ELIC. The kinetics of deactivation and desensitization of ELIC wild-type and pore mutants were studied in fast-perfused outside-out patches of membrane (solution-exchange time of 90 s, <150 μs) excised from Xenopus oocytes. The pH on both sides of the membrane was 7.4. According to the prime-numbering system, residues 240 and 247 correspond to positions 9′ and 16′ of M2, respectively (Fig. S1 and S1 Materials and Methods). (A and B) Kinetics of deactivation. The displayed time courses (shown normalized) were fitted with monoeponential functions; instead, extent-of-desensitization values (mean ± SEs) are shown. (C) Normalized time courses of currents elicited by the application of cysteamine for 5 s. (D)Extent of desensitization. Because the macroscopic current responses of the mutants decay negligibly upon exposure to high concentrations of cysteamine, their time courses could not be fitted to exponential-decay functions; instead, extent-of-desensitization values (mean ± SE) were calculated. (E and F) Time courses of currents elicited by the application of cysteamine for 1 min. (G) Extent of desensitization values (mean ± SE) corresponding to the macroscopic traces in E and F. (H) Time course of the current elicited by the application of cysteamine for 20 min. To prolong the lifetime of the patch of membrane, the potential (~80 mV) was applied only briefly during the 20-min application of agonist, as indicated above the current trace. For all other panels, the potential was held constant at ~80 mV. The color code is the same for all panels.](image-url)
most-slowly activating eukaryotic members of the superfamily, such as the α7nACh receptor (3.1 ± 0.2 ms; n = 5; jumps in ACh concentration from 0 to 1 mM) and the serotonin type 3A receptor (7.3 ms; jumps in serotonin concentration from 0 to 100 μM) (25), upon exposure to comparably fast agonist applications. To make sure that 10 mM cysteamine was high enough a concentration, we increased the concentration of agonist to 100 mM (Fig. 2 C and D), and no changes in the activation time course were observed (the values of cysteamine concentration given here correspond to those estimated colorimetrically by determining the amount of 5-thio-2-nitrobenzoate released upon reaction with GSH). To rule out a possibility that problems with the perfusion system slowed down the time course of the recorded responses, we transfected HEK-293 cells with cDNA coding the (adult-type) muscle AChR, and estimated the kinetics of activation. As expected for this fast synaptic channel, we found that activation proceeds very rapidly upon stepping the concentration of ACh from 0 to 100 μM (Fig. 2 C and D), to the extent that its time course is likely to be limited by the kinetics of the solution exchange at the tip of the patch pipette. Finally, for comparison, we analyzed the activation time course of GLIC upon stepping the extracellular pH from 7.4 to 4.5 (Fig. 2 C and D) and found that the time constant of a monoexponential fit is 18.9 ± 5.6 ms (n = 11). We conclude that the slow activation of ELIC is an intrinsic property of this channel.

In an attempt to better understand the “nondesensitizing” phenotype of the L9′A mutant, we turned to single-channel recordings (Fig. 3). At the single-channel level, the activity of wild-type ELIC in the presence of 10 mM cysteamine occurs as series of several openings in quick succession interrupted by long shuttings. This grouping of consecutive openings from individual channels in the presence of high concentrations of agonist (“clusters”) is highly reminiscent of the single-channel behavior of the eukaryotic members of the superfamily (27), and thus, it is tempting to identify the longer shut intervals as sojourns in desensitized conformations. With this in mind, we examined single-channel recordings from the ELIC L240A mutant and found that openings still occur as clusters. Our limited understanding of this channel precludes us from estimating how many of the channels in the group are closed, how many are open, and how many are desensitized. Certainly, among the known eukaryotic members of the superfamily (27), and thus, it is tempting to identify the longer shut intervals as sojourns in desensitized conformations.

The lining of the extracellular half of ELIC is unique in that it contains a full ring of phenylalanines at the pore-facing position 16′ (4) (Fig. S1A). Certainly, among the known eukaryotic members of the superfamily, a full ring of phenylalanines is

**Fig. 2.** ELIC currents activate slowly. (A–D) Kinetics of activation studied in fast-perfused outside-out patches of membrane at ~80 mM. The pH on both sides of the membrane was 7.4. The time courses (shown normalized) were fitted with monoexponential functions, and the corresponding time constants (± SE) are shown.

