Comment on “On the Functional Annotation of open-channel structures in the glycine receptor”

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Running Title: Comment on the functional annotation of the glycine receptor

Abstract (150)

Recently, we reported the simulation of a stable open state of the glycine receptor. Central to the stability of the simulations was the behaviour of the highly conserved leucine residues at the 9' gate, which were found to rotate into adjacent pockets thus providing a structural rationale for decades of biochemical observations. In contrast, a previously reported model from Cerdan *et al*, resembled a more collapsed state. However, in support of their model, they draw attention to the agreement between calculated and experimental conductance measurements and argue that our model tends to overestimate ion flow. Here, we argue that there are many pitfalls with this approach and that the apparent agreement most likely reflects a fortuitous cancellation of errors. The computed values are highly sensitive to very small changes in model parameters. It is also likely that polarization effects will be very significant and these have not yet been considered.

Introduction

Progress in the solving of ion channel structures, particularly via the use of cryo-electron microscopy (cryo-EM), has meant that structural explanations may be found for experimental observables like conductance rates and selectivity. However, assigning an observed conformational state to a known functional state is non-trivial (Klesse et al., 2019) due, in part, to the precise balance of physical forces that become important in determining the functional state. Furthermore, the balance of these forces can also be subtly influenced by experimental conditions and how carefully atomic models are built. This was highlighted by efforts to functionally annotate the suggested open-state of the Glycine Receptor (GlyR), where under standard molecular dynamics simulation conditions the channel was reported to "collapse" into a new conformational state (Dämgen and Biggin, 2020, Cerdan et al., 2018).

Cerdan *et al*. published a model that used MD to relax the structure and presented agreement between calculated conductance rates of anions and experimental data as supporting evidence that they had captured a physiologically relevant open state (Cerdan et al., 2018). However, in structural terms this model has a pore-profile that is almost as closed as the desensitized state. In contrast, we recently presented a different MD-derived model (Dämgen and Biggin, 2020), that allowed the 9' leucines to occupy discrete interfacial pockets that stabilized the receptor in a state that was slightly more closed than the original open state captured by cryo-EM in the presence of detergents (Du et al., 2015), but more open than the model from Cerdan *et al.* In their letter to the editor, they comment that the computed conductance of a single representative snapshot from our model gives a conductance of 382 – 480 pS, which they argue is too large to correspond to the physiologically relevant open state (86 pS).

We previously argued (Dämgen and Biggin, 2020) that the model by Cerdan *et al*. was simply another collapsed state, a state which we can readily reproduce and has a low number of permeation events under 0 mV and equilibrium conditions. The agreement of their model with conductance is however extremely alluring and surprisingly good. However, there are several reasons why we believe this may actually just reflect a fortunate confluence of conflicting errors and that there are numerous issues to resolve before conductance can be estimated accurately computationally such that we can correctly annotate functional states of ion channels. Indeed, many of these issues were the reason we refrained from computing the conductance in our original paper (Dämgen and Biggin, 2020). In what follows we

outline several aspects that we believe the community needs to be aware of in order to critically assess the validity of computationally obtained conductance estimates.

Are we comparing like with like?

The experimental measurements are made on the full-length receptor, but Cerdan *et al.* use truncated systems that use a short linker between the M3 and M4 helices instead of the full intracellular domain. Furthermore, the conductance calculations are based on a model of the TMD only. Conductance is determined by the rate-limiting step of the permeation process. It is conceivable that in the open state, the rate limiting step could be in the ICD or the ECD. Thus, exclusion of the ICD or ECD in the receptor model might influence the conductance measurements. For the specific case of the α1 homomeric GlyR, the rate limiting step seems to lie within the TMD, because removal of the ICD does not seem to affect single channel conductance (Moroni et al., 2011) and replacing the ECD with the ECD from GLIC displays the same conductance as the full-length wild type GlyR α1 including a number of sub-conductive states (Moraga-Cid et al., 2015). However, this might not generalize to other members of the pLGIC superfamily and in any computational electrophysiology efforts applied to other pLGICs, this should be considered.

In the full-length cation-selective branch of pLGICs, ions are thought to exit the channel via lateral fenestrations in the ICD, an idea recently support by MD simulations on the albeit cation-selective α3β4 nAChR (Gharpure et al., 2019). Work on the related nACh and $5HT_3$ receptors, shows strong and substantial evidence that the ICD influences channel conductance (Hales et al., 2006, Kracun et al., 2008) where for example, the replacement of three arginine residues in the ICD of the $5HT_{3A}$ subunit results in a dramatic increase in single-channel conductance (Kelley et al., 2003), although for $5HT_3$ it has been argued that the ICD does not affect size selectivity (McKinnon et al., 2011). Although, there are similarities between the overall architecture between cation and anion pLGICs, it has also been argued that the ICD of anion-conducting pLGICs is significantly different from cation-conducting channels (Pandhare et al., 2019).

As for the ECD, simulations on the $5HT_3$ receptor have suggested that ions can enter/exit the ECD through gaps at the subunit interfaces on the side at the ECD-TMD interface, rather than through the

pore defined by the channel axis (Di Maio et al., 2015). In the case of eukaryotic pLGICs, the role of glycosylation both on or within the main ECD vestibule (see for example the $GABA_A$ receptor structures (Zhu et al., 2018, Phulera et al., 2018, Laverty et al., 2019)), which could possibly block the ion entry into through the main ECD vestibule, has not been investigated. Furthermore, it has been demonstrated that the ECD of pLGICs can exert substantial effects on conductance (Hansen et al., 2008, Moroni et al., 2011)

Another important aspect is that the experimentally obtained quantitative values for selectivity in GlyR show dramatic variation such that a dependence on a single report at this stage would seem premature. For example, if one compares the data from and (Bormann et al., 1987) and (Lee et al., 2003), the differences in selectivity are quite significant as summarized in **Table 1**.

