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Coupling of activation and inactivation gate in a K⁺ channel: potassium and ligand sensitivity

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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.)

1st Editorial Decision

05 May 2009

Thank you for submitting your manuscript for consideration by the EMBO Journal. First, please let me apologise for the unusually long time taken for us to get back to you with a decision. However, I have now received the comments of three referees, which are enclosed. As you will see, all three referees find your work interesting and are broadly in favour of publication. However, a number of issues - primarily requiring changes to the text rather than additional experiments - first need to be resolved in a revised version of your manuscript. In particular, I would like to draw your attention to the comments of referee 2 regarding the accessibility of your manuscript to the non-specialist. In addition, all three referees highlight a number of areas that would benefit from further discussion. Given that your manuscript is currently significantly under our length limit, it should not be too much of a problem to extend the discussion of your results along the lines suggested by the referees.

I would therefore like to invite you to submit a revised version of the manuscript, addressing all the comments of all three reviewers. I should add that it is EMBO Journal policy to allow only a single round of revision. Acceptance of your manuscript will thus depend on the completeness of your responses included in the next, final version of the manuscript.

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

Yours sincerely,

Editor
The EMBO Journal

REFEREE REPORTS:

Referee #1 (Remarks to the Author):

This study by Ader et al directly addresses a fundamentally important concept in K⁺ channel gating i.e. the link between the gate at the selectivity filter and the helix-bundle crossing (HBC) gate. A powerful combination of electrophysiological and ssNMR data are combined to propose an attractive hypothesis for a gating cycle. They observed protonation of E118/120 at the HBC indicating an important role for these residues in the pH-gating mechanism and the study also highlights an important mechanistic difference in the way KTX and porphyrin block the channel.

The title suggests that the authors are able to directly visualize ions in the inactivation gate, but this is not the case. They detect a K⁺ sensitivity to the processes they measure. However, they do not demonstrate that it is the direct effect of K⁺ ions within filter that exerts this effect. It may well be the most logical explanation, but has not been shown directly in this study. I would suggest the title be rephrased to take this into account and perhaps emphasize the link between the two gates. Also, are the authors able to correlate their results on K⁺ sensitivity with the ionic sensitivity for KcsA which has been reported previously. Although this point is mentioned it is not discussed in any great detail.

A major weakness of the study is that they are unable to provide any direct structural mechanism for the observed linkage between the filter and HBC gates. It has been known for some time that functional linkage between these gates exists, but so far no structural explanation has been presented. The current study goes further than many previous studies in this area as it provide direct evidence for a correlated movement of these gates, but does not propose a clear explanation of how they are linked. The authors detect movement of residues 99-101 which are intriguingly close to the T/V residues of the selectivity filter which are observed to collapse during inactivation. Is it possible that a direct physical interaction between this part of TM2 and the pore-helix is responsible for a direct mechanical coupling of the two gates? This intriguing possibility has not been properly explored or discussed.

Referee #2 (Remarks to the Author):

The manuscript by Ader et al. seeks to describe the gating of a KcsA-Kv1.3 chimera and its dependence on permeant ions and to demonstrate coupling between inactivation and activation gates. The authors spectroscopically (ssNMR) identified several major gating states of the channel and attempted to correlate them with electrophysiological data. The premise of the study is interesting, the combination of techniques seemed promising, but the manuscript did not meet expectations mainly because the data is not well presented and explained, and as a result the message of the paper is vague and unclear.

First, the authors use electrophysiological current recordings to show that the gating of the chimera is affected by internal and external K. External K appears to slow down pH-induced inactivation while internal K accelerates it. The former is interpreted to mean that occupancy of an external K binding site keeps the channel from collapsing while the latter suggests that occupancy of an internal K binding site is responsible for closing the channel activation gate rather than the inactivation gate (this interpretation is not supported by the data presented to this point). These sided pH and K-induced electrophysiological changes at the gates are further probed with ssNMR chemical shift mapping. It is concluded that acidic pH leads to conformational changes at the gate and selectivity filter only in the absence of K. The protonation states of the filter and activation gate glutamates appear to also be K-sensitive, suggesting that in the presence of K, the activation gate is always closed, independent of pH. This conclusion is a bit odd, since it is known that one can record from KcsA in lipid bilayers in high K (above 50 mM K) and the P_o is pH-dependent. The authors need to discuss how their results compare with already existing data on KcsA and how the conditions of their NMR experiments correlate with E. Perozo's EPR studies on KcsA (they also probed activation gate conformations). Furthermore, the authors need to briefly describe the

chimeric channel they are working with and how it was constructed. The reader should not need to go to a different paper to figure out what KcsA variant the paper is on.

