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The Pore Structure and Gating Mechanism of K2P Channels

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(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.)

1st Editorial Decision 13 April 2011

Thank you for submitting your manuscript for consideration by The EMBO Journal. Three referees have now evaluated it and their comments are shown below. You will see that the referees are generally positive about your work and that they would support its ultimate publication in The EMBO Journal after appropriate revision. I would thus like to invite you to prepare a revised manuscript in which you need to address or respond to the points raised by the referees in an adequate manner.

I should add that it is EMBO Journal policy to allow only a single round of revision, and acceptance of your manuscript will therefore depend on the completeness of your responses in this revised version.

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.

Yours sincerely,

Editor
The EMBO Journal

REFEREE COMMENTS

Referee #1
EMBO J-2011-77604

This paper by Piechotta and colleagues starts to explore the gating mechanism of three K2P channels (TREK1, TASK3, TRESK) that generate large currents and were ideal for assessing intracellular block of the operative gate(s) by QA ions. The paper concludes that the operative gate in these K2P channels is the selectivity filter gate and not the helix bundle crossing gate. 5 major points are made:

1) Increasing the length (i.e. hydrophobicity) of the alkyl chain in symmetrical QA ions resulted in a large increase in their intracellular potency for all three K2P channels and altered their block kinetics [Fig. 1];

2) The QA block of these K2P channels showed little voltage dependence and was weakly affected by extracellular K, similar to Shaker Kv channels and unlike Kir channels [Fig. 2]. Moreover, the homology model that best fit the TPenA affinity of TREK1 and its mutants of the entire P1-M2 and P2-M4 regions was the one based on the KvAP structure [Figs. 3 and Table S1];

3) Based on the model built using the KvAP structure, 5 residues stood out as potentially interacting directly with TPenA: P1(T157), TM2(I182 and L189), P2(T266), TM4(L304) [Fig. 4];

4) Exploiting the slow kinetics of THexA block compared to the fast activation and deactivation kinetics of proton and mechanical gating of TREK1, the authors provided evidence that the K2P channel is blocked in both closed and open states (unlike the open state block of the Shaker Kv). Moreover TPenA inhibition (IC50) for TREK1 showed no dependence on NPo, unlike a clear decrease for the Shaker Kv, consistent with the notion of QA block occurring in both states for TREK1 but only in the open state for the Shaker Kv [Figs. 5 and 6].
These results are consistent with the KvAP model of the Helix Bundle Crossing gate being open for TREK1, not limiting the QA blocker to bind at its site while the channel is closed [Fig. 6G];

5) The T157C mutant as well as mutants around but not at the T266C mutant (i.e. L264C, T265C, and I267C) affected significantly the pH EC50, lending further evidence that the selectivity filter serves as the pH gate that is blocked by the QA ions [Fig. 7]. Moreover, substitution of K by Rb stabilized the filter gate increasing the pH EC50, while Tl did the opposite [Fig. 8].

This is a well-designed and well-performed study that addresses an important question in the K2P field and would be of interest to many investigators in the ion channel field, as gating is the most actively pursued area of research in ion channel biophysics. My comments are meant as suggestions to further improve the impact of this valuable study.

Major Points:

1. One of the major points made is that the Helix Bundle Crossing gate is not operative in the K2P channels examined and this conclusion is primarily reached by comparing state-dependent QA block of TREK1 versus the Shaker Kv. It would greatly increase the impact of the work, if another independent experimental approach tested this hypothesis and provided further supporting evidence. One suggestion maybe introducing Pro residues at the "Gly hinge" region of TM2 or TM4 to force the Helix Bundle Crossing gate open as it does in many other channels. I would pursue this experiment in silico first (with the KvAP-based homology model) to determine which residue might

be most effective in inducing the largest kink before pursuing it experimentally. A positive experimental control maybe needed to show that the mutation enables access through the Helix Bundle Crossing (not sure whether part of the TOctA reduced efficiency is due to getting through this gate. If so, this QA blocker maybe a good choice). The expectation from such an experiment would be that both the QA block and proton gating of the channel ought to not be affects by the Pro mutation.

2. It should be fairly straightforward and informative to test QA block in the way the experiments shown for pH gating were done to assess the effects of Rb and Tl (i.e. compare behavior of WT TREK-1 in K solutions -as in Fig. 3C - to Rb and Tl solutions as was done for pH in Figure 8).

3. I want to commend the authors for the systematic mutagenesis profiling of the P1-M2 and P2-M4 regions and the parallel modeling effort to select the best model that is consistent with the experimental data. Yet, I feel that the discussion on page 9 about how residues in TM4 may be affecting TPenA affinity is speculative and as such it does not belong in the Results section. I would move this discussion to the Discussion section on page 16, around where the authors state "...but whether this extends beyond the non-identical nature in the side chains of the two pores is not clear...". Exploring this issue further in this or future work by mutating residues of TM2 into those in TM4 and vice-versa will indeed be very interesting and provide greater insight into the determinants of the asymmetric behavior of these channels.

