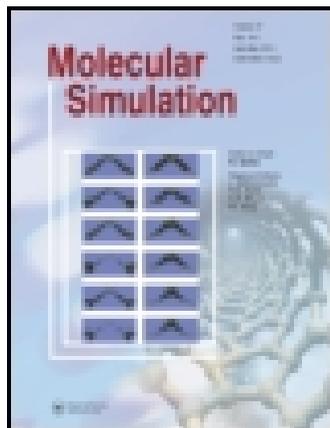


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Molecular Simulation

Publication details, including instructions for authors and subscription information:

<http://www.tandfonline.com/loi/gmos20>

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Reza Salari^{ab}, Sruthi Murlidaran^a & Grace Brannigan^{ab}

^a Center for Computational and Integrative Biology, Rutgers University, Camden, NJ, USA

^b Department of Physics, Rutgers University, Camden, NJ, USA

Published online: 17 Apr 2014.



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To cite this article: Reza Salari, Sruthi Murlidaran & Grace Brannigan (2014): Pentameric ligand-gated ion channels: insights from computation, *Molecular Simulation*, DOI: [10.1080/08927022.2014.896462](https://doi.org/10.1080/08927022.2014.896462)

To link to this article: <http://dx.doi.org/10.1080/08927022.2014.896462>

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RECENT ADVANCES IN MOLECULAR BIOLOGY

Pentameric ligand-gated ion channels: insights from computation

Reza Salari^{a,b}, Sruthi Murlidaran^a and Grace Brannigan^{a,b*}

^aCenter for Computational and Integrative Biology, Rutgers University, Camden, NJ, USA; ^bDepartment of Physics, Rutgers University, Camden, NJ, USA

(Received 11 December 2013; final version received 12 February 2014)

Pentameric ligand-gated ion channels (pLGICs) conduct upon the binding of an agonist and are fundamental to neurotransmission. New insights into the complex mechanisms underlying pLGIC gating, ion selectivity and modulation have recently been gained via a series of crystal structures in prokaryotes and *Caenorhabditis elegans*, as well as computational studies relying on these structures. Here, we review contributions from a variety of computational approaches, including normal-mode analysis, automated docking and fully atomistic molecular dynamics simulation. Examples from our own research, particularly concerning interactions with general anaesthetics and lipids, are used to illustrate predictive results complementary to crystallographic studies.

Keywords: molecular dynamics simulation; neurotransmitter receptor; Cys-loop receptor

1. Introduction

Pentameric ligand-gated ion channels (pLGICs) are a family of integral membrane proteins that increase their permeability to certain ions upon agonist binding. pLGICs can be found in many organisms from bacteria to complex vertebrates; in humans, pLGICs serve as neurotransmitter receptors and are abundant in many locations, including central and peripheral nervous systems and neuromuscular junctions. They can be either excitatory (cationic) such as the nicotinic acetylcholine receptor (nAChR) and 5HT-3 receptor or inhibitory (anionic) as in γ -aminobutyric acid receptor type A receptor (GABA(A)R), glutamate-gated chloride receptor (GluCl) and glycine receptor (GlyR). Dysfunctional pLGICs have been implicated in serious diseases and conditions such as Alzheimer's disease,[1] Parkinson's disease,[2] epilepsy,[3] schizophrenia,[4–6] myasthenia gravis,[7] smoking addiction [8] and alcohol dependence.[9,10] They are also likely targets of numerous drugs, including nicotine, alcohol, anaesthetics and benzodiazepines.[11–15]

Because of their significance and complexity, there have been extensive experimental, theoretical and computational efforts in the community over the past 30 years to unravel the structural and functional mysteries of pLGICs. These efforts have tremendously improved our understanding of these channels and have been summarised in several excellent reviews.[16–18] In particular, the availability of atomic structures for a number of pLGICs has provided an unprecedented opportunity for many fields, including computational biophysics, to identify

molecular mechanisms involved in the pLGIC function and modulation. Here, we briefly review the most recent computational studies regarding various molecular aspects of pLGICs.