**Fig. 3.** Single-channel behavior. (A–D) Stretches of single-channel currents recorded in the outside-in configuration (Xenopus oocyte membranes) at ~80 mM, in the continued presence of cysteamine. The pH on both sides of the membrane was 7.4. Channel openings are downward deflections. Display f, ~5 kHz. The calibration bars are the same for all traces (with the exception of those in the Insets). (E) Intracluster open probabilities (mean ± SE). In the absence of a more detailed understanding of the single-channel behavior of these constructs, clusters were defined (somewhat arbitrarily) as series of openings separated by shuttings shorter than 200 ms. Our conclusions would not change if we defined clusters using a critical time of 20 ms, instead.
present at this position only in a few channels from nematodes whose functional properties remain unexplored. Engineering such a ring at position 16′ of the mouse-muscle AChR causes the channel to adopt a highly stable refractory state that seems to be distinct from the well-known desensitized conformation (4). We considered, then, the possibility that the presence of these aromatic side chains in ELIC could compensate for the effect of shortening the leucines at 9′, thus preventing the leucine-to-alanine mutation from increasing the gating equilibrium constant of this channel. To test this idea, we mutated the phenylalanine at position 16′ of ELIC to leucine, one of the most common amino acids at this position in the cation-selective subset of eukaryotic nicotinic-type receptors (Fig. S1A). Electrophysiological characterization of this double mutant revealed little, if any, additional effect of the mutation at 16′ (Figs. 1–3): most importantly, deactivation remains faster than the wild-type’s and desensitization remains remarkably slow, with little (if any) decay of the current response on application of 10 mM cysteamine for 20 min (Fig. 1H). The negligible effect of this mutation on the kinetics of deactivation is unexpected too, because, for example, mutating the ring of four leucines and one phenylalanine at position 16′ of the mouse-muscle AChR to a full ring of alanines slows down channel deactivation by a factor of ∼18 (Fig. S3). Furthermore, in the zebrafish-muscle AChR, mutating (only) the two N-terminal leucines at this position to proline slows down deactivation by a factor of ∼4 (28).

Other substitutions at position 9′ of ELIC give rise to, essentially, the same results. Indeed, substituting a serine at this position (in the background of the phenylalanine-to-leucine mutation at position 16′) (Figs. 1–3) yields a mutant that, like the alanine-substituted channel, deactivates faster than the wild-type. In stark contrast, a leucine-to-serine mutation at position 9′ of the mouse-muscle AChR causes a dramatic increase in the gating equilibrium constant, leading to a much-prolonged mean duration of bursts of single-channel openings (e.g., ref. 13). It is worth emphasizing that the muscle AChR is merely used here as an example: mutation of the 9′ aliphatic side chain to a shorter or polar one has been shown to lead to a “gain-of-function” phenotype in all eukaryotic members of the superfamily in which the effect of this type of mutation has been tested.

Peculiarities of the bacterial channels aside, the results above led us to identify a combination of mutations that, in the continued presence of a high concentration of cysteamine, greatly prolong the occupancy of the open-channel conformation. Of course, we cannot rule out the possibility that these mutants desensitize completely upon even longer incubations with agonist of the sort that is needed for protein crystals to grow. Whatever the case might be, though, it seems sensible to conclude that the probability of ELIC occupying the closed-channel state is much lower for the ligand-bound mutants than it is for the unliganded wild-type channel. Because we wanted to obtain structural information about states other than the closed-channel conformation, we set out to crystallize the two double-mutant variants of ELIC (L9′A + F16′L and L9′S + F16′L) in the presence of cysteamine.

The best-diffracting crystals were those grown in the presence of 10 mM cysteamine at pH 6.5 to 6.9. To assess the effect of this lower pH on channel function (note that the electrophysiological characterization presented above was carried out at pH 7.4), we recorded currents from the L9′A + F16′L double mutant in the presence of an external solution of pH 6.5 containing the same proton buffer as that used in the reservoir solution [that is, N-(2-acetamido)iminodiacetic acid; see SI Materials and Methods]. Interestingly, we found that the change of external pH from 7.4 to 6.5 (in the presence of 10 mM cysteamine) increases the intracluster open probability of the double mutant from ∼0.44 to ∼0.93 (Figs. 3 C and E and 4 A and B); the desensitization time course remains unaffected (Fig. 4 C and D). This effect of pH on channel-gating is highly advantageous in the context of this work because it increases the contribution of the open-channel state to the mixture of interconverting conformations. At pH 7.4, on the other hand, even a concentration of cysteamine as high as 50 mM only elicits an open probability of ∼0.75 (Fig. S4). A more detailed analysis of the effect of protons (and, perhaps, of the proton buffer itself) on the cysteamine-gated activity of ELIC is needed. However, with cysteamine pK_{a1} and pK_{a2} values of ∼8.60 and ∼10.75, it is clear that the effect of low pH on the L9′A + F16′L double mutant cannot be ascribed to the increased concentration of the fully protonated form of this ligand. Certainly, the latter is expected to account for almost all of the cysteamine present in solution already at pH 7.4 (e.g., ∼9.4 mM for a total concentration of 10 mM).

