THE EMBO JOURNAL

Manuscript EMBO-2012-82654

Structural basis for ion permeation mechanism in pentameric ligand-gated ion channels

Ludovic Sauguet, Frédéric Poitevin, Samuel Murail, Catherine Van Renterghem, Gustavo Moraga-Cid, Laurie Malherbe, Andrew W. Thompson, Patrice Koehl, Pierre-Jean Corringer, Marc Baaden and Marc Delarue

Corresponding author: Marc Delarue, Institut Pasteur

Review timeline:

Submission date:	13 July 2012
Editorial Decision:	05 September 2012
Revision received:	08 November 2012
Editorial Decision:	05 December 2012
Revision received:	03 January 2013
Accepted:	07 January 2013

Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

Editor: Anne Nielsen

1st Editorial Decision 05 September 2012

Thank you for submitting your manuscript for consideration by The EMBO Journal. It has now been seen by three referees whose comments are shown below. I would like to take this chance to apologize for the long reviewing time for your manuscript, which was cased by difficulties finding available referees during to the summer holiday season.

As you will see from the reports, all three referees express great interest in your manuscript and highlight the fundamental importance of your findings. However, while they would thus all support publication after appropriate revisions, referees #1 and #2 both emphasize that the manuscript will have to be shortened and rewritten for clarity, especially in the section addressing the MD simulations. In addition to this point, all three referees also raise a number of minor issues that you will have to address before submitting a revised version.

Given the referees' positive recommendations, we offer you the opportunity to submit a revised version of the manuscript, addressing the comments of all three reviewers. I should add that it is EMBO Journal policy to allow only a single round of revision, and acceptance or rejection of your manuscript will therefore depend on the completeness of your responses to the full satisfaction of the referees in this revised version. Please do not hesitate to contact me if you have questions related to the review process and the requests made by the referees.

When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Peer-Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process, please visit our website: http://www.nature.com/emboj/about/process.html

We generally allow three months as standard revision time. As a matter of policy, competing manuscripts published during this period will not negatively impact on our assessment of the conceptual advance presented by your study. However, we request that you contact the editor as soon as possible upon publication of any related work, to discuss how to proceed.

Thank you for the opportunity to consider your work for publication. I look forward to your revision.

REFEREE REPORTS:

Referee #1:

This paper reports a GLIC structure at sufficient resolution (2.4 ≈ I/sigma 1.7) to allow for the first time identification of mechanistically important waters, and from anomalous difference Fourier maps, ions and detergent molecules. This provides the starting point for an insightful series of MD experiments, which combined with the X-ray data lead to a model for ion permeation in GLIC, and most probably other pentameric ligand gated ion channels. New insight into the role of Ser 6 in ion permeation, previously identified in prior work, is achieved from MD experiments in conjunction with single channel recording.

In combination this paper reports significant and important advances that greatly advance our understanding of ion permeation in an important family of ion channels. I have high enthusiasm for this work.

In parts the manuscript is difficult to follow due to excessive detail and likely could be made accessible to a wider audience if this was addressed.

Major points

1) The authors need to consider if the presence of a membrane electric field (i.e. a physiological membrane potential) would alter any of their detailed analysis of ion permeation and water movements discussed ion pages 15-19.

2) Several of the Figures are going to be very difficult to see in a type set manuscript due to the small size of lettering, and large amounts of white space. Figure 1B, Figure 4C lower panels and Figure 8 lower panels are especially problematic.

Minor points

Summary Page 2. Meaning unclear ... a second wider water pentagon at the level of Thr 2', in continuous exchange with Glu -2'... this reads as if water and Glu2 exchange. Also, the crystal structure cannot reveal that the waters are in exchange.

Page 5: yields a precise assignment of ions. Perhaps change to yields a more precise assignment of ions

Page 8: meaning unclear ... ii) in fine they may prove to be ... What does 'fine' indicate?

Fig 2 and page 9. It is unclear if the Cs and Rb difference maps identified ions at both positions, or if Cs was detected at one site, and Rb at the other; if the latter is true does this have functional significance. It is unclear from Fig 4 and the text on page 10 how the Cs and Rb sites are related to binding of Na.

Page 10 paragraph 3 and 4: "Some density is observed for the crystallographic water pentagon ... " This is difficult to understand in relationship to the preceding text, which implies strong density peaks at Ser 6. Next, in paragraph 4 it is stated that there is a "tight water pentagon" although in paragraph 3 it is stated that the pentagon is not present. The confusion is probably because the authors are in some cases discussing crystallographic structures, and in other MD averages. This should be made clear (the text on page 11 is much easier to follow).

Page 15. "... the ion was pulled linearly down the central axis of the channel" ... The ion remained close to the center of the pore ..." Is position of the ion simply a consequence of the imposed trajectory, or could it move away from the symmetry axis?