2. Structure and activation

During the past decade, several structures of pLGICs with atomic resolution have been reported. These include a medium-resolution cryo-EM structure of *Torpedo* nAChR in the absence of agonist,[20] a crystal structure of a pLGIC from *Erwinia chrysanthemi* (ELIC) in the closed state,[21] a presumably open-state crystal structure of a pLGIC from *Gloeobacter violaceus* (GLIC) [22,23] and most recently the crystal structure of a glutamate-gated chloride channel from *Caenorhabditis elegans* (GluCl) in the open-state bound to the potent modulator ivermectin. [19] Although these channels belong to evolutionarily distant organisms and share low sequence identity,[24] their three-dimensional architecture reveals striking conservation of the overall structural features of pLGICs from bacteria to complex eukaryotes (Figure 1).

As their name suggests, pLGICs consist of five subunits that are arranged in (pseudo) C5 symmetry around a central pore (Figure 1). Many eukaryotic pLGICs are heteromeric with multiple homologous subunit species labelled α , β and so on, and further divided into subtypes: α_1 , α_2 and so on. All subunits contribute to the two main parts of the channel: the extracellular domain (ECD) and the transmembrane domain (TMD). The ECD has a β -

*Corresponding author. Email: grace.brannigan@rutgers.edu

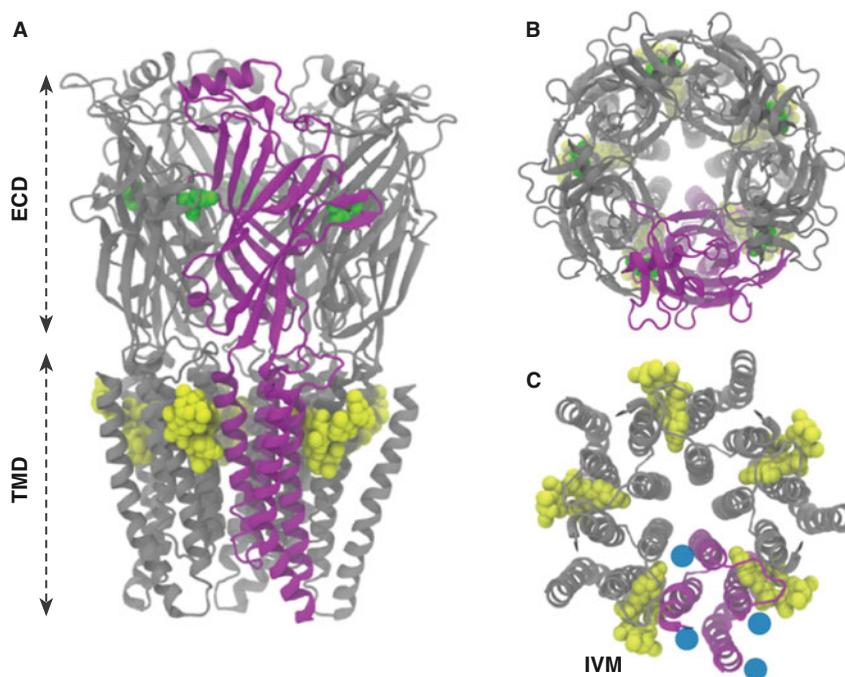


Figure 1. (Colour online) The general structure of pLGICs. (A) View along the axis perpendicular to the membrane, based on GluCl, [19] showing the ECD and TMD, with a single subunit shown in purple. The agonists ivermectin and glutamate are shown in yellow and green, respectively, as reported in the GluCl structure. (B) View from the ECD, looking towards the membrane. (C) View of the TMD, looking towards the membrane from the ECD, with the M1–M4 helices labelled.

sandwich immunoglobulin-like structure while the TMD is composed of four helices (M1–M4) that span the membrane (Figure 1). Eukaryotic receptors are gated by an agonist that typically binds to the ECD at the interface between adjacent subunits.