The crystals from the two double mutants diffracted to 3.8 Å (L9′S + F16′L) and 4.2 Å (L9′A + F16′L), a resolution that, albeit low, is comparable to that of other X-ray crystal structures of agonist-bound (10) and full-length (29, 30) bacterial channels. Evidently, this resolution is too low to allow the determination of the conformation of individual side chains or to detect the rearrangement of loop regions unambiguously, but it is enough to detect large changes in the orientation of the transmembrane α-helices of the kind that would make the pore of ELIC look like that of GLIC. A comparison of the structural models of the mutants with that of unliganded wild-type ELIC, however, failed
the pore (mean ± SE of all subunits) for residues in andanking the trans-
membrane α-helices of the M2 segments M2 of the four constructs compared in
position 20 of the pore mutants. The value of zero along the distance axis corresponds to
the center of the pore. The value of zero along the distance axis corresponds to
the center of the pore. The color code is the same as that in C. The profile of GLIC in the
presence of a desensitizing concentration of protons (PDB ID code: 3EAM; in
purple) is also included. (F) Distances from the Cα atoms in the long axis of the pore (mean ± SE of all subunits) for residues in and flanking the trans-
membrane segments M2 of the four constructs compared in E. The color code is the same as that in E. The distance profile of the AChR from
Torpedo in the absence of activating ligands (PDB ID code: 2BG9, in black) is also included; this profile is nearly indistinguishable from that of GLIC. The three profiles of ELIC (corresponding to the unliganded wild-type and the two double mutants in the presence of 10 mM cysteamine at pH ~6.5) are also indistinguishable from each other.

Gonzalez-Gutierrez et al. VOL. 109, NO. 16, 2012 | 6335

PHYSIOLOGY

Fig. 5. X-ray crystal structures of two mutants of ELIC in the presence of 10 mM cysteamine. (A and B) Ribbon representation of the transmembrane region of the indicated structural models. The mesh shows the solvent-accessible surface. The side chains corresponding to pore-facing positions 9, 16, and 20 (that is, the side chains forming the three narrowest con-
strictions of the pore in unliganded wild-type ELIC) are shown in ball-and-stick representation. The front subunit was removed, for clarity. (Upper) Extracellular. (Lower) Intracellular. The corresponding representation of unliganded wild-type ELIC is shown in Fig. S5A. (C) Aligned Cα traces. Only one subunit per construct is shown. (D) Distance between aligned Cα atoms. The Cα atoms of each subunit in the structural models of the mutants L9A +
F16L and L9S + F16L in the presence of 10 mM cysteamine were aligned with those of the unliganded wild-type ELIC (mutant chain A with wild-type chain A, mutant chain B with wild-type chain B, and so on, for all 10 subunits in the asymmetric unit), and the distances between Cα atoms were calcu-
lated and averaged. (E) HOLE profiles (31) of ELIC wild-type and the two pore mutants. The value of zero along the distance axis corresponds to
position 20. The color code is the same as that in C. The profile of GLIC in the
presence of a desensitizing concentration of protons (PDB ID code: 3EAM; in
purple) is also included. (F) Distances from the Cα atoms in the long axis of the pore (mean ± SE of all subunits) for residues in and flanking the trans-
membrane segments M2 of the four constructs compared in E. The color code is the same as that in E. The distance profile of the AChR from
Torpedo in the absence of activating ligands (PDB ID code: 2BG9, in black) is also included; this profile is nearly indistinguishable from that of GLIC. The three profiles of ELIC (corresponding to the unliganded wild-type and the two double mutants in the presence of 10 mM cysteamine at pH ~6.5) are also indistinguishable from each other.

to reveal such changes (Fig. 5 and Fig. S5A). Similar results were
obtained when the structural models of the two double mutants in
the presence of 10 mM cysteamine were compared with the model of the unliganded F16L single mutant (Fig. S5 B–D), the struc-
ture of which we solved at 3.5 Å resolution. Thus, in the absence of more detailed information about the conformation of side chains, we are compelled to conclude that the pore structure of the two double mutants of ELIC in the presence of a high
concentration of cysteamine is essentially the same as that of the
unliganded wild-type channel, at least in the crystalline state.

This result is quite puzzling because our electrophysiological characteriza-
tion of the two double mutants led us to expect, perhaps naively, that they would be crystallized in the open or, eventually, in some desensitized conformation. However, there are other similar examples of structural models having a tenuous connection to electrophysiological data. For example, Fig. 5F shows that the distances between the Cα atoms of the M2 seg-
ments and the long axis of the pore in the GLIC open-channel
model are indistinguishable from those in the muscle-type AChR model from Torpedo (PDB ID code: 2BG9), even though the latter was built from data collected in the complete absence of cholinerigic ligands (32), and hence, is expected to represent the closed-channel conformation.