Table 1. Permeability ratio P(X)/P(Cl-) for various anions

Species	Bormann *et al* (table 2) (Bormann et al., 1987)	Lee *et al* (table 1) (Lee et al., 2003)
acetate	0.035	0.09
nitrate	1.9	1.32
bicarbonate	0.11	0.13

Limitations of the Models

Assuming that the TMD alone was a sufficient model to estimate conductance for the homomeric α1 GlyR, i.e. assuming there are no rate-limiting steps outside the TMD, the following issues cast doubt on Cerdan *et al.*'s interpretation. Firstly, the computations are performed with position restraints on the protein backbone (and sometimes even the side chains) and consequently prevent correct sampling of the true ensemble of the physiological open state. A single structure is not representative of the whole ensemble and cannot reproduce the correct thermodynamic properties of the physiological open state. One of the key observations from our previous work (Dämgen and Biggin, 2020) is that the -2' Pro gate region is highly dynamic as evidenced by much higher than usual root mean squared fluctuation (RMSF)

values for the Cα atoms in this region as well as by the poor density of the cryo-EM map of 3JAE (Du et al., 2015). Thus, to reflect this correctly, position restraints should be avoided and much longer simulations would also be required to ensure correct sampling of permeation through this region. This would be particularly important if this region is in fact the rate-limiting step, where the presence of fluctuations may reduce the number of conductance events. It has already been shown that this position plays a vital role in selectivity (Lee et al., 2003).

Secondly, the use of an additive fixed charged model is known to have limitations and the particular water model used can influence the results significantly. Indeed, as Cerdan *et al*. originally demonstrated, TIP4P (which can be considered a more accurate water model than TIP3P) predicts a much lower conductance for the wide-open structure (3JAE) of 228 pS compared with 413 pS when using TIP3P – an almost 50% drop in conductance. Thus, different water models can give vastly different results. Furthermore, it is emerging that polarization effects may have a very pronounced effect on conductance predictions, as demonstrated for gramicidin for example (Peng et al., 2016). Moreover, Klesse has recently demonstrated (Klesse, 2019) that under a polarizable treatment, chloride ions exhibit high affinity to uncharged residues. This would be relevant here not only in the context of the -2'P gate, but also in other rather hydrophobic regions of the pore, where one would expect to see increased friction between the ions and protein, with consequent reduction in ion permeation rates. The inclusion of polarization effects into molecular mechanics force-fields has been challenging, but is steadily receiving more attention (Lin and MacKerell Jr, 2020).

Thirdly, the conductance was estimated by only two measurements at two different membrane potentials. Most importantly, a linear least squares fit based on only two different membrane potentials is statistically insufficient to yield an accurate conductance estimate. A related point is that the error estimate for the permeation events could be improved by performing more than just two measurements per membrane potential. In this context, it is important to keep in mind that the correct stochastic model for ion permeation is the Poisson-distribution, which was also used by Cerdan *et al*. for uncertainty estimation. However, we would like to point out that for this distribution, the variance equals the mean. Therefore, at higher voltages, when more permeation events are expected to be observed, the uncertainty increases. Hence, our recommendation would be to increase the number of independent

simulation repeats with increasing voltage in order to improve the accuracy of these data points relative to the ones obtained at lower voltages.

An additional minor point is that the simulations were performed at 300 K (26.85 °C), but the experiments are reported at 21-25 °C and thus the simulations would be expected to slightly overestimate the conductance.

Significance of the Leu Gate

The authors compare the two models with more recent cryo-EM data (Yu et al., 2019). They argue that their structural model is in better agreement with the new open GlyR structures from this preprint than our model - a conclusion reached by cherry-picking one structural aspect (the tilt of M2 helix), while ignoring other key structural properties, such as 9'L orientation, the -2'P gate or the overall pore profile. In fact, the images from (Yu et al., 2019) suggest that the taurine-bound (open) state agrees with Dämgen and Biggin in that the leucine at the 9' position is directed away from the pore. Furthermore, comparison to structures from another group (Kumar et al., 2019) (Fig. 1 therein) also suggests that the leucines at the 9' are indeed facing away from the luminal pore, in total agreement the model from Dämgen and Biggin and also in agreement with the open-state cryo-EM structure of the $5HT_3$ receptor (6HIN) (Polovinkin et al., 2018). In contrast to this, two of the five 9'L residues of Cerdan *et al*.'s model (Cerdan et al., 2018) are not accommodated in their hydrophobic pockets but oriented towards the pore centre (see Fig. 4c in Dämgen and Biggin, 2020). If these new structures with the leucine in the same position as suggested by us are not the open state, the question then becomes: what state do they represent, if any? Interestingly, the constriction at the -2'P in the recent open (Yu et al., 2019, Kumar et al., 2019) with 2.8-3.2 Å appears to be somewhat in between the model by Cerdan *et al.* (2.5 Å) and our model (3.6 Å).

Conclusion

Much progress has been made on the functional annotation of ion channels, but accurate prediction of conductance and which conformational state that corresponds to, remains challenging. Because of the issues outlined above, in our opinion, the approach by Cerdan *et al*. cannot estimate conductance accurately mainly due to the use of a reduced model, the lack of receptor flexibility and the omission of

explicit polarizability. To estimate conductance more accurately, polarizable force fields are likely to be a necessity.

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Declaration of Interests

The authors declare no competing interests.

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