Upon agonist binding, a cascade of conformational changes begin from the ECD and propagate about 50 Å away in the TMD, where they lead to the opening of the central pore formed by the M2 helices. Channel gating, therefore, likely involves events on multiple timescales. Various computational techniques, such as molecular dynamics (MD), [25–27] targeted MD (TMD), [28] steered-MD (SMD), [29] elastic-network models, [30] normal mode analysis (NMA) [31–34] or a multi-scale combination of them [35] have been employed to elucidate different stages of the transition, mainly from open to closed state. Recent studies have indicated that these conformational changes include rearrangement in the ECD, [27,36] ECD–TMD interface, [27,30] tilting and rotation of M2 helices [27,30,37] and tertiary and quaternary changes. [26,27,31,33] Experimental cysteine cross-linking has identified locally closed allosteric intermediates for GLIC [38] and α_1 GlyR [39]; the structure for locally closed GLIC has been reported and shown to be stable over the course of 100-ns MD simulation. [38]

Analysis of correlated motions of pLGICs using NMA [31,33,40] has indicated several low-frequency motions,

including a quaternary twist where the ECD and TMD rotate in opposite directions around an axis normal to the membrane. This iris-like motion, first described using models of nAChR, has been proposed to dominate channel opening. [31–34] Comparison of the structures of ELIC (in a closed state) and GLIC (in an open state) later confirmed the presence of such a twist, along with a tertiary motion within the ECD. [22] MD simulations of GLIC and GluCl have also shown the emergence of such a twist during the course of channel closure. [26,27] Several computational studies have used this motion to generate the open-state of pLGICs. [37,41]

In a microsecond MD study, Nury et al. [26] followed the sequence of events during GLIC closure at neutral pH, at which the acid-sensitive channel was expected to be closed. They found that upon adjusting the ionisation state of amino acids for pH 7, the channel rapidly closes, mainly due the protrusion of the upper part of the M2 helix. [26] However, it has since been shown [42,43] that GLIC closes rapidly (within 50–100 ns) under the CHARMM22 force field [44,45] in recent versions of NAMD even at pH 4.6, for which the channel is expected to remain open; such closure is not observed using the AMBER [46] force field with GROMACS. [47] Flat-bottom conformational restraints can be used to maintain an open GLIC conformation in CHARMM. [42]

Yoluk et al. [48] observed a rapid closure of GluCl in a microsecond-long study for GluCl when the agonist ivermectin (bound to the TMD) was removed. They observed that upon removal of ivermectin the inter-subunit cavities shrink relative to control simulations, which was accompanied by a subtle reduction in pore radius.[48] We have recently [49] proposed that in cholesterol-rich membranes, occupation by cholesterol may offset such shrinkage in some pLGICs. In another study, Calimet et al. [27] used the reaction coordinates extracted from GLIC and ELIC structures to monitor the events during the GluCl closure upon removal of ivermectin. They proposed that the sequence of quaternary twist, agonist unbinding and reorientation of the M2–M3 loop causes the closure of the pore.

3. Ion selectivity

Computational methods have also been used to study the location of the gate and the origin of the ion selectivity in pLGICs. The employed methods range from fast implicit models to full-atomistic simulations and include Poisson–Nernst–Planck theory,[50] Brownian dynamics simulations,[37,50,51] all-atom MD simulations using various enhanced-sampling techniques, such as umbrella sampling [52–54] or adaptive biasing force,[55,56] and all-atom unrestrained MD simulations with persistent electrochemical gradient [57] which resembles single-channel electrophysiology.

Computational studies [52,55] of nAChR based on a medium-resolution cryo-EM structure, imaged in the absence of agonist,[20] have supported the presence of a hydrophobic gate around Leu9' on the M2 helix. The hydrophobic gate is proposed to act by imposing significant dehydration penalty for ions going through the pore so that even when the pore is not physically occluded, the channel is functionally closed. Using MD simulations it has been shown that model hydrophobic pores become functionally closed (i.e. dried) when the radius is below a critical limit, [58] and also possess intrinsic ion selectivity features.[59] Hydrophobic gates in the TMD were also found to be the dominant factor in specifying the ion selectivity in the case of a homology model of the anionic channel GlyR α_1 , where sodium ions crossed an approximately 6 kcal/mol energy barrier near residue Leu9' while there was no significant barrier for chloride ions.[55] An experimental chimera of the ECD of GLIC and the TMD of α_1 GlyR is a proton-gated anion-selective channel, thus supporting the hypothesis that major components of ion selectivity for GlyR α_1 are located in the TMD,[60] while the ECD plays a more dominant role in agonist sensing (in this case protons).