Further analysis of the X-ray experimental data revealed addi-
tional electron density within the transmembrane domain at the level of the ring formed by the five asparagines at position 20. Tentative-
ly, we modeled this signal (in the F–J pentamer of the L9A +
F16L double mutant and in both pentamers in the asymmetric unit of the F16L single mutant) as arising from a Na+ ion, but we
cannot rule out the possibility that this density actually corre-
sponds to NH4+, the other cation present at a high concentration in the crystallization milieu. In the case of the crystals grown in the
presence of cysteamine, additional electron density can also be
observed at some of the five subunit–subunit interfaces in the
region expected to harbor the agonist-binding sites (Fig. S6).

However, because of the limited resolution of the data, no attempt
was made to model cysteamine molecules at these locations.

Discussion

By no means should our results be taken to imply that mutations,
in general, are ineffective at favoring the crystallization of specific
conformations of nicotinic-type receptors. Rather, the results show that many aspects of membrane-protein crystallization remain a
matter of trial and error, we suggest that other closed-channel
destabilizing mutations should be tested as well. However, it is
likely that approaches other than point mutations or ligand
binding will be needed to coax ELIC to crystallize in its different
functional states. Perhaps, the lack of a phospholipid membrane,
the presence of osmolytes and precipitants, and the limitations
imposed by the periodic packing of the protein in the crystal
lattice alter the energetics of ELIC’s conformational equilibria to
the extent that the effect of mutations on the occupancy proba-
bilities in the crystallization milieu cannot be predicted from
electrophysiological recordings. In fact, these factors have been
shown to affect the conformational dynamics of BtuB (the
Escherichia coli outer-membrane transporter for vitamin B12
(33–35) and the kinetics of the photocycle in bacteriorhodopsin
(36), for example. In addition, of particular relevance to this
article, the effect of membrane lipids on the relative occupancies
of the closed, open and desensitized states of the AChR from
Torpedo’s electric organ has been well documented (3, 37, 38).

Alternatively, it may well be that only one conformation of ELIC
crystallizes under the tested conditions, in which case all efforts to
crystallize specific functional states of the channel by appropri-
ately tilting the free-energy landscape would be futile.

As for the kinetic properties of ELIC, we think that its un-
characteristically slow activation time course and the noncanonical
effect of pore mutations on some of its functional properties
should be regarded as intriguing features (rather than as disappointing findings) that may help unlock some of the secrets that this superfamily of ion channels still holds. The high single-channel conductance, the tractable kinetics, and the relative ease with which crystals form justify the use of this bacterial channel for further studies.

Materials and Methods

Current recordings from ELIC, GLIC, and the mouse-muscle AChR (adult type) heterologously expressed in Xenopus laevis oocytes (ELIC) or HEK-293 cells (ELIC, GLIC, and AChR) were performed at −22 °C using the outside-out or the cell-attached patch-clamp configuration, as indicated. In the outside-out configuration, agonist was applied to the excised patches of membrane as rapid jumps (solution-exchange time <100 μs) by switching the bathing solutions (differing only in the presence or absence of agonist) flowing from either barrel of a piece of t-tube capillary glass mounted on a piezo-electric device (Burleigh-LSS-3100; Thorlabs). The patch-pipette solution consisted of: 110 mM KF, 40 mM KCl, 1.0 mM CaCl2, 11 mM EGTA, and 10 mM Hepes/KOH, pH 7.4, whereas the agonist-free solution flowing through one of the barrels of the theta-type tubing consisted of: 142 mM KCl, 8.4 mM MgCl2, 1.7 mM MgSO4, 10 mM Hepes/KOH, pH 7.4. The concentration of agonist in the second-barrel solution was 100 μM ACh for recordings from the AChR and 10–100 μM cysteamine for recordings from ELIC. For some of the latter, the pH of both t-tube solutions was adjusted to 6.5 with KOH using 25 mM N-(2-acetamido)imino diacetic acid (ADA), instead of Hepes, as the proton buffer; the agonist-containing solution also contained 10 mM cysteamine. For recordings from GLIC, the pH values of both t-tube solutions differed; these were adjusted to 4.5, 7.4, or 9.0 with KOH using 10 mM acetic acid, Hepes or N-Tris(hydroxymethyl) methyl-4-amino butane-sulfonic acid, respectively, as the proton buffer. For detailed information on the patch-clamp setup, see SI Materials and Methods.