Lastly, the authors claim they can understand coupling between the activation and inactivation gates by spectroscopically observing channels complexed with either porphyrin or kaliotoxin, that stabilize either a non-conductive or a conductive conformation of the selectivity filter, respectively. The porphyrin-bound channel appears to be in a closed-collapsed form at pH 7.5 and open-collapsed at pH 4. The experimental evidence for gate coupling (interactions between the side chains of the residues in the filter) needs to be explained better as it is not clear to this reviewer. This appears to be a major point of the paper and should be given more attention. Furthermore, the kaliotoxin-channel experiments are not well presented either. It appears that the activation gate is always closed, even at pH 4, in these channels forced in a conductive conformation by kaliotoxin. However, the message is unclear, and the effects are not readily apparent from a figure.

Other major points:

- 1) The authors need to make an effort to present their results and discuss them in a more integrated and focused way as they go along. Right now, the paper appears disjointed since the electrophysiology and the NMR are presented separately, with few parallels made.
- 2) The authors try to make an important point that in the presence of K the activation gate is predominantly closed (using ssNMR). This needs to be discussed a bit more because it is not clear what the authors think is going on and how to relate this to electrophysiology.
- 3) One predicts to also see a less collapsed filter in high K than in low K. The authors do not discuss how their spectroscopic results correlate with the external K effects on inactivation seen electrophysiologically.
- 4) There is a histidine that was also implicated in pH sensing by an NMR study and an electrophysiology study. The authors only focus on the glutamates. The changes in the protonation states of this histidine and its K and pH dependence need to be briefly discussed.
- 5) Page 10 top: "these results also demonstrate that potassium sensitivity of the channel pore domain changes remarkably with the lipid or detergent environment". This sentence appears out of the blue. I don't see where these results are shown.

Referee #3 (Remarks to the Author):

In this work, the authors studied the activation and inactivation gating states of the chimeric KcsA-Kv1.3 channel using inside-out patch clamp electrophysiology from proteoliposomes and solid state NMR in lipid bilayers by tracking K⁺- and pH-dependent changes in protein conformation and side chain protonation. In addition, using the external pore blockers (1) tetraphenyl porphyrin derivative, which traps a collapsed, non-conductive conformation of the selectivity filter and (2) kaliotoxin, which stabilizes a conductive filter-conformation, they probed the coupling of the lower activation gate (helix bundles) and the upper inactivation gate (selectivity filter) by ssNMR. The results show that pH-induced activation is correlated with protonation of glutamate residues at or near the activation gate. In the absence of K⁺, acidic pH opens the KcsA-Kv1.3 channel and renders the selectivity-filter vulnerable to inactivation. In the presence of 50 mM K⁺, acidic pH stabilizes, a closed activation gate and a conductive filter upper gate, suggesting that the probability of activation-gate opening at acidic pH is K⁺-sensitive. The authors suggest that the two gates are coupled and that effects of the permeant K⁺ ion on the inactivation gate modulate activation gate opening.

This manuscript is interesting and represents an important work since it addresses the issue of the coupling between the activation and inactivation gates of a K⁺ channel. The data reflect an extensive study that is technically well done and clearly described. The experiments are well-controlled and most of the interpretations of the data are warranted. The findings are a valuable contribution to the knowledge of the coupling between ion channel permeation and gating. However, I have some concerns with specific issues that should be clarified.

1- Based on their electrophysiological data shown in Figure 1 and previous ssNMR results (Ader et al, 2008), the authors conclude that occupancy of an internal K⁺-binding site regulates activation-gate closure. However, the authors clearly show that increasing intracellular K⁺ accelerates the inactivation rate of the channel, which implies that the internal K⁺ binding site also regulates the inactivation gate closure. This issue should be clarified since ssNMR and inside-out recording

represent different experimental settings.