Minor Points

1. On page 4 in the first paragraph, the Clarke et al., paper out of Jackie Gulbis' lab in 2010 published in Cell ought to be mentioned here and used in the Discussion (see below).

2. On page 6 in the second paragraph, the reference to the figure is incorrect. Change "...of about 60 mM (Figure 1B/D)." to "...of about 60 mM (Figure 1B/E)."

3. On page 7 in the second paragraph, change "reliable" to "reliably" ["However, for THexA and smaller QA ions, inhibition could be reliably determined and in Figure..."]

4. On page 7 in the last sentence, change "similar" to "similarity" ["greater similarity with Shaker than Kir1.1 (Figure 2D). This remarkable similarity in the..."]

5. On page 8 in the second paragraph, add the word "the" before "inner pore" ["...position within the inner pore and that the data from Figure 3A could be used to distinguish..."]

6. On page 8 in the third paragraph, add the word "the" before "inner pore" ["...the nitrogen atom was positioned at the centre of the inner cavity, and TPenA aligned with the...]

7. On page 9 in the second paragraph, reference to P183 is mislabeled in Figure 4 as P182.

8. On page 10 in the first two lines, I would remove the statement: "Furthermore, the next best fit models, based upon MthK (3LDC) and Kv1.2 (2R9R), are also open at the bundle crossing." The Kv1.2 (2R9R) structure is a bad as the KcsA closed (1K4C) structure (3 hits, 3 false positives).

9. On page 15 in the third paragraph, change the "are" into "is" ["...here that the intracellular pore of K2P channels is blocked by QA ions in a very similar...]

10. On page 17 in the third paragraph, correct the English in the sentence: "This gating behavior most closely resembles CNG channels with are although thought to gate exclusively at the selectivity filter..."

11. On page 19 in the second paragraph, I feel that your discussion would benefit by bringing in the tight coupling of the gates (as suggested by Clarke et al., 2010 and Cuello et al., 2010a; Cuello et al., 2010b), regardless of whether the gates are operative or not.

12. On page 20 in the Figure 3 legend indicate what the light green bars represent and also what ND represents for the G296C mutant.

Referee #2

This paper uses an appealing combination of mutagenesis, biophysical pharmacology and molecular modeling to generate a rendering of the TREK-1 channel pore and, more importantly, to establish that the bundle crossing is non-restrictive in closed channels and that the gate for H/pressure activation is likely in the selectivity filter.

Overall, this is a very nice paper. The experiments appear to have been carefully performed, the paper is well written and well illustrated and the conclusions are well supported. A few minor concerns and/or corrections are noted.

General: Although generally convincing, the suggestion that QA ions are blocking in K2P channel pore seems to rely largely on arguments by analogy (i.e., shared properties with QA block of Kv channels). The mutagenesis also supports the pore block idea but those types of studies are not definitive. Can the authors provide a more conclusive experiment to demonstrate that QA inhibition is truly due to a classic pore block mechanism? This would be helpful since the main conclusion that the bundle crossing is not restrictive, even when the channel is closed, relies on the assumption that the QA binding site is located in the pore, above the crossing. The rationale used for picking the best fit model also presumes this site of action.

p. 6 "The IC50 for QA inhibition for TRESK increased sharply ..." should be decreased

p. 8 ".... produced an approximately 500 fold reduction in TPenA block" Is this a 500 fold increase in IC50? or decrease in sensitivity? It does not seem likely that the block itself could be reduced 500 fold.

p. 16, 2nd and 3rd paragraph. The text indicates that the Striet et al 2011 paper modeled TASK-3 but it looks like that paper dealt with TASK-1.

Referee #3

General comments

In this study, Piechotta et al. have used a range of quarternary ammonium ions (with different length alkly chains) to investigate the pore structure and gating mechanism of Trek-1 potassium channels. Having established that QA ions block a range of 2PK channels from the intracellular side and that the binding site within the pore is close to the selectivity filter (using mutagenesis and homology modeling), they then provide compelling evidence that THexA can access its binding site equally well when the channel is closed or open. This demonstrates that the bundle crossing gate in TREK-1 channels has to allow passage of large QA ions even in the "closed state" and the authors conclude that the primary gating mechanism in K2P channels, unlike KcsA and Kv channels, resides close to or within the selectivity filter.

Specific comments

1. Discussion, p17, 3rd paragraph, 3rd sentence.
Based on the results presented in this study, the authors should be careful about concluding that the inner pore cavity does not undergo any major structural rearrangements during activation.
The data in this study shows two things: a) the binding site for QA ions near the selectivity filter does not undergo substantial changes between the "open" and "closed" states and b) the pore aperture at the bundle crossing has to be sufficiently wide in both the "open" and "closed" states to allow free access of large QA ions. This is not quite the same as saying that the pore cavity does not undergo any major structural rearrangements. It may not do but that is yet to be proven. The authors should tone down this statement.