ACKNOWLEDGMENTS. We thank Luis Cuello, Vivian González-Pérez, and Emad Tajkhorshid for expert advice; Steven Sin for wild-type muscle AChR subunit cDNA; Keith Brister, Joseph Brunelle, David Smith, and Zdzislaw Wawrzk at the Life Sciences Collaborative Access Team (21 ID–F) at Argonne National Laboratory (Advanced Photon Source) for technical assistance during crystallographic data collection; and Mas Pasquinzi and A. Holmstrom for assistance with cell culturing. This work was supported by National Institutes of Health Grants R01 GM095660 (to S.K.N.) and R01 NS042169 and corresponding American Recovery and Reinvestment Act of 2009 supplement (to C.G.); and National Institutes of Health Training Grant T32GM008276 (to D.P.).

Supporting Information

Gonzalez-Gutierrez et al. 10.1073/pnas.1119268109

SI Materials and Methods

Expression of ELIC, the Nicotinic-Type Channel from Erwinia chrysanthemi, in Xenopus Oocytes. The ELIC-coding sequence (UniProt E0SJQ4) was taken from the published genome of Erwinia chrysanthemi, also known as Dickeya dadantii (1). The sequence differs from that described in previous work (2, 3) in that a glycine insertion occurs between positions 163 and 164. The sequence further differs from Uniprot entry P1C177 in that an asparagine (instead of a methionine) residue occupies position 289 (Fig. S1). The presence of an additional residue shifts the numbering of the amino acid sequence: for example, the residue occupying position 9 of M2 in wild-type ELIC is leucine 240 (not 239) and that occupying position 16 is phenylalanine 247 (not 246). The sequence was codon-optimized for protein overexpression in Escherichia coli and was commercially synthesized (Integrated DNA Technologies). For functional assays in oocytes, the sequence of the mature wild-type ELIC was subcloned in an in-house modified pGEM vector optimized for expression in Xenopus laevis; the signal-peptide coding sequence was that of the chicken α7 AChR. Point mutations were engineered using a QuickChange site-directed mutagenesis kit (Stratagene) and were confirmed by dideoxy sequencing (ACGT). Wild-type and mutant DNA were linearized by digestion with HindIII (New England Biolabs), and capped mRNA was synthesized using the mMESSAGE mMACHINE kit (Ambion). Defolliculated oocytes (Ecoocyte Bioscience) were injected with 15–20 ng of capped mRNA in a total volume of 50 nL (World Precision Instruments), and electrophysiological recordings were performed 2–5 d after injection. During injection, the oocytes were placed in a Ca2+-free solution containing: 82.5 mM NaCl, 2.5 mM KCl, 1.0 mM MgCl2, 2.0 mM MgSO4, and 5.0 mM Hepes/NaOH, pH 7.4. After injection, the oocytes were kept at 18 °C in ND-96 solution (96 mM NaCl, 2.0 mM KCl, 1.8 mM CaCl2, 1.0 mM MgCl2, 5.0 mM Hepes/NaOH, pH 7.4) supplemented with 2.5 mM sodium pyruvate and 100 μg/mL gentamycin sulfate. Right before experiments, oocytes were exposed for 2–3 min to a hypotonic solution containing: 200 mM potassium glutamate, 20 mM KCl, 1.0 mM MgCl2, 10 mM EGTA, and 10 mM Hepes/KOH, pH 7.4, after which the vitelline membrane was removed manually.

Expression of ELIC, GLIC, and the Muscle AChR in HEK-293 Cells. HEK-293 cells were transiently transfected with complementary DNAs (cDNA) coding ELIC, GLIC, or the mouse-muscle AChR (α1-, β1-, δ-, and ε-subunits) using a calcium-phosphate precipitation method, as described previously (4). ELIC-coding cDNA was prepared by inserting a commercially synthesized stretch of DNA (Integrated DNA Technologies), consisting of the signal peptide of the chicken α7 AChR followed by the sequence of the mature ELIC channel, into the pcDNA3.1 vector.