Page 17 paragraph 1: Very difficult to follow; meaning and significance unclear: " ... Thr 2 ... adopts a slightly increasing proportion of the conformation, with almost 90 % of conformation when the ion is at the same level" and later: "... this behavior displayed a high variability".

Typos that need to be corrected - there are probably more.

Page 3 ... but also sophisticate ... Should be but also sophisticated

Page 3 ... following the pioneer work ... Should be following the pioneering work.

Page 4 ... Early study on the Torpedo ... should be Early studies on the Torpedo

Page 5 ... requires to document ... should be requires documenting

Page 12 ... under two-electrodes ... should be under two-electrode

Page 12 ... 4 different stoechiometries ... should be 4 different stoichiometries

Referee #2:

The manuscript by Sauguet et al. on the ion permeation pathway of pLGIC channels is based on a 2.4 ≈ resolution crystal structure of GLIC, electrophysiological studies, and extensive MD simulations addressing hydration and cation permeation mechanisms of the pore.

The results are very interesting to a broad membrane transport community and generally appealing to structural biologists, revealing a role of the pentameric symmetry on function. It is of course not unpexpected that pentameric features are observed along the permeation pathway, but how it links to function is key.
The crystallography is very solid and represents a significant step forward with proper assignment of hydration, H-bond interactions and DDM ligands of the structure and allows a comprehensive study on Na+/Cs+/Br- and SO4(2-) site localizations. The electrophysiology is also well performed and validates a key point. The MD is based on a previously reported structure refined at 2.9 ≈ resolution (3EAM) immersed in a DOPC bilayer.

The combination of data is very appropriate to reach a strong scientific point, and the overall train of arguments is also fine. However, the manuscript is too long as the MD simulations take an unproportional space and appear almost incomprehensible to the reader, overwhelming the very clear results obtained from the crystal structure and electrophysiology. An extensive rewriting, avoiding a long tail of arguments primarily from MD alone, would therefore improve the manuscript.

Specific points:

1) The crystallography appears excellent - my only concerns go towards validation: i) did the test set selection for Rfree take NCS into account?, ii) how many reflections were selected?, and iii) were overlapping or individual test sets used for the different data sets/structures reported in the table? Furthermore, iv) structure validation, preferably by MOLPROBITY, is missing - Ramachandran outliers etc.

2) The MD section has issues to consider. i) the simulations are performed in DOPC, which is described as a "native environment". In fact it is very far from any native environment, which will always be an asymmetric bilayer with a potential, and bacteria do not have PC lipids in any case. ii) On the same line of moderation, statements like "...probably due to the particular conditions encountered in the crystal" might as well say "probably due to the limitations of the MD simulations" when discussing inconsistent findings of simulations and experiments. iii) it is understandable that MD studies were performed on the 2.9 ≈ structure reported earlier, but at least some simulations should be presented to demonstrate consistency as several details on side chain rotamers and solvation models will have changed - e.g. the Na+ pulling simulation - otherwise the combination seems weird, or the high-res structure shoudl be used more clearly as validation of the MD. v) the MD assumes that the DDM cluster can be omitted. What if this plug consists of lipids in the native environment - seems very likely given the unusual observation of a DDM cluster in the permeation pathway. Could an active role of lipids in gating be considered, perhaps in response to the membrane potential?

3) the introduction starts out a bit weird - stating that not only high resolution structures but also sophisticated and costly computational approaches are required to reveal mechanisms. Period. I think most people would consider functional studies in appropriate cellular and in-vitro models as being foremost critical.

4) the MS is missing page numbers. The prime notation used occasionally is difficult to comprehend for the non-specialist reader and shoudl be replaced by ordinary residue names, perhaps indicating at the same time equivalent residues in GLIC (when used for other pLGIC channels), the model in

question here. Also the Cys-loop receptor family is suddenly referred to (top of intro page 2) without introduction of this term.

Referee #3:

Understanding ion permeation and selectivity is an important issue in ion channel research. The ms of Sauguet et al. addresses this issue for ion conduction in the pore of a pentameric pH-gated ion channel (GLIC) combining crystal structural work with some electrophysiological experiments and molecular dynamical simulations. The authors present a 2.4 A resolution GLIC structure with a precise alignment of water molecules, ions and detergents within the GLIC pore. Interestingly, the structural data indicated two water pentagons with one hydrated sodium ion between them. The two pentagons are situated at Ser6' and Thr/Glu 2', pore residue positions well known for their importance in ion conduction. Mutations of Ser6' alter the conductance of Na+ ions through the pore. Subsequent MD-based simulations suggest that the Ser6' side chain plays a crucial role in determining hydration geometry and its dynamics within the GLIC pore. The simulation results hint at an interesting anisotropy in the hydration profile, when Na+ crosses the constriction point, being most pronounced at positions Ile 9' and Thr 2'. The authors conclude from their observations that the positions in the crystal structure occupied by water pentagons are important for maintaining the hydration shell of the permeating Na+ ion while it travels past positions 9' and 2'. The ms presents an enlightening view of the dynamic role that the specific organization of hydration shell and water molecules has as the cation passes the barrier within the conduction pathway. In summary, this is a very nice and informative piece of work on a fundamental issue in ion channel function.