2- The authors conclude page 9 that the probability of activation-gate opening at acidic pH is K⁺-sensitive and they link the observed internal K⁺ binding site to the closed conformation of the activation gate with previous electrophysiological data showing a low open probability ($P_o = \sim 0.06$) for the activated KcsA-Kv1.3 channel at steady-state (Ader et al, 2008). In the work of Cordero-Morales et al (NSMB, 2006), the open probability of KcsA determined at 200 mM K⁺ (in symmetrical solutions) is found to match to the voltage-dependence of inactivation (see Figure 1 of the paper). Hence, a voltage-driven relief of steady-state inactivation accounts for an increase in open probability resulting from voltage depolarization. Cordero-Morales et al (NSMB, 2006) propose a mechanism for voltage-dependent gating at the selectivity filter. In the current manuscript, it will be important that the authors address the issue of the K⁺-sensitivity versus voltage-sensitivity of the inactivation gate.

Minor: In supplementary figures 1 and 2, the activation and inactivation time constants are inappropriately expressed as (s⁻¹) and should be rather expressed as (s).

1st Revision - authors' response

29 June 2009

A: Ref 1 asks us to change the title

We have modified the title to:

Coupling of activation and inactivation gate in a K⁺ channel: potassium and ligand sensitivity

B: Ref. 1 suggests additional comments about the K⁺ sensitivity in reference to the ionic strength
We fully agree with the reviewer that both parameters are of critical relevance. This aspect is now further discussed on pages 16/17, where we write:

Note that the overall ionic strength, which has been shown to influence channel open probability (Heginbotham et al, 1998), was kept constant in our experiments. Such a strategy also eliminates other side effects, for example related to surface potential or mechanical stability of the proteoliposome. Therefore, the observed K⁺ effects on channel gating can be traced to potassium itself. Previous reports attributed low channel open probability at acidic pH to an open-inactivated state as seen in ssNMR experiments in low (< 1 mM) K⁺ concentrations (Blunck et al, 2006; Cordero-Morales et al, 2006b; Liu et al, 2001; Perozo et al, 1999). In light of the results presented here, the exact potassium concentrations, most likely also the lipidic environments used (Valiyaveetil et al, 2002), seem of crucial relevance for comparing results of different structure-based functional studies.

C: Reviewers 1 and 2 ask us to extend our discussion of the structural aspects of gate coupling in relationship to our ssNMR analysis.

Firstly, we discuss now in further detail the structural implications of the chemical-shift changes seen in our ssNMR spectra. In particular, we distinguish between backbone and side-chain chemical-shifts. For the latter ones, a direct structural interpretation is not yet possible. Nevertheless, as a spectroscopic parameter that probes the local chemical environment, side-chain chemical shifts are sensitive to local structural rearrangements. Furthermore, we have previously found that both chemical-shifts and through-space distances speak in favor of close resemblance of the pore conformations of KcsA and KcsA-Kv1.3 in the closed state. With these observations in mind, we now write on page 12:

Side chain resonances of the lower selectivity filter and pore helix (Thr72-Thr75) are only

seen to shift together with resonances originating from the gating hinge. This suggests that these side chains are part of an interaction network coupling inactivation and activation gate.

on pages 13/14:

The data indicate that KTX binding and high K⁺ concentrations have analogous effects on KcsA-Kv1.3 gating states, preserving the channel in a closed-conductive conformation in the steady state even at pH 4.0. Table 1 provides a summary of gating and protonation states observed for free and ligand-bound KcsA-Kv1.3 under different [K⁺] and pH conditions.

and finally on page 17/18:

We have previously shown that ssNMR chemical-shift and through-space distance data obtained on KcsA-Kv1.3 at pH 7.5 (Ader et al, 2008) are in good agreement with the closed-conductive (C) state of the KcsA channel as seen in the crystal structure (PDB ID 1K4C). There, side chains of residues in pore-helix and lower selectivity filter on the one hand, and TM2 gating hinge region on the other hand are in close spatial proximity, possibly forming an interconnected network crucial for gate coupling. We observed in our ssNMR data synchronized pH-dependent chemical-shift changes in side chain nuclei of residues from both regions, e.g. Ile100 C δ 1 and Thr75 C δ 2 which are less than 5 Å apart in the KcsA X-ray structure (Zhou et al, 2001). This suggests that the respective amino acids are part of such a coupling network and that conformational rearrangements during opening of the activation gate, where the inner helices swing open, are conveyed to the inactivation gate via the gating hinge region. While our data at this stage cannot provide a high-resolution structural view of the coupling mechanism, we were able to identify residues that are key players for gate coupling based on chemical-shift changes, suggesting that the two gates interact sterically on the side chain level.