Electrophysiological Recordings and Analysis. Currents were recorded at 22 °C in the outside-out or the cell-attached configuration of the patch-clamp technique using an Axon Axopatch 200B amplifier (Molecular Devices). Agonist was applied to the external aspect of outside-out patches as rapid jumps as indicated in Materials and Methods. Previous work (4) has underscored the importance of a fast solution exchange to prevent the kinetics of ligand perfusion from distorting the time course of the recorded macroscopic currents. The composition of the patch-pipette and external-perfusion solutions is indicated in Materials and Methods. For all tested constructs, the peak of the macroscopic current responses occurred within the agonist-application intervals (“pulses”). To estimate the kinetics of deactivation, the decaying phase of the elicited transients were fitted between the end of the agonist application and the end of the response with monoexponential or (in some patches containing wild-type or mutant ELIC channels) double-exponential functions. For those ELIC-deactivation time courses requiring a second exponential component, only the faster one was considered for the calculation of the mean and standard-error values shown in Fig. 1B; for this subset of recordings, the amplitude of the faster component was >70%. Comparing the monoexponential deactivation time courses as a group and the double-exponential time courses as a separate group leads to the same conclusion: regardless of the number of exponential components required to fit the data best, the mutations in M2 studied here speed up the deactivation of ELIC. To estimate the kinetics of entry into desensitization, the decaying phase of the transients were fitted between the peak of the current response and the end of the agonist application with monoexponential functions. To estimate the kinetics of activation, the transients were fitted between the beginning of the agonist application and the peak of the response with monoexponential functions. The effective bandwidth before data analysis was DC–5 kHz. Recordings were analyzed using pClamp 9.0 (Molecular Devices), QuB (5, 6) and in-house-developed programs.

ELIC Overexpression and Purification. The DNA sequence of the mature wild-type ELIC (see above) was subcloned into an in-house modified version of the pET-28 vector (Novagen) in such a way that, upon translation, a maltose-binding protein (MBP)-ELIC fusion protein was generated. The entire DNA insert coding the PolB signal peptide and His6-Tag, MBP, a second (His)6-Tag, the tobacco etch virus (TEV) protease site, and the ELIC channel. Point mutations were engineered and confirmed as indicated above. BL21 (DE3) competent cells (Agilent Technologies) were transformed with the wild-type or mutant constructs and grown at 37 °C in Terrific Broth medium (Invitrogen) supplemented with 50 mg/mL kanamycin. Protein overexpression was induced with 0.4 mM isopropyl-β-D-1-thiogalactopyranoside overnight at 20 °C. All subsequent steps were performed at 4 °C. Cells were harvested, resuspended in buffer A (50 mM sodium phosphate, 150 mM NaCl, pH 7.9) supplemented with a protease inhibitor mixture (Roche), and ruptured using a high-pressure homogenizer (EmulsiFlex–CS; Avestin). Membranes were isolated by ultracentrifugation at 200,000 × g for 2 h, the pellet was solubilized overnight under gentle agitation in buffer A supplemented with 40 mM n-undecyl-β-D-maltoside (UDM; Anatrace), and the solubilized fraction was cleared by a second ultracentrifugation step at 200,000 × g for 2 h. The supernatant was collected and His-tagged proteins were purified by affinity chromatography on His Trap HP columns (GE Healthcare) using buffer A supplemented with 3 mM UDM and 300 mM imidazole as the elution buffer. The eluted fractions of MBP-ELIC fusion protein were desalted using a PD-10 column (GE Healthcare) and digested overnight with in-house purified TEV protease. A second step of affinity chromatography was performed to remove the released His-tagged MBP. ELIC protein was eluted in buffer B (10 mM phosphate buffer, 150 mM NaCl, pH 7.9) supplemented with 3 mM UDM and 30 mM imidazole, concentrated and loaded onto a Superdex 200 column (GE Healthcare). The elution buffer of this final purification step was buffer B supplemented with 1.2 mM UDM. The fractions corresponding to the pentameric form of ELIC were pooled, concentrated to 9–10 mg/mL, supplemented with 0.5 mg/mL E. coli polar lipids (Avanti Polar Lipids), and used for crystallization trials.
Crystallization and X-Ray Diffraction. Purified ELIC mutants were crystallized by vapor diffusion in hanging drops at 4 °C. Concentrated protein was mixed in a 1:1 (vol/vol) ratio with reservoir solution consisting of 200 mM (NH₄)₂SO₄, 10–12% (wt/vol) of PEG 4000 and 50-mM N-(2-acetamido)iminodiacetic acid (ADA), pH 6.5–6.9. Protein crystals grew within 1–2 d, after which they were kept in the hanging drops for an additional 2–6 d before being harvested. For cryoprotection, crystals were transferred for a few seconds into reservoir solution supplemented with 30% (vol/vol) ethylene-glycol and were subsequently flash-frozen in liquid nitrogen. Diffraction data were collected at 100 K at the 21 ID–F/G beamline at the Advanced Photon Source (Argonne National Laboratory, Argonne, IL). The data were indexed, integrated and scaled using the XDS package (7). The structures were solved by molecular replacement using Phaser (8) with PDB deposition 2VL0 as a search model, followed by iterative cycles of refinement in Refmac (9) and modification of the model in Coot (10). No attempts were made to refine regions of poor electron density as defined in the starting model. Because of the low resolution of the X-ray data, all models were rebuilt with Auto Build in Phenix (11) using the “Rebuild in Place” option. Cross-validation used 5% of the data in the calculation of the free R factor. Crystal parameters, data-collection parameters, and refinement statistics are indicated in Table S1. HOLE software was used to generate pore-radius profiles (12), Visual Molecular Dynamics to calculate distances (13), and PyMOL to make molecular images (The PyMOL Molecular Graphics System, Version 1.3; Schrödinger).