I have but a few points of criticism which the authors should address before their ms might be published.

Electrophysiology: The authors state that activation kinetics of GLIC channels with a higher proportion of Ser6'Gly were slowed, 'resulting in a right shift of the proton's concentration-response curves'. These data should be shown.

The single channel data also give some information on open probability, open times and possibly closed times if properly executed. This important information should be included in the conductance discussion.

MD-simulations: pLGIC sequences often contain a Thr 6' instead of Ser 6'. Interestingly, Ser appears to be paired with Ile 9', Thr 6', however, with Leu 9'. The distinct pairs of these important pore residues are likely to have conformational reasons, which may also involve the organization of water molecules within the pore. An MD-based simulation with Thr 6' paired to Leu 9' is a crucial computational experiment to validate the conclusions drawn from the data shown in Figure 6. In particular, one would like to know what the radius of the pore is after Ile has been replaced by Leu. A double GLIC mutant (Ser6'-Thr/Ile9'-Leu) would be very informative concerning the hypothesis that an interplay between Thr6' methyl side chain and Ile9' side chain orientation is responsible for a reduction of pore radius and an alteration in the dynamic organization of pore water molecules.

1st Revision - authors' response 08 November 2012

Queries by Referee #1 :

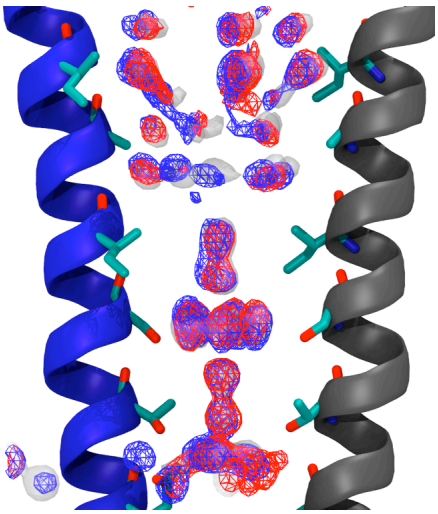
Q1 : In parts the manuscript is difficult to follow due to excessive detail and likely could be made accessible to a wider audience if this was addressed.

Concerning the MD simulations, the level of details provided in the main text was significantly reduced in order to make the manuscript accessible to a wider audience (see p.12-18). In particular, the Discussion section has been completely rewritten (see p. 18-23 of the revised manuscript). Some necessary controls are now described in Supplementary Material rather than in the main text, so that an expert reader will be able to find this information but the main text is easier to read.

Q2 : 1) The authors need to consider if the presence of a membrane electric field (i.e. a physiological membrane potential) would alter any of their detailed analysis of ion permeation and water movements discussed on pages 15-19.

As now briefly mentioned in the manuscript, application of a -50/+50mV membrane potential (in the MD protocol) does not significantly alter the hydration of the channel.

This is illustrated by the figure shown below that reveals that the hydration density observed before (white surface) and after (red) applying a -50/+50mV membrane potential are very similar (red and blue wireframe, respectively).



Furthermore, as recently demonstrated, the conductance of the channel is virtually independent of the voltage and is mainly determined by the protein (See: F. Zhu & G. Hummer, Theory and simulation of ion conduction in the pentameric GLIC channel, J.C.T.C. (2012)).

Q3 : 2) Several of the Figures are going to be very difficult to see in a type set manuscript due to the small size of lettering, and large amounts of white space. Figure 1B, Figure 4C lower panels and Figure 8 lower panels are especially problematic.

All figures that were problematic have been corrected (See Figures 1, 5, 6, 7, 8).

Q4 : Summary Page 2. Meaning unclear ... a second wider water pentagon at the level of Thr 2', in continuous exchange with Glu -2'... this reads as if water and Glu2 exchange. Also, the crystal structure cannot reveal that the waters are in exchange.

Summary has been rewritten and this particular sentence removed (see p.2). The apparent exchange between the "loose" pentagon and the side chain of Glu -2' is now discussed in more details in the result section and in the discussion of the revised manuscript (see p.8 – paragraph 1 and p.20-paragraph 1, respectively).

Q5 : Page 5: yields a precise assignment of ions. Perhaps change to yields a more precise assignment of ions

Done.