Single-ion potential of mean force calculations for GLIC, which was crystallised in low-pH and, therefore, likely to be in the open state and permeable to cations, showed that in addition to the hydrophobic gate, a ring of

five glutamic acid residues near the intracellular opening of the channel form another energy barrier for chloride ion diffusion.[53,56] Ion conductivity is sensitive to the protonation state of Glu222, and it is likely that some of the five Glu222 residues are in the protonated state, as otherwise the free energy minimum formed by the channel would be too deep for permeation of sodium ions.[56] Similar studies on GluCl, an ion-selective channel crystallised in the open state, showed two sources for ion selectivity: three rings of lysine residues in the ECD, as well as a selectivity filter located at the Pro-2' residue with a free energy barrier that is about $4 k_B T$ higher for a sodium ion relative to a chloride ion.[37]

Recently, high-resolution structures of an open form of GLIC at 2.4 Å [61] were solved that revealed two water pentagons (at Ser6' and Thr2') in the GLIC channel, with a sodium ion lodged between them. Atomistic MD and SMD simulations totalling over 2 μ s were used to further characterise the microscopic interactions underlying conduction, including the evolution of hydration profiles and the critical role of the Ser6' hydroxyl.[61]

4. Lipid–protein interactions

Eukaryotic pLGICs can be highly sensitive to the contents of the lipid membrane. Reconstituted nAChRs require cholesterol for native levels of function, with multiple studies indicating specific interactions (for reviews see [62–65]). GABA(A)R function also displays complex effects of cholesterol enrichment or depletion [66] and successful reconstitution procedures for GABA(A)Rs have also included cholesterol.[67,68] A recent study [69] reports that the barrier separating the conducting and non-conducting states of the nAChR is sensitive to the hydrophobic thickness of the surrounding membrane, and suggested a potential mechanism in which upright orientation of the M4 helices is essential to achieving a coupled state [70] capable of conducting ions.

Relatively few computational studies have investigated interactions between pLGICs and cholesterol, however. Baier et al. [71] identified potential annular cholesterol sites using recognised cholesterol motifs and automated docking. Cheng et al. [72] investigated interactions of $\alpha_4\beta_2$ nAChRs with membrane cholesterol and anionic lipids in simulations lasting over 10 ns, observing penetration of inter-subunit cavities by phosphatidic acid acyl chains. Slow mixing equilibration in the membrane, however, precludes atomistic studies of pLGICs with non-random lipid mixtures, which may represent a more realistic configuration for the membrane.

The possibility of more deeply buried sites has been explored as well. The TMD of the nAChR structure (Protein Data Bank: 2BG9) shows numerous gaps between helices in the cryo-EM image that have similar density to

the membrane.[20,73] Using docking calculations and MD simulations, we showed that these gaps could represent the presence of unresolved cholesterol in the cryo-EM structures.[74] Three sites were proposed, labelled as A (superficial), B (inter-subunit) and C (intra-subunit) (Figure 2). The presence of the cholesterol in the gaps is necessary to maintain the experimentally determined overall structure during MD simulations, with occupation of all 15 sites yielding the structures most consistent with the cryo-EM structures after 25 ns of simulation.[74]

Using a model of the GABA(A)R based on GluCl (with ivermectin removed), we found [49] that automated docking identified the inter-subunit (ivermectin) sites as potential cholesterol sites, with orientations consistent with distinct regions of ivermectin. MD simulations lasting 200 ns were run using multiple groups of initial coordinates and multiple models for the GABA(A)R. Over the course of the simulations cholesterol was observed to bind in one of the two main orientations, with the rough side facing either the M1 or M3 helices; exchange was observed in both directions, as was unbinding and spontaneous rebinding of cholesterol to the sites (Figure 2).

The universality of specific interactions with various lipids across pLGICs is unknown; both of these studies followed similar approaches and yielded distinct predictions for cholesterol sites on the nAChR and GABA(A)Rs, with predicted sites on the GABA(A)R less deeply buried and more exposed to the lipid membrane. The two studies used structural templates with numerous differences, including experimental methodology, resolution, lipid environment, presence/absence of agonists and expected conformational state, in addition to the differences in sequence between nAChR and GABA(A)Rs. However, we note that the location of the backbone helices in the two templates (2BG9 and 3EAW) overlaps closely, so that the

reduced protein density in the nAChR 2BG9 structure reflects the presence of smaller residues rather than a larger overall footprint of the TMD. This observation, based entirely on backbone coordinates consistent between the two models as well as known sequence differences, suggests that significant differences in protein density between nAChR and GABA(A)R may yield qualitatively different binding modes for cholesterol.