Fig. S1. ELIC’s amino acid sequence. (A) Alignment of M2 α-helical segments. UniProt codes are given in parentheses. The leucines and isoleucines occupying positions 9’ and 16’ are indicated in red. (B) Sequence of ELIC used in this work. The differences between this sequence (UniProt E0SJQ4) and that deposited as UniProt P0C787 are indicated in red (SI Materials and Methods). ELIC, GLIC, the α1- and α7-subunits of the AChR, and the A subunit of the serotonin type 3 receptor (5-HT3AR) form, or are part of, cation-selective channels. The α1-subunits of the γ-aminobutyric acid receptor (GABAR), the glycine receptor (GlyR), and the glutamate receptor (GluClR) form anion-selective channels.
Fig. S2. Effect of canonical gain-of-function mutations on GLIC. The kinetics of deactivation and desensitization of GLIC wild-type and the I9′A pore mutant (1) were studied at −80 mV in fast-perfused outside-out patches of membrane excised from HEK-293 cells. (A) Kinetics of deactivation upon stepping the pH of the external solution from 4.5 to 7.4 (in the case of wild-type GLIC) or 9.0 (in the case of the 9′ mutant). (B) Kinetics of desensitization upon stepping the pH of the external solution from 7.4 to 4.5 (in the case of wild-type GLIC) or from 9.0 to 4.5 (in the case of the 9′ mutant). For both panels, the pH of the patch-pipette solution was 7.4.

Fig. S3. Effect of mutations at M2 position 16' on the mouse-muscle AChR. The kinetics of the wild-type AChR and a pore mutant (in which the wild-type ring of four leucines and one phenylalanine at position 16' of M2 was replaced by a full ring of alanines) were studied at –80 mV in fast-perfused outside-out patches of membrane (macroscopic recordings) and in the cell-attached configuration (single-channel recordings). Both constructs were expressed in HEK-293 cells. (A and B) Kinetics of deactivation. The time courses (shown normalized) were fitted with monoexponential functions, and the corresponding time constants (± SEs) are shown. (C–F) Single-channel data recorded in the continued presence of ACh. Note the different time scales in C and E. Openings are downward deflections. Display f, ~5 kHz. The distribution of burst durations corresponding to the wild-type AChR was best-fitted with one exponential component, whereas that corresponding to the mutant was best-fitted with two. Bursts of openings were defined as series of openings separated by shuttings shorter than a given value (a “critical time”). The latter was calculated for each individual recording as indicated in ref. 1. For the particular recordings shown in this figure, the critical time was 1.19 ms for the wild-type AChR and 0.77 ms for the 16' mutant. (G) Burst durations (mean ± SE). For the mutant, only the mean of the slowest component of the distribution is given. As expected (2), the values of the (macroscopic) deactivation time constant are in agreement with the means of the slowest component of the (single-channel) distribution of burst durations.

Fig. S4. Effect of cysteamine concentration on intracluster open probability. (A) Stretches of single-channel currents recorded in the outside-out configuration (Xenopus oocyte membranes) at −80 mV, in the continued presence of the indicated concentration of cysteamine. The pH on both sides of the membrane was 7.4. Channel openings are downward deflections. Display $f_r$ ~5 kHz. (B) Intracluster open probabilities. Clusters were defined as series of openings separated by shuttings shorter than 200 ms.

Fig. S5. X-ray crystal structures of the unliganded wild-type ELIC (from ref. 1) and the unliganded F16′L mutant. (A and B) Ribbon representation of the transmembrane region of the indicated structural models. The mesh shows the solvent-accessible surface. The side chains corresponding to pore-facing positions 9', 16', and 20' (that is, the side chains forming the three narrowest constrictions of the pore) are shown in ball-and-stick representation. The front subunit was removed, for clarity. (Upper) Extracellular. (Lower) Intracellular. (C) Aligned Ca traces. Only one subunit per construct is shown. The structural model of the unliganded wild-type ELIC is not included in this comparison. (D) Distance between aligned Ca atoms. The Ca atoms of each subunit in the structural models of the double mutants L9′A + F16′L and L9′S + F16′L in the presence of 10 mM cysteamine were aligned with those of the unliganded F16′L mutant in a pairwise manner, and the distances between Ca atoms were calculated and averaged.