Q6 : Page 8: meaning unclear ... ii) in fine they may prove to be ... What does 'fine' indicate?

« *In fine* » (latin expression) was replaced by « Ultimately ».

Q7 : Fig 2 and page 9. It is unclear if the Cs and Rb difference maps identified ions at both positions, or if Cs was detected at one site, and Rb at the other; if the latter is true does this have functional significance. It is unclear from Fig 4 and the text on page 10 how the Cs and Rb sites are related to binding of Na.

This issue is now clarified in the revised version of the article (see p.9, paragraph 2). Cs^+ and Rb^+ are detected in two distinct sites of the pore, respectively named *cation site 1* and *cation site 2*. *Cation site 1* is also occupied by a Na^+ ion (always present in the crystallization solution) in the GLIC_2.4 structure and in the GLIC structure in complex with Rb^+.

Q8 : Page 10 paragraph 3 and 4: "Some density is observed for the crystallographic water pentagon ... " This is difficult to understand in relationship to the preceding text, which implies strong density peaks at Ser 6. Next, in paragraph 4 it is stated that there is a "tight water pentagon" although in paragraph 3 it is stated that the pentagon is not present. The confusion is probably because the authors are in some cases discussing crystallographic structures, and in other MD averages. This should be made clear (the text on page 11 is much easier to follow).

The entire description has now been rewritten and we tried to avoid any such confusion in the revised manuscript (see p. 13-14).

Q9 : Page 15. "... the ion was pulled linearly down the central axis of the channel" ... The ion remained close to the center of the pore ..." Is position of the ion simply a consequence of the imposed trajectory, or could it move away from the symmetry axis?

The ion is free to move away from the symmetry axis. We clarified this in the text by indicating: « Four independent 50 ns MD simulations were carried out pulling the ion linearly down the central axis of the channel across its 40Å-spanning TMD, leaving it free to move away from the symmetry axis. » (see p.15, paragraph 3).

Q10 : Page 17 paragraph 1: Very difficult to follow; meaning and significance unclear: " ... Thr 2 ... adopts a slightly increasing proportion of the alpha conformation, with almost 90 % of alpha conformation when the ion is at the same level" and later: "... this behavior displayed a high variability".

This complicated description of the correlation between sidechain conformational minima and ion hydration was completely rewritten and this particular part removed in the revised manuscript (see p.16, paragraph 3).

Q11 : Page 3 ... but also sophisticate ... Should be but also sophisticated

Done.

Q12 : Page 3 ... following the pioneer work ... Should be following the pioneering work.

Done.

Q13 : Page 4 ... Early study on the Torpedo ... should be Early studies on the Torpedo

Done.

Q14 : Page 5 ... requires to document ... should be requires documenting

Done.

Q15 : Page 12 ... under two-electrodes ... should be under two-electrode

Done.

Q16 : Page 12 ... 4 different stoechiometries ... should be 4 different stoichiometries

Done.

Queries by Referee #2:

Q17 : However, the manuscript is too long as the MD simulations take an unproportional space and appear almost incomprehensible to the reader, overwhelming the very clear results obtained from the crystal structure and electrophysiology. An extensive rewriting, avoiding a long tail of arguments primarily from MD alone, would therefore improve the manuscript.

We followed the referee's recommendation and have extensively rewritten the manuscript, reducing the detail on the MD simulations and clarifying the descriptions (see also the comments to the editor).

Q18 : 1) The crystallography appears excellent - my only concerns go towards validation: i) did the test set selection for Rfree take NCS into account?, ii) how many reflections were selected?, and iii) were overlapping or individual test sets used for the different data sets/structures reported in the table? Furthermore, iv) structure validation, preferably by MOLPROBITY, is missing - Ramachandran outliers etc.

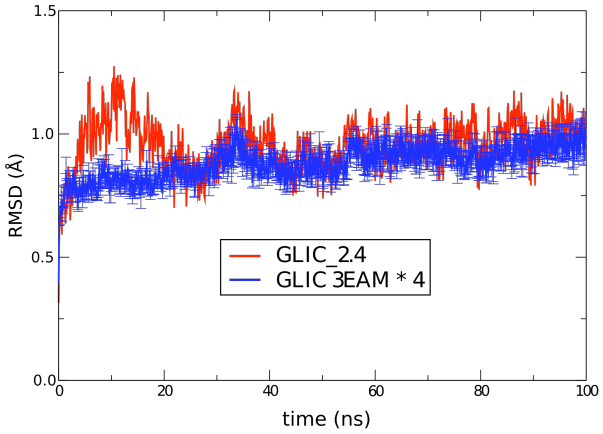
5% reflections were randomly selected for Rfree but did not take NCS into account. The test sets were overlapping for the different datasets presented in this structure. As a control, an independent Rfree set encompassing 10% of the data was selected randomly for the GLIC_2.4 dataset and the refinement process was reinitiated and conducted from the same initial structure. The final refinement statistics (R/Rf 20.6/21.7%) were very close to the one observed for the previous Rfree set (R/Rf 20.5/21.6 %).