(...)

Hence, we found that KTX binding similar to high [K⁺] stabilizes a closed activation gate at acidic pH. K⁺ binding sites 2-4 of the selectivity filter, which are affected by porphyrin but not by KTX binding (Fig. 5), are prime candidates for the internal high-affinity binding site that influences activation gating as seen by electrophysiological and ssNMR experiments.

For further clarification, we also added Table 1 in our revised version. This table summarizes our ssNMR-based analysis of channel conformation and protonation states that we conducted for free and ligand bound KcsA-Kv1.3 under different [K⁺] and pH conditions. The corresponding table caption reads as

Table 1. Summary of channel states observed under different [K⁺] and pH conditions for free and ligand-bound KcsA-Kv1.3 reconstituted in asolectin liposomes. n.d.: not determined, a: chemical shift changes indicate adaptation of the selectivity filter conformation due to toxin binding as reported previously (Lange et al, 2006).

D: Refs. 1-3 ask us to further comment on the relationship between ssNMR experiments and electrophysiological data, in particular regarding the voltage sensitivity of gating

We strongly agree with the reviewers that such a clarification is necessary. Correspondingly we have added on page 17 an entire paragraph:

It is also important to note that electrochemical gradients as present in electrophysiology have so far not been reproduced in structural studies. For this reason, we have not attempted to study the voltage sensitivity of KcsA-Kv1.3 gating (Cordero-Morales et al, 2006a) by ssNMR on a structural level. Conversely, an electrochemical gradient generated either by a gradient of K⁺ or voltage across the lipid bilayer is obligatory for functional studies by

electrophysiology. Thus, our strategy was to combine functional experiments on KcsA-Kv1.3 in asymmetric [K⁺] and transmembrane voltage conditions with ssNMR data on K⁺-, pH- and ligand-dependence of KcsA-Kv1.3 steady-state conformation. This allowed us to delineate the influence of K⁺ on activation and inactivation gates both under equilibrium and nonequilibrium conditions, yielding a coherent picture of KcsA-Kv1.3 channel gating. While potassium channel gating proceeds via an open-inactivated state, as established in electrophysiological experiments (Fig. 1 a; (Chakrapani et al, 2007a; Kurata & Fedida, 2006)), our data show that, in the absence of transmembrane voltage, the steady-state conformation of KcsA-Kv1.3 in asolectin liposomes is closed-conductive even at pH 4.0 if millimolar (10 mM) K⁺ concentrations are present.

Furthermore, we emphasize during the discussion of the K⁺-sensitivity of Glu71 protonation on pages 15/16 that this residue also entails voltage sensitivity to the inactivation gate as found by Perozo and co-workers.

Substitution of Glu71 by alanine prevents entry into the inactivated state and essentially abolishes the voltage sensitivity of KcsA gating (Cordero-Morales et al, 2006a; Cordero-Morales et al, 2007). Using ssNMR spectroscopy, we could directly show that protonation of Glu71 correlates with inactivation gate closure, consistent with a crucial role of this residue in pH-, K⁺-, and voltage-sensitivity of KcsA inactivation.

E: Ref. 2 asks us to compare our data to published work on KcsA, for example by Perozo et al.

While previous electrophysiological investigations of KcsA mostly employed high millimolar [K⁺] in the presence of electrochemical gradients, the buffer conditions used for structural investigations of KcsA channel opening varied or were not reported. Perozo et al. described buffer conditions used for channel opening such as 50 mM citrate phosphate buffer (Science, 1999 and Nature Structural Biology, 2001) or PBS buffer (J. Gen. Physiol. 2006). From this information, no conclusions about [K⁺] and the related K⁺-dependency of steady-state channel opening can be drawn. Our present study investigated steady-state channel states systematically as a function of [K⁺] and emphasizes the need to consider [K⁺] if structural studies are compared. Therefore, we included the following statement on page 17:

Previous reports attributed low channel open probability at acidic pH to an open-inactivated state as seen in ssNMR experiments in low (< 1 mM) K⁺ concentrations (Blunck et al, 2006; Cordero-Morales et al, 2006b; Liu et al, 2001; Perozo et al, 1999). In light of the results presented here, the exact potassium concentrations, most likely also the lipidic environments used (Valiyaveetil et al, 2002), seem of crucial relevance for comparing results of different structure-based functional studies.