Fig. S6. Additional electron density attributable to bound cysteamine. (A) Ribbon representation of the structural model corresponding to the L9S + F16L double mutant in the presence of 10 mM cysteamine. (Inset) Some of the side chains that line the cavity where cysteamine appears to bind are shown in ball-and-stick representation with the carbon atoms colored according to the color assigned (in the ribbon representation) to the subunit they belong to. The Fo–Fc omit electron density, tentatively corresponding to a molecule of cysteamine, is shown as a yellow mesh contoured at 3.0 $\sigma$. (B) View of the structural model from the extracellular side.

Fig. S7. Time course of reduced cysteamine at 4 °C. The decline in the concentration of cysteamine as a function of time because of oxidation of its thiol group was estimated colorimetrically as indicated in Materials and Methods. The time course was fitted with a monoexponential function, and from the corresponding time constant, the half-life of reduced cysteamine was calculated to be $\sim$ 20 d.
### Table S1. Data collection and refinement statistics

<table>
<thead>
<tr>
<th>Data collection and refinement</th>
<th>ELIC L240A + F247L, 10 mM cysteamine</th>
<th>ELIC L240S + F247L, 10 mM cysteamine</th>
<th>ELIC F247L</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Data collection</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Space group</td>
<td>P2₁</td>
<td>P2₁</td>
<td>P2₁</td>
</tr>
<tr>
<td>No. of molecules in asymmetric unit</td>
<td>10</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td><strong>Cell dimensions</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(a, b, c) (Å)</td>
<td>105.57, 267.43, 111.16</td>
<td>105.38, 266.45, 110.91</td>
<td>105.35, 266.94, 110.84</td>
</tr>
<tr>
<td>(\alpha, \beta, \gamma) (°)</td>
<td>90.00, 108.64, 90.00</td>
<td>90.00, 109.47, 90.00</td>
<td>90.00, 109.60, 90.00</td>
</tr>
<tr>
<td>Resolution (Å)</td>
<td>20–4.2 (4.31–4.2)</td>
<td>20–3.8 (3.9–3.8)</td>
<td>20–3.5 (3.59–3.5)</td>
</tr>
<tr>
<td>No. of unique reflections</td>
<td>42,057</td>
<td>56,196</td>
<td>71,891</td>
</tr>
<tr>
<td>(R_{merge})</td>
<td>0.179 (0.829)</td>
<td>0.137 (0.691)</td>
<td>0.113 (0.707)</td>
</tr>
<tr>
<td>(I/\sigma I)</td>
<td>16.6 (4.17)</td>
<td>11.8 (2.94)</td>
<td>17.6 (3.39)</td>
</tr>
<tr>
<td>Completeness (%)</td>
<td>99.4 (98.7)</td>
<td>99.1 (99.5)</td>
<td>99.1 (99.3)</td>
</tr>
<tr>
<td>Redundancy</td>
<td>15.3</td>
<td>6.4</td>
<td>8.5</td>
</tr>
<tr>
<td><strong>Refinement</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Resolution (Å)</td>
<td>4.2</td>
<td>3.8</td>
<td>3.5</td>
</tr>
<tr>
<td>(R_{crys})</td>
<td>22.0</td>
<td>21.5</td>
<td>21.5</td>
</tr>
<tr>
<td>(R_{free})</td>
<td>24.9</td>
<td>24.4</td>
<td>23.9</td>
</tr>
<tr>
<td>R.m.s. deviations</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bond lengths (Å)</td>
<td>0.012</td>
<td>0.013</td>
<td>0.015</td>
</tr>
<tr>
<td>Bond angles (°)</td>
<td>1.638</td>
<td>1.570</td>
<td>1.747</td>
</tr>
<tr>
<td>No. atoms</td>
<td>Protein</td>
<td>24,990</td>
<td>25,000</td>
</tr>
<tr>
<td></td>
<td>B-factors</td>
<td></td>
<td>25,020</td>
</tr>
<tr>
<td></td>
<td>Wilson plot</td>
<td>122.5</td>
<td>90.0</td>
</tr>
<tr>
<td></td>
<td>Protein</td>
<td>153.0</td>
<td>127.7</td>
</tr>
<tr>
<td></td>
<td>Na⁺</td>
<td>50.0</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>PDB ID code</td>
<td>3UQ5</td>
<td>3UQ7</td>
</tr>
</tbody>
</table>

Gonzalez-Gutierrez et al. www.pnas.org/cgi/content/short/1119268109