Structure validation was checked using Molprobity and the corresponding values were added to the statistic table (See **Table 1**). All structures have over 98 % residues in the most favoured regions of the Ramachandran plot and are within the 99th and the 100th percentile among the best structures refined at a comparable resolution. This information is now included in the method section.

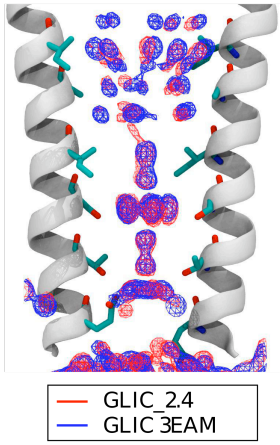
Q19 : 2) The MD section has issues to consider. i) the simulations are performed in DOPC, which is described as a "native environment". In fact it is very far from any native environment, which will always be an asymmetric bilayer with a potential, and bacteria do not have PC lipids in any case. ii) On the same line of moderation, statements like "...probably due to the particular conditions encountered in the crystal" might as well say "probably due to the limitations of the MD simulations" when discussing inconsistent findings of simulations and experiments. iii) it is understandable that MD studies were performed on the 2.9 A structure reported earlier, but at least some simulations should be presented to demonstrate consistency as several details on side chain rotamers and solvation models will have changed - e.g. the Na+ pulling simulation - otherwise the combination seems weird, or the high-res structure should be used more clearly as validation of the MD. v) the MD assumes that the DDM cluster can be omitted. What if this plug consists of lipids in the native environment - seems very likely given the unusual observation of a DDM cluster in the permeation pathway. Could an active role of lipids in gating be considered, perhaps in response to the membrane potential?

We have applied the moderation suggested by the referee and toned down the statements that were indicated (see p.12). We also carried out controls using the high resolution 2.4 Å structure directly as a starting point rather than the 2.9 Å structure, which showed no difference as the two structures are almost identical (calculated r.m.s.deviations over 1555 residues is 0.25 and 0.38 Å for Cα and all heavy atoms, respectively).

M2 RMSD



Water density



An active role of the lipids in gating is an interesting hypothesis, especially as lipids are known allosteric modulators of the pLGICs. However, it is difficult to relate it to the presence of a stable cluster of DDM in the pore of GLIC. We believe that the length of DDM's aliphatic tail (C12) is crucial in order to stabilize the detergent cluster. Phospholipids display a much longer aliphatic tail (typically, C18) that would prevent them from forming a stable cluster like DDM does. In addition, detergents like DDM are soluble in water (up to the CMC concentration), while lipids are not. We believe that DDM reaches the pore through the outer vestibule, as a lateral diffusion through the transmembrane domain would require large deformation of the bundle of helices. We thus think it is unlikely that lipids might access and thereby plug the pore in native conditions. Anyway, this interesting hypothesis cannot be totally rejected, even though more experiments, probably beyond the scope of this study, will be required to document that point further.

Q20 : 3) the introduction starts out a bit weird - stating that not only high resolution structures but also sophisticated and costly computational approaches are required to reveal mechanisms. Period. I think most people would consider functional studies in appropriate cellular and in-vitro models as being foremost critical.

This particular sentence has been replaced in the revised version of the manuscript by << Structural details at atomic resolution are often difficult to obtain for such trans-membrane proteins. A large number of structural studies has been undertaken for the tetrameric potassium channel KcsA, complemented by computational approaches to simulate the dynamic aspects of permeation following the pioneering work of MacKinnon, Roux and others. >> (see p. 3).

Q21 : 4) the MS is missing page numbers. The prime notation used occasionally is difficult to comprehend for the non-specialist reader and should be replaced by ordinary residue names, perhaps indicating at the same time equivalent residues in GLIC (when used for other pLGIC channels), the model in question here. Also the Cys-loop receptor family is suddenly referred to (top of intro page 2) without introduction of this term.

Page numbering has been introduced in the revised manuscript. We believe that it is important to keep the prime notation in the manuscript because it is universal to the pLGIC family. Nevertheless, the ordinary residue names have also been incorporated in the text (see p. 4) as well as in all the figures. Reference to Cys-loop receptor family has been replaced with pLGIC family.

Queries by Referee #3:

Q22 : Electrophysiology: The authors state that activation kinetics of GLIC channels with a higher proportion of Ser6'Gly were slowed, 'resulting in a right shift of the proton's concentration-response curves'. These data should be shown.

These data are now shown in a novel supplementary Figure (**Figure S3**).

