nature portfolio

Peer Review File

Structural insights into light-gating of potassium-selective channelrhodopsin

Corresponding Author: Professor Oliver Ernst

This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The authors employed single-particle cryo-electron microscopy to determine structures of the slow-cycling HcKCR1 mutant C110A in the dark and upon illumination. The comparison of the structures revealed no major conformational changes, but a reorientation of the Schiff base NH bond from the extracellular to the cytoplasmic side and a series of side-chain reorientations, which led to the formation of a continuous intramolecular tunnel in the laser-flash-activated structure. MD simulations suggest that the intramolecular tunnel could be filled with water molecules and permeates potassium ions. Moreover, the authors further investigated the functional roles of putative pathway-forming residues by structure-guided mutagenesis and patch-clamp analysis. The manuscripts provides important findings, which may contribute to a comprehensive mechanistic description of light-gated potassium channels, and which may facilitate engineering of improved KCR variants for optogenetic applications.

Major comments:

1. The prerequisite for a precise description of light-induced changes in side-chain orientations are structures with sufficiently high resolutions. From Table 1 and the local resolution maps shown in Supplementary Fig. 2 and Supplementary Fig. 5, it however becomes apparent that the 7TM domains of the dark-adapted structure and the structure upon continuous illumination only reach a resolution of ~3 Å (compared to a resolution of up to ~2.6 Å for the 7TM domain of the structure upon laser-flash-illumination). The authors may discuss this limitation in their description of side-chain orientation changes upon illumination in the main text and/or provide further evidence for the validity of their results.

2. The authors have shown in their own previous work on HcKCR1 that the reversal potential for the peak photocurrent differs from the reversal potential for the stationary photocurrent (https://doi.org/10.1038/s41593-022-01094-6), which indicates the likely formation of at least one, less potassium selective, additional open state upon absorption of a second photon. That important information is however missing in the authors´ structure-guided mutagenesis and patch-clamp analysis approach to assess the role of putative pathway-forming residues (Fig.5). It cannot be ruled out that the contribution of certain residues to channel selectivity differs in distinct open states. For better assessment of the role of certain residues in channel selectivity, the authors should show both, the reversal potentials for the peak photocurrents and the reversal potentials for the stationary photocurrents and discuss the results accordingly.

3. In lines 353-356 of the manuscript the authors claim: “We did not observe large photoinduced helical movements in HcKCR1_C110A and propose that subtle local changes are sufficient for light-gated K+ conductance in KCRs, as for active H+ translocation by haloarchaeal pumps48.” Yet the extent of photoinduced conformational changes in haloarchaeal pumps is still subject of controversial discussions (e.g. https://doi.org/10.1016/j.sbi.2009.07.009). The authors should provide a more balanced view on literature.

4. Fig. 5 e displays “Photocurrent amplitudes normalized to the WT recorded in the same experiment.” to show the effect of mutations in putative pathway-forming residues on photocurrent size. The normalization procedure does not correct for the transfection trial dependent variability in the expression of the HcKCR1 variants. For proper assessment of the effect of the mutations on photocurrent size, the authors may compare HcKCR1 variant photocurrent densities from at least 3 different transfections instead.

Minor comments:

a) When hypothesizing on the role of a putative H-bonded water chain in facilitating a H+ conductance in HcKCR1 the authors refer to an original work that inter alia describes a H+ conductance at very low pH values in HcKCR2 (lines 248-250, DOI: 10.1126/science.adj9696). The manuscript would benefit from the determination of the HcKCR1 proton permeability (e.g. PH+/PK+ ratio) by additional patch-clamp experiments.

b) Is Fig 2 c showing normalized values? The authors may provide this information in the figure caption or add a y-axis to the figure. Aside from that, the light intensity or pulse length seems to be not high or long enough to measure stationary HcKCR1 wt photocurrents, which compromises the comparison to the mutants.

Reviewer #2

(Remarks to the Author)
Kalium channelrhodopsin 1 from Hyphochytrium catenoides (HcKCR1) is a light-gated potassium-selective channelrhodopsin that has emerged as a valuable tool for optogenetics. Morizumi et al. have solved the structures of a slow-cycling HcKCR1 mutant in a dark-adapted resting state and in illuminated states using single particle cryo-EM. These structures combined with molecular dynamics simulations and electrophyiological experiments using mutants of channel-forming residues provide molecular insights into the mechanism of K+ conductance, including gating and selectivity.