5. Pharmacology

Numerous drugs have been shown to modulate the function of pLGICs and activate, potentiate or inhibit the channels. Crystallography has revealed various sites on GLIC, ELIC or GluCl for the competitive acetylcholine antagonist pancuronium,[75] the benzodiazepine flurazepam,[76] the general anaesthetics (GA) propofol, desflurane[77] and bromoform,[78] the local anaesthetic lidocaine,[79] the channel blocker picrotoxin[19] and the anti-parasitic ivermectin.[19] However, the universality of binding sites across pLGICs is generally unknown; for instance, desflurane and propofol are shown to bind to intra-subunit sites in GLIC [77] but evidence from mutagenesis and photoaffinity labelling suggests that GAs may act on GABA(A)Rs and nAChRs via inter-subunit sites as well.[13–15,80] Furthermore, a single mutation can switch the binding of GAs from intra- to inter-subunit sites in GLIC.[81] Consequently, for many modulator/pLGIC combinations, the relative affinities of the modulators for various proposed sites are unknown.

Computational studies have primarily focused on interactions of pLGICs with GAs. Modulation of eukaryotic [13–15] and prokaryotic [82] pLGICs by GAs is well established. In general, at clinical concentrations, GAs inhibit excitatory channels (such as nAChR) but potentiate inhibitory channels (such as GABA(A)R);

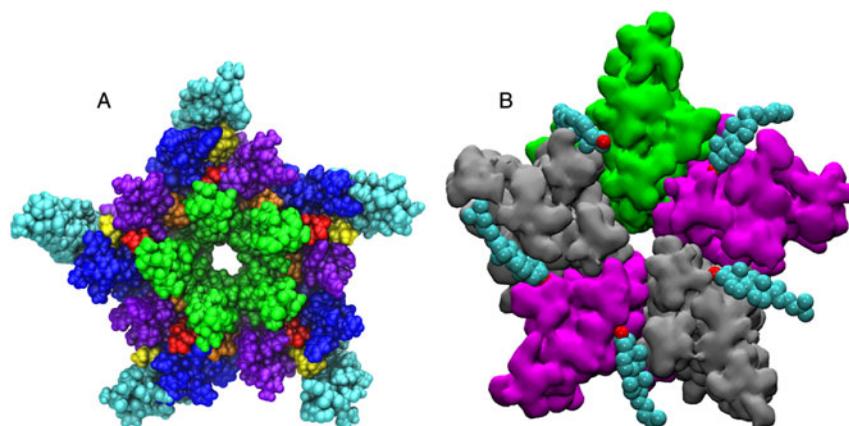


Figure 2. (Colour online) Potential embedded sites for cholesterol on nAChR (left) and GABA(A)R (right). Left: nAChR is coloured by helix (purple – M1, green – M2, blue – M3 and cyan – M4) with cholesterol in proposed sites A (yellow), B (orange) and C (red). Right: cholesterol (coloured by name) orientations after 200 ns of simulation of a GABA(A)R-cholesterol complex, with GABA(A)R coloured by subunit (γ – green, α – purple and β – silver).

both effects are consistent with the desired clinical effect. Experimental approaches such as photoaffinity labelling and site-directed mutagenesis have yielded three likely classes of sites in the TMD: intra-subunit, inter-subunit and pore.[80] Although no crystal structures of any vertebrate pLGICs have been reported, several groups have built homology models of the TMD based on available structures.[41,49,83,84]

5.1 Unbiased searches for binding sites

Computational searches for potential binding sites have largely used automated docking, unrestrained MD simulations including ‘flooding’ simulations, or some combination of the two. Automated docking has been used to provide initial coordinates for further unrestrained simulations,[43,85,86] which has primarily indicated sites on the surface of the protein or at the interface between the ECD and TMD. The alternative flooding approach [87,88] begins the simulation with a (usually high) concentration of the anaesthetic dispersed randomly in the water and allows it to partition into the membrane and protein sites. Flooding MD simulations are a more expensive alternative to docking with the advantage of being fully atomistic, including water molecules and lipids, and fully flexible. However, they require long simulation times to ensure equilibrium partitioning and sufficient phase-space exploration.