F: Ref. 2 asks us to introduce KcsA-Kv1.3

For this reason, we included an additional statement on page 5:

KcsA-Kv1.3 contains a high affinity binding site for the scorpion toxin kaliotoxin (KTX) which was generated by replacing eleven amino acid residues in the extracellular loop of the KcsA pore domain by those of Kv1.3 (Legros et al, 2000; Legros et al, 2002).

G: Ref. 2 asks us to present electrophysiological and ssNMR data in a more joint manner

We feel that presenting results obtained by different biophysical techniques in a concerted manner can be challenging. After careful consideration we believe that reporting results in a

successive manner and linking together their common implications in the discussion session is most appropriate in our case. To emphasize the experimental differences but also the joint consequences of our work we added or changed several paragraphs in the result and discussion sections of our manuscript.

On page 8 we reason why the distinct K⁺ events apparent from electrophysiology necessitate a structural characterization by ssNMR.

The data shows that KcsA-Kv1.3 channel inactivation is over 100 fold more sensitive to [K⁺]_{in} than to [K⁺]_{out}. It suggested that occupation of an internal and external K⁺ binding site modulates KcsA-Kv1.3 channel gating. This implies that equilibria between activated and inactivated channel states, which are influenced by changes in [K⁺]_{in} and [K⁺]_{out}, are correlated with K⁺-sensitive conformational rearrangements in the KcsA-Kv1.3 channel. To investigate this K⁺ sensitivity of KcsA-Kv1.3 conformational states on a structural level, we employed ssNMR spectroscopy to obtain information on KcsA-Kv1.3 in the presence of different K⁺ concentrations.

On page 9 joint implications of functional and structural studies are stated.

These ssNMR results had two important implications. First, acidic pH, which opens the KcsA-Kv1.3 channel, renders the selectivity-filter vulnerable to inactivation. Second, the probability of activation-gate opening at acidic pH is K⁺-sensitive. The data demonstrate that the prevailing KcsA-Kv1.3 conformation observed at pH 4.0 shifts from the open-collapsed (I) state to the closed-conductive (C) state of the channel (Fig. 2D) in the presence of high millimolar K⁺-concentrations. This complements our electrophysiological studies on the K⁺ dependency of KcsA-Kv1.3 channel inactivation and shows that both activation and inactivation gate of KcsA-Kv1.3 respond to changes in potassium concentration at a structural level.

On page 15, we added a paragraph, indicated under D, discussing important experimental differences between the techniques employed and outline our strategy allowing us to interpret functional and structural data in a joined manner.

H: Ref. 2 mentions His25 as an additional residue implicated in pH sensing.

Indeed, NMR and mutational data suggest a role of His25 in pH sensing. Due to spectral overlap, it was not possible to investigate the role of this residue by ssNMR. We have included on page 10 a corresponding statement:

The histidine residue in the outer transmembrane helix (His25), which was shown to be involved in activation gating (Takeuchi et al, 2007; Thompson et al, 2008), could not be resolved in ssNMR spectra of KcsA-Kv1.3 (Schneider et al, 2008).

I: Ref. 3 indicates a mistake in annotating rate units

We apologize for this mistake, which has been corrected. Finally, we agree with reviewer 2 that lipid/detergent aspects would require a more detailed discussion which is outside the scope of our work. We hence have decided to remove the sentence in question. We modified the text on pages 4-7, 10-13, 15, 18, and 19 so as to enhance the overall clarity of our work.

Many thanks for sending your revised manuscript. It has now been seen again by referee 2, who is satisfied with the changes (his/her comments are copied below). I am therefore pleased to tell you that your manuscript is now ready to be accepted, and you will receive the formal acceptance message shortly.

Referee 2 comments:

The authors did a good job revising the manuscript and I believe their responses and revision are appropriate.