2. p.6 Bottom 3 lines describe a qualitative assessment of the kinetics of QA binding and unbinding. Whilst the time-constant for "unbinding" is simply a reciprocal of the off-rate (assuming a simple bi-molecular binding mechanism) the on-rate is a function of both the time constants of binding and unbinding. Thus an apparently fast rate of binding can be misleading if the rate of unbinding is also fast. It is an easy matter to calculate the on-rates and off rates (assuming a bi-molecular binding mechanism) and these should be reported rather than the qualitative arguments used in the text

1st Revision - authors' response 30 May 2011

Thank you for the positive feedback regarding the above ms which we recently submitted for consideration by The EMBO Journal. We note that all three referees responded enthusiastically, and we address their specific comments below:

Referee #1

Major Points
The referee has made several helpful suggestions for future studies and we thank them for these interesting ideas.

1. The introduction of proline residues into the TM helices could indeed be used to attempt changes in width of the bundle crossing opening. However, the precise structural consequences of such profound changes in the backbone of the TM helices are unpredictable especially because modelling cannot take into account the restraints imposed by the cytoplasmic domains. Any results obtained would therefore be open to many different possible interpretations, whether or not they affected QA sensitivity. Therefore at this stage we do not feel that such experiments would enhance the present study.

2. We have now determined the effect of exchanging K^+ for Rb^+ on QA inhibition. As expected, and in contrast to pH activation, QA inhibition was not affected by the permeant ion. This is now mentioned on page 14 and also helps address the concern of Referee#2.

3. The speculative comments in the results section have been removed so that this section is now simply descriptive of the results observed.

Minor Points
All of these minor comments have now been addressed and typos corrected throughout the ms. We thank the referee for the care taken in spotting these errors and omissions.

1. References now included
2. Typo corrected
3. Typo corrected
4. Typo corrected
5. Typo corrected
6. Typo corrected
7. Typo in figure 4 corrected
8. Statement now clarified.
9. Typo corrected
10. Typo corrected
11. Mention is now made to tight coupling of gates and references included.
12. Figure legend clarified.

Referee#2

General comment - *Can the authors provide a more conclusive experiment to demonstrate that QA inhibition is truly due to a classic pore block mechanism?*

One distinctive feature of intracellular pore blockade is the effect of extracellular K^+ on blocker affinity (external K^+ knock-off). As shown in Fig. 2C, raising the extracellular [K^+] from 4 to 120 mM resulted in an 1.4 fold decrease of the IC_{50} for TPenA inhibition for TRESK. A similar outcome (2.1 fold decrease) is shown in Fig2C for TPenA inhibition in the Shaker Kv channels, for which the pore blocking mechanism of QA has been established beyond all reasonable doubt. This K^+ knock-off effect adds further support to our structural model for a QA binding site within the K2P pore, as does the lack of effect of the permeant ion on QA block (see response to referee #1).

The typographical errors noted on p6,8 and 16 have all now been corrected.

Referee#3

1. The statement on p17 has now been clarified, and this qualification is also repeated on p18 where we address this question of movement of the TM-helices in more detail. We state clearly that the TMs must exhibit some degree of movement in order to transduce the intracellular gating signal to the filter.

2. *p.6 Bottom 3 lines describe a qualitative assessment of the kinetics of QA binding and unbinding. Whilst the time-constant for "unbinding" is simply a reciprocal of the off-rate (assuming a simple bi-molecular binding mechanism) the on-rate is a function of both the time constants of binding and unbinding. Thus an apparently fast rate of binding can be misleading if the rate of unbinding is also fast. It is an easy matter to calculate the on-rates and off rates (assuming a bi-molecular binding mechanism) and these should be reported rather than the qualitative arguments used in the text.*

Certainly, it would be possible to report rates instead of the time constants in Fig. 5. However, we intentionally have chosen to report the time constant for two reasons. Firstly, the tau_{off} values for TButA, TPenA as well as the tau_{on} value for 5 µm THexA were within the range of the solution exchange and, therefore, cannot be regarded as the reaction rates for blocker binding and unbinding (thus reporting rates here would be misleading). Secondly, in Fig. 5 C the intention was to compare the time course of QA inhibition and release from inhibition with the speed of solution exchange, and the latter is traditionally (and most appropriately) parameterised by tau values. For these reasons, we would strongly prefer to stick to the time constants.

2nd Editorial Decision 24 June 2011

Thank you for sending us your revised manuscript. Our original referee 1 has now seen it again, and you will be pleased to learn that in his/her view you have addressed the criticisms in a satisfactory manner. Still, there is one editorial issue that needs further attention. Please add the statistical details including the number of independent repeats into all relevant figure legends including the supplementary material.

Please let us have a suitably amended manuscript as soon as possible. I will then formally accept the manuscript.

Thank you very much again for considering our journal for publication of your work.

Yours sincerely,

Editor
The EMBO Journal

REFEREE COMMENTS

Referee #1

The authors have adequately addressed my concerns and comments in the revised version.