Previous flooding simulations with GAs have used small inhalational anaesthetics such as halothane [87] or isoflurane [88]; the approach is less successful with highly insoluble anaesthetics such as propofol. We found [88] that 100 isoflurane molecules required 400 ns to fully partition into membrane–pLGIC systems containing torpedo nAChR or GLIC; intra-subunit cavities were found in both receptors, while inter-subunit sites were only observed in nAChR (Figure 3). Two isoflurane molecules bound to the pore of both channels, which would have a clear ion-blocking effect. [88] Flooding approaches have also been used for predicting sites for ethanol on GlyR, in which ethanol was observed to bind to both inter- and intra-subunit sites, with inter-subunit sites having the highest occupancy.[89]

The number of binding sites found using flooding approaches will be highly sensitive to the concentration of the drug present in the surrounding solvent. In order to locate sites within a feasible simulation time, relatively high concentrations of the drug are typically used. Comparison of such concentrations to clinical concentrations used for inducing anaesthesia is not straightforward, however, as the equilibrium concentration present in the aqueous phase of the simulation may be significantly lower than that of the lipid phase; for instance, in the flooding simulations of Ref. [88], no isoflurane remained in the water by the end of the simulation. A more direct approach to predict the concentration dependence of site

occupancy relies on the computational measurement of binding affinity, which is more efficient than running multiple flooding simulations with varying concentrations.

5.2 Binding affinity prediction

Computational methods for estimating binding affinities range from the inexpensive scoring functions provided by automated docking programs to the sophisticated, thermodynamically exact alchemical free energy perturbation (AFEP) methods, but all have the advantage that they can be used to estimate affinities for isolated sites, rather than averages over the entire pLGIC. Although automated docking methods cannot typically be used to measure an absolute binding affinity, Bertaccini et al. [90] demonstrated that automated docking scores of propofol analogues display log-linear correlation with EC_{50} s for GABA(A)R potentiation, indicating that such scores can be used to estimate relative affinities of similar compounds. Liu et al. [85,86] used AFEP to estimate absolute binding affinities of halothane for both open and closed conformations of an $\alpha_4\beta_2$ model of a neuronal nAChR, finding that shallow binding sites tested were of low-affinity but a deeper inter-subunit site indicated by experiments was of moderate to high affinity. AFEP calculations of isoflurane and propofol binding to two locations in GLIC (Figure 4) the pore and the allosteric intra-subunit site indicated by crystal structures, predicted that isoflurane has higher affinity for the pore while propofol has similar affinity for the pore and intra-subunit site.[42] Based on the calculated free energies, the GLIC pore was predicted to be blocked by the anaesthetics at micromolar concentrations as observed experimentally.[42] Due to occupation of the GLIC pore by detergents, the possibility of GAs binding to the pore has not been addressed using crystallography. Similar methods were used to show that a single-site mutation at the F14' site in the GLIC TMD causes desflurane and chloroform to bind with higher affinity to inter-subunit sites than intra-subunit sites; the mutation is also associated with a reversal of their effect on GLIC function.[81]

5.3 Effect of binding on protein structure and dynamics

Effects of GA binding are typically subtle over accessible timescales, and efforts to predict the effects on function from unrestrained MD simulations can be challenging (with the exception of observed pore block). Flexible docking of the GA halothane to homology models of $\alpha_4\beta_2$ nAChR in open [85] and closed [86] states indicated several binding sites, primarily at the ECD–TMD interface, with subsequent MD simulations showing that halothane has a more pronounced effect on the global structure and dynamics of the open conformation. Since many of the binding sites were of low affinity, it was proposed from these calculations that