Q23 : The single channel data also give some information on open probability, open times and possibly closed times if properly executed. This important information should be included in the conductance discussion.

The single-channel study described on GLIC S6'G heteropentamers constitute a notable achievement from a technical point of view. Indeed, the small single channel conductance of the GLIC channel (9 pS) and of the heteropentamer emphasized in **Figure 4** (4 pS) are near the limit of resolution of the patch-clamp technique. This makes time resolution very poor due to necessary filtering. In addition, we are most probably recording single channel data from a mixed population of several types of heteropentamers (as visible in **Figure 4**), which would need to be analysed individually. Trying to derive open state probabilities, or open- or closed- state dwell times distributions, from the single channel data described in this paper would be extremely difficult and not directly linked to the question asked in this study, which is studying the effect of mutation at the Ser 6' position on channel conductance.

Q24 : MD-simulations: pLGIC sequences often contain a Thr 6' instead of Ser 6'. Interestingly, Ser appears to be paired with Ile 9', Thr 6', however, with Leu 9'. The distinct pairs of these important pore residues are likely to have conformational reasons, which may also involve the organization of water molecules within the pore. An MD-based simulation with Thr 6' paired to Leu 9' is a crucial computational experiment to validate the conclusions drawn from the data shown in Figure 6. In particular, one would like to know what the radius of the pore is after Ile has been replaced by Leu. A double GLIC mutant (Ser6'-Thr/Ile9'-Leu) would be very informative concerning the hypothesis that an interplay between Thr6' methyl side chain and Ile9' side chain orientation is responsible for a reduction of pore radius and an alteration in the dynamic organization of pore water molecules.

Reviewer 3 pertinently questions if the apparent pairing between the residues at the 6' and 9' positions of the pore has conformational reasons. Following the reviewer's recommendations, the double GLIC mutant (S6'T-I9'L) was tested by electrophysiology. The results of functional tests on the double mutant (see p.15 - paragraph 1, **Figure 4** and **Figure S2A and S8**) and the related discussion (see p. 21) are included in the new manuscript. While the S6'T single-mutant displays no current, the second I9'L mutation rescued the activity of the channel, thus strongly suggesting that an interplay between the methyl of Thr 6' and the side chain of Ile 9' might explain the loss-of-function phenotype of the GLIC S6'T mutant. MD simulations were also performed on this double-mutant but were not conclusive in the sense that the I9'L mutation did not increase the pore radius at the level of the 9'. This is most likely due to the fact that this double mutation may induce a local conformational change that cannot be observed in a 100 ns time-scale simulation. More extensive MD simulations would be required to document this point further. In that sense, we decided not to include this simulation in the manuscript.

2nd Editorial Decision 05 December 2012

Thank you for submitting your revised manuscript to The EMBO Journal. It has now been seen by two of the original referees, whose comments are included below. While both referees find that all major criticisms have been sufficiently addressed, referee #1 highlights a few minor points that remain to be clarified before we can officially proceed towards acceptance of the manuscript. In addition, we would also need you to look into the following editorial issues:

It is EMBO Journal policy that key materials and methods are included in the main manuscript and not in the supplementary information. I understand that including a full description of the simulations performed in your study would be too extensive, but I have to ask you to include a description of the crystallization conditions, electrophysiology measurements and immunofluorescence in the main manuscript file.
I also have to ask you to add a scale bar to the immunofluorescence images in fig 4 with indication of size in the figure legend and to also provide information on the statistics and number of replicates underlying the error bars in fig 4F.
Thank you again for giving us the chance to consider your manuscript for The EMBO Journal, I look forward to your revision.

REFEREE REPORTS:

Referee #1:

The revised manuscript is substantially improved and is much more accessible for the wide readership of The EMBO J. My concerns about the Figures have also been largely addressed, with the exception of Fig. 8, for which it is still very difficult to see details in panel A.

As noted in my review of the original submission this paper marks a substantial advance in our understanding of ion permeation in an important class of ion channel. This was achieved via solving an X-ray crystal structure for GLIC at sufficient resolution to allow identification of mechanistically important waters, which combined with anomalous difference Fourier maps to locate ions and detergent molecule provided the starting point for an insightful series of MD experiments. A nice example of the level of insight that can be achieved is the restoration of permeation for the S6'T/I9'L double mutant, and the interpretation of the results in terms of conformational coupling.

1) The text on pages 8, 10 and 18 concerning waters in the loose pentagon involved in the hydration shell of Na is unclear and should be revised. On line 9 a value of d=3.67{plus minus}0.23 Angstrom is reported. Is this the distance between the water oxygen atoms alone, or is it the sodium - oxygen distance, or both? Figure 1 shows long bond distances for sodium-oxygen interactions. Are these distances consistent with the conventional understanding of the inner hydration shell of Na for which a bond distance of 2.4 Angstrom would be expected? A nice paper on this was published by Mahler and Persson (2012) Inorg Chem 51: 425-438. These details are very important since it leads to a consideration of whether ion hydration in an ion channel pore is different from that in bulk solvent, perhaps not surprising given the ordered water pentagons that the paper already clearly addresses.