While the overall structure of HcKCR1 and the structural basis of ion selectivity have been described before, this work provides valuable insight into how photoisomerisation of the retinal cofactor triggers the opening of the K+-conductive channel. Capturing light-activated rhodopsins in an active state is crucial to understanding their mechanisms, but very challenging due to their short photocycles. The authors have managed to solve the structure of HcKCR1 in an open state by taking great care to thoroughly characterise the illuminated sample prior to data collection and through careful analysis of the structures. Molecular dynamics simulations and electrophysiology experiments clearly confirm the functional consequences of the conformational changes between the dark-adapted and light-activated states, i.e., opening of a continuous water-accessible channel and K+ conductance.

The paper is very clear and well-written, structural and functional experiments have been meticulously performed and analysed to a high standard. Description of experimental procedures are detailed and allow others to reproduce them. The results are robust and strongly support all of the authors' conclusions. This study provides significant insights into the mechanism of channelrhodopsins, which will be valuable for the broader microbial rhodopsin community but in particular for optogenetic applications involving HcKCR1. Based on the high quality of the research and the significance of the findings, I recommend publication of this paper in Nature Communications. I have some questions which I would like the authors to address and a few minor points which will improve the clarity of the paper.

1) It should be made clearer how the final models were obtained. While the methods specify that C3 symmetry was applied for the final refinement of the dark-adapted structure, imposed symmetries should also be indicated for the other two structures in the methods and results sections and in Supplementary Figures 2 and 5 illustrating the cryo-EM workflows. It should also be clearly mentioned if symmetry relaxation was used in the final refinements (see also the next point).

2) I appreciate the analysis of the symmetry relaxed structure of the laser-flash-illuminated state, demonstrating the presence of three different retinal forms, this is very valuable. However, why did the authors not deposit a model of this pseudo-symmetrical structure with the different retinal configurations? Furthermore, it appears that the deposited structures, based on the PDB validation report and the provided files, seem to be monomeric. While this is acceptable for crystallographic structures, a full trimeric model should be deposited for cryo-EM structures. Especially, if there are potential differences between the protomers.

3) In addition to reference-based motion correction for the final refinement in Cryosparc, have the authors tried local and global CTF refinement (per-particle defocus, beam-tilt, trefoil, spherical aberration, tetrafoil) to improve the final resolution?

4) Was a special cholesterol-producing Pichi pastoris strain used for protein expression? To my knowledge they primarily produce ergosterol, which makes the choice to model cholesterol rather than ergosterol questionable. If a special strain was used, this should be clearly stated in the methods.

5) Is there a structural basis that could explain the observed increase in K+ selectivity of the M20 mutants?

6) Could the presence of 11-cis retinal be related to a potential desensitised state? Has this been observed elsewhere and is anything known about the rate of decay compared to 13-cis retinal?

7) I am curious why the authors chose to solve the structures in 150 mM NaCl. Clearly this would be outside the scope of the paper, but would the presence of KCl be expected to make a difference and have the authors tried this?

Minor points:

- Figure 2: WT dark-adapted structure not from this study, indicate PDB ID.
- Table 1: Indicate map sharpening method, include model-to-map validation statistics such as CCmask/ligand, Q-score/EMRinger score, model-to-map FSC. I would also recommend including Ramachandran Z-scores for better assessment of model quality.
- Local resolutions in Supplementary Figs. 2 and 5 do not match with reported resolution range in Table 1 and the mentioned 2.6Å in the first results part (line 119).
- Supplementary Figure 2: Add corrected curve in FSC plots
- Consider illustrating H-bonds in Figure 4 for easier interpretability, in particular those that change between dark and illuminated states (e.g., D116-R244).
- There seems to be a mixture of 3 and 1 letter codes used to describe amino acid residues in the manuscript, which can be confusing at times.
- Potentially flesh out what specific properties could be engineered for optogenetic applications and how the new structural insights would facilitate this.

Reviewer #3

(Remarks to the Author)
This manuscript by Morizumi and colleagues investigates the light-gating of a potassium-selective channelrhodopsin. The authors used single-particle cryo-electron microscopy (cryo-EM) to solve the Kalium channelrhodopsins (KCR1) structures, in dark-adapted and laser-flash-activated states, from Hyphochytrium catenoides. Classical molecular dynamics (MD) and computational electrophysiology simulations were applied to study the hydrogen bond networks and the potassium ion translocation mechanism. The conclusions drawn from simulations are interesting, but poorly supported.