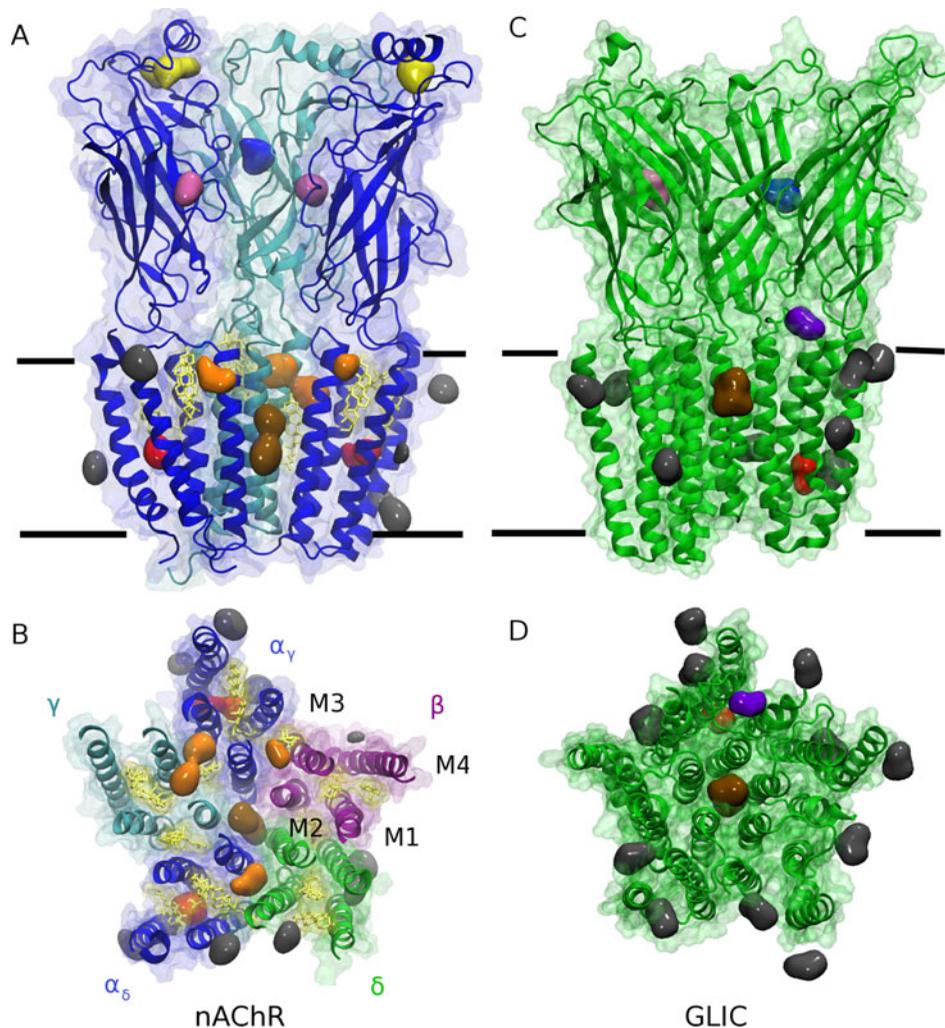


Figure 3. (Colour online) Regions of persistent occupation by isoflurane. (A and B) Isoflurane binding sites in the nAChR. Protein is coloured by subunit: α – blue, β – purple, δ – green and γ – cyan. Embedded cholesterol is yellow. Coloured blobs represent an isoflurane density isosurface averaged over the last 100 ns of a 400 ns simulation; large blobs represent occupation over at least most of that period, whereas a few much smaller blobs represent occupation for less than half of that period. (A) Side view of the α and γ subunits, as well as isoflurane sites contacting those subunits. Isoflurane binding sites in the TMD are coloured as follows: superficial/annular sites (grey), intra-subunit sites (red), inter-subunit sites (orange) and pore site (brown). Isoflurane binding sites in the ECD are pink, blue and yellow, corresponding with binding to the agonist site, β sandwiches and α_1 helices, respectively. (B) View of the nAChR TMD, looking down on the membrane from the extracellular region. (C and D) Isoflurane binding sites in GLIC. Protein is green and blobs are coloured as in A, with the addition of isoflurane in the loop site (purple). (C) Side view of three chains of GLIC. (D) TMD of GLIC, with isoflurane in loop site, intra-subunit site, pore site and annular sites. Reprinted from Ref. [88]

numerous low-affinity sites could result in significant effects on dynamics.[86]