Related to this, what bond distance cutoff was used in Figure 7 to calculate the coordination state for Na, and was this the same for all Na ligands?

2) The clarification of the location and occupancy of cation binding sites on page 9 paragraph 2 raises important questions concerning ion occupancy and permeation. The crystallographic data imply that both Na (cation site 1) and Rb (cation site 2) bind simultaneously in a single file pore. Is this correct? The alternative would be partial occupancy at each site. Can Na occupy both sites?

Also, as asked in the prior review, do the different locations of the Cs, Rb and Na ions imply different selectivity at these sites?

3) Figure 8 is incomprehensible at the resolution shown and should be rethought, or perhaps move to supplemental information

Minor points

Page 4 paragraph 2 line 13; change to: the underlying mechanism of this particular...

Page 9 line 5: change i) they allow to: i) they validate

Page 9 paragraph 2 line 3, change 'must be' to: is likely

Page 10 paragraph 3 line 2, as written this implies that BHK cell recordings were performed under two electrode voltage clamp, which while perhaps possible, is very unlikely.
Page 10 paragraph 3 lines 5-6. Meaning unclear. The same results were obtained?

Pages 10 last line and pages 11: The description of whole cell recording for BHK cells and TEVC for oocytes is mixed up is several sentences.

Pages 14 and 17: why multiple paragraphs, some with only one sentence?

Page 21 paragraph 3 line 3, should be: an intact but inactive pentamer.

Fig 1 legend: presumably the maps are maximum likelihood 2mFo-DFc? Please correct. Why is there no density for the Na ion in the lower left panel?

Referee #2:

The authors have met the remarks and answered well to the questions raised by the reveiwers. The manuscript provides a ramarkable insight of pentameric ion channels at a significantly improved resolution that important aspects of solvent interaction controlling the ion permeation pathway can be described and discussed.

Besides minor details to be picked up by the editorial process the manuscript is fit for publication.

2nd Revision - authors' response 03 January 2013

Editorial issues:

While both referees find that all major criticisms have been sufficiently addressed, referee #1 highlights a few minor points that remain to be clarified before we can officially proceed towards acceptance of the manuscript. In addition, we would also need you to look into the following editorial issues:

1) First of all it is EMBO Journal policy that key materials and methods are included in the main manuscript and not in the supplementary information. I understand that including a full description of the simulations performed in your study would be too extensive, but I have to ask you to include a description of the crystallization conditions, electrophysiology measurements and immunofluorescence in the main manuscript file.

Ok. The key material and methods concerning protein expression, crystallography, electrophysiology and fluorescence microscopy are now included in the main manuscript.

2) In addition, the literature references should be presented in the format described in our guidelines

Ok.

3) The main article text file should be uploaded in doc format rather than pdf.

Ok.

4) I also have to ask you to add a scale bar to the immunofluorescence images in fig 4 with indication of size in the figure legend and to also provide information on the statistics and number of replicates underlying the error bars in fig 4F.
Ok.

5) We would also need you to provide an outline of the author contributions,

Ok.

6) a conflict of interest statement

Ok.

7) The PDB coordinates for the reported crystal structure.

All PDB coordinates and structure factors related to this study have been deposited on the PDB. PDB entries are provided in table 1.

Point-to-point answer to Referee #1:

The revised manuscript is substantially improved and is much more accessible for the wide readership of The EMBO J. My concerns about the Figures have also been largely addressed, with the exception of Fig. 8, for which it is still very difficult to see details in panel A.

Figure 8 has been improved by removing unnecessary details and by transferring part of the

information to a novel supplementary Figure S9.

As noted in my review of the original submission this paper marks a substantial advance in our understanding of ion permeation in an important class of ion channel. This was achieved via solving an X-ray crystal structure for GLIC at sufficient resolution to allow identification of mechanistically important waters, which combined with anomalous difference Fourier maps to locate ions and detergent molecule provided the starting point for an insightful series of MD experiments. A nice example of the level of insight that can be achieved is the restoration of permeation for the S6'T/I9'L double mutant, and the interpretation of the results in terms of conformational coupling.