The authors performed classical MD simulations of the dark-adapted and laser-illuminated trimers, obtained from the cryo-EM experiments, to then analyze the H-bond networks. The relative method section “Graph-based analyses of the protein-water H-bond networks” lacks critical details for the simulations reproducibility. It is unclear what equilibration protocol was used, the simulations time length, and how many replicates were performed.

Regarding the results section “Formation of a transient water chain”, it is difficult for the reader to interpret the results having two long supplementary tables. It might be useful to have a histogram graph (with relative error bars), or a time series of distances, to compare the most important hydrogen bonds discussed in the main manuscript. Moreover, the node labels in Supplementary Figure 6b, c and d are too small and hard to read.
The sentence “the RSB could transiently connect to the H-bonded Ser70/Asp116 cluster in the intracellular segment via a chain of three H-bonded water molecules” (at line 246-247) is not clear to me. As far as I understand, Lys233 is establishing a water-mediated h-bond with Ser70 only in one protomer of the laser-illuminated trimer with a 54% occupancy. I do not think this is statistically significant.

The results section “Rearrangement of the aromatic cluster” describes a perturbation of a cluster of aromatic residues, but it is not clear whether this claim is also supported by simulations.

The authors used the computational electrophysiology technique to investigate the potassium ion flow through the channel. However, reading the relative method section “Computational electrophysiology”, it is not clear if they simulated two copies of the whole trimer or two copies of a protomer. Then, they should clarify which barostat and pressure coupling were used for the equilibration and production phases. Lastly, a description of the procedure for the transmembrane voltage calculation is also needed.

The results reported in the “The K+ flow through the channel” section are missing statistical quantifications. These data are only qualitatively described and cannot support any claim.
For instance, the authors could quantify ion permeation and conductance (similar to what has been done in Figure 2 and Supplementary Figure 1 in Zhuang Y et al., 2022 https://doi.org/10.1073/pnas.2208081119).
They should also quantify the contacts established between the permeating ions and the protein residues, showing that the reported interactions are statistically meaningful. This could support the claim that the described residues are involved in the channel gating.

As a general remark, the authors should dedicate a couple of supplementary figures to show the stability of the systems in both computational settings.

Finally, I have to point out that no simulation input or output data have been shared, significantly reducing the reported study's reproducibility.
To improve both the reliability and reproducibility of the present study, I suggest following the "Reliability and reproducibility checklist for molecular dynamics simulations" (https://www.nature.com/articles/s42003-023-04653-0) included in the Nature Communication Computational tools reporting guidelines https://www.nature.com/ncomms/submit/resources.

Reviewer #4

(Remarks to the Author)
This is a nice, clearly written paper reporting the structure and structural changes of a molecule that can be used in

ontogenetic studies. For publication in Nature Communications a bit more effort should be made to highlight why knowing the structure of this protein can be of wider interest. There are connections in the paper to the wavelength of excitation and the rate of the channel opening and closing. Can having the detailed open and closed structure help make changes to the protein that would make it more useful for different uses.
The resolution is at the border of high resolution (≈2.7-3.1Å). The main conclusion is that side chain motions are most important. It would be useful to discuss the positional uncertainty at this resolution.
Line 153. It would seem to be worth the effort to make a stab at identifying and differentiating K+ and water. In cryoEM my understanding ions scatter differently depending on their charge. Also a census of hydrogen bonds or nearby charges might help identify ion binding. In addition, the MD calculations could find which sites, if any, prefer K+.
It seems clear that this protein differs from standard channel rhodopsins in that it does not undergo large conformational changes to open a channel. Is there a better correspondence with halorhodopsin which is a Cl pump or is this protein functioning in a mode different from ChR or halorhodopsin?
Dehydration of an ion can be difficult. Is the K+ dehydrated through its full path?
Figure 5 shows serious constrictions in the channel. Can a figure from the MD be added that shows the channel width in individual snapshots when a K is transiting through each constriction. Figure 5 would also be more useful if key amino acids were marked along the path.

Line 57: Halorhodopsin is an ion channel which has similar generic structure so this is protein is not that unique
Unclear what is the job of the protein in its native state. E.g. line 66 should give the conductance of the natively used ion.
Line 85: It is important that the ion goes through the monomer rather than the pore being formed in the center of sever monomers. But with the monomer channel you have here it's not so clear the importance of the protein assembling as dimers, trimers, or tetramers. More important that the multimer level is the same in the cell and in the nonodisk.
The C110A structure is used, which seems like a fine choice. It would be useful to know what region of the protein this residue is found in when the mutation is first mentioned. It's good