Effects of isoflurane on the anaesthetic-sensitive GLIC in both the crystallographically identified [77] sites for desflurane/propofol, as well as those suggested by docking and subsequent MD, were investigated [43] using unrestrained MD simulations and principal component analysis; results indicated that interactions at the interface between the ECD and TMD were weakened in the presence of isoflurane, and that isoflurane had significant effects on channel dynamics. Further simulations suggested that asymmetric binding of propofol to the crystallographically identified

sites caused pore closure, while symmetric binding (five propofol) was observed to have little effect. [91]

GLIC has also been used as a model to study the action of alcohol on pLGICs. Ethanol was shown to reduce the root-mean-squared-deviation of a model of GlyR relative to a control simulation.[89] Methanol and ethanol weakly potentiate GLIC, while larger alcohols inhibit it.[92] A point mutation at Phe14' on the M2 helix (to Ala or Cys) converts GLIC to be much more sensitive to ethanol potentiation, comparable to the eukaryotic relatives.[92] MD simulations of the Phe14'Ala mutant in the absence of alcohol showed that the mutation causes an increased kink

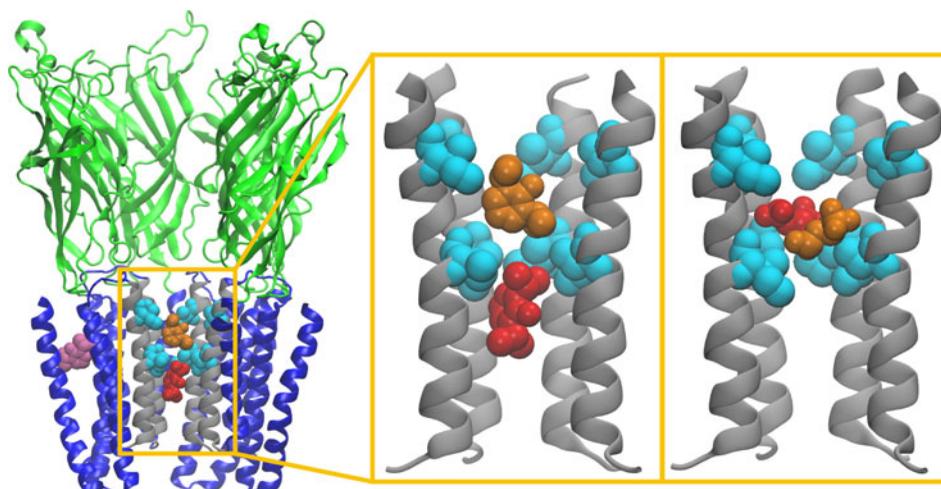


Figure 4. (Colour online) Binding of propofol and isoflurane to the GLIC pore. Left: view of the GLIC channel with two propofol molecules blocking a pore restrained to be open (shown in red and orange), and one bound in the crystallographic binding site (purple). Centre: the two propofol molecules bound to the pore formed by M2 helices (grey). Right: analogous magnification of two isoflurane molecules in the pore. Isoleucines bounding the hydrophobic gate (I232 and I239) are shown in cyan. To reveal the pore interior, only four of the five GLIC subunits are shown. Reprinted from Ref. [42].

in the M2 helix at the 9' position, spreading the subunits away from each other and increasing the volume of the inter-subunit cavity and linking tunnel (between the intra- and inter-subunit cavities).[92,93]

6. Conclusion

pLGICs are an important category of ion channels that are involved in the pathogenesis of many diseases (such as Alzheimer's disease and epilepsy) and are the target of many drugs acting on the central nervous system (such as GAs). Detailed molecular understanding of such complex multi-domain membrane proteins is challenging and requires a multidisciplinary approach. Computational simulations can complement experimental findings and in certain cases can be predictive. The emergence of crystal structures of several pLGICs along with advances in computational power has provided an opportunity to study channel gating, ion selectivity factors and the mechanisms underlying modulation by drugs and lipids. Future increases in accessible timescales as well as improvements to signal-to-noise ratios in finite temperature simulations remain essential for the process of ranking numerous computationally observed interactions in order of functional relevance.

Acknowledgement

This work was supported by National Institutes of Health [P01 GM55876] and the National Science Foundation [MCB1330728].

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