1) The text on pages 8, 10 and 18 concerning waters in the loose pentagon involved in the hydration shell of Na is unclear and should be revised.
   We revised the text concerning the loose pentagon:
- page 8, §2, line 9: we added the mean distances between the water molecules of the loose pentagon and the Na+ ion and/or the oxygen atom of Thr 2' in order to clarify the observation that the water molecules of the loose pentagon interact more tightly with the Na+ ion and Thr 2' hydroxyl rather than with each other (see also the answer to point 2).
- Page 10, §1, lines 2 and 3: the text was revised.
- Page 17, §2 and page 18. The discussion section concerning the loose pentagon has been rewritten and is now hopefully more clear. (see also the answer to point 3).

2) On line 9 a value of d=3.67{plus minus}0.23 Angstrom is reported. Is this the distance between the water oxygen atoms alone, or is it the sodium - oxygen distance, or both?

The corresponding text has been revised in the manuscript. d=3.67 Angstrom is the mean distance measured between the oxygen atoms of the five water molecules composing the "loose pentagon". This rather large distance suggests that the water molecules do not interact tightly with each other in the "loose pentagon" but rather with the Na^+ and the oxygen atom of Thr 2' for which the main values respectively are 3.45 ± 0.21 and 3.25 ± 0.18 Angstrom.

3) Figure 1 shows long bond distances for sodium-oxygen interactions. Are these distances consistent with the conventional understanding of the inner hydration shell of Na for which a bond distance of 2.4 Angstrom would be expected? A nice paper on this was published by Mahler and Persson (2012) Inorg Chem 51: 425-438. These details are very important since it leads to a consideration of whether ion hydration in an ion channel pore is different from that in bulk solvent, perhaps not surprising given the ordered water pentagons that the paper already clearly addresses.

This is an interesting observation, which is now discussed in the manuscript (see page 18, §2). "The observed mean distance between the Na^+ ion and the water molecules from the "loose pentagon" (3.45±0.21Å) are higher than that of the bulk (2.43Å) (Mahler and Persson, 2012). This suggests that ion hydration in the selectivity filter region of the pore differs significantly from that of the bulk and is reminiscent with the MD Na^+-pulling experiment showing that the distribution of water molecules around the permeant ion becomes strongly anisotropic in this most constricted region of the pore."

4) Related to this, what bond distance cutoff was used in Figure 7 to calculate the

coordination state for Na, and was this the same for all Na ligands?

The cut-off distance is 3.2 Angstrom and it was the same for all Na ligands. This is now detailed in the corresponding figure legend.

2) The clarification of the location and occupancy of cation binding sites on page 9 paragraph 2 raises important questions concerning ion occupancy and permeation. The crystallographic data imply that both Na (cation site 1) and Rb (cation site 2) bind simultaneously in a single file pore. Is this correct?

Yes, this is correct. Furthermore, the B-factors for each cation are very close to that of the neighbouring protein residues suggesting that both sites are fully occupied.

The alternative would be partial occupancy at each site. Can Na occupy both sites?

In principle yes, Na could occupy both sites. Nevertheless, so far, we have only observed Na binding in cation site 1.

Also, as asked in the prior review, do the different locations of the Cs, Rb and Na ions imply different selectivity at these sites?

This is a seducing hypothesis regarding the different hydration properties of these ions, but it might be too speculative as GLIC is non-selective channel to monovalent cations. Indeed, GLIC has been shown to conduct, Na+, K+ and Cs+.

3) Figure 8 is incomprehensible at the resolution shown and should be rethought, or perhaps move to supplemental information

Figure 8 has been improved by removing unnecessary details and by transferring part of the figure to a novel supplementary Figure S9.

Minor points

Page 4 paragraph 2 line 13; change to: the underlying mechanism of this particular...

Ok. Done

Page 9 line 5: change i) they allow to: i) they validate

Ok. Done

Page 9 paragraph 2 line 3, change 'must be' to: is likely

Ok. Done

Page 10 paragraph 3 line 2, as written this implies that BHK cell recordings were performed under two electrode voltage clamp, which while perhaps possible, is very unlikely.
Page 10 paragraph 3 lines 5-6. Meaning unclear. The same results were obtained?
Pages 10 last line and pages 11: The description of whole cell recording for BHK

cells and TEVC for oocytes is mixed up is several sentences.

The electrophysiology part of the result section has been substantially revised and these concerns were all assessed (See pages 10-11)

Pages 14 and 17: why multiple paragraphs, some with only one sentence?

These sentences were fused in one single paragraph

Page 21 paragraph 3 line 3, should be: an intact but inactive pentamer.

Ok. Done

Fig 1 legend: presumably the maps are maximum likelihood 2mFo-DFc? Please correct.
Why is there no density for the Na ion in the lower left panel?

The maps are the maximum likelihood 2mFo-DFc maps and this is now mentioned in the figure legend.

The density for the Na ion is shown on the side view of the pore but not on the lower left panel where it has been removed for clarity to prevent it from masking the electron density of the loose pentagon that lies below the Na ion. This is now mentioned in the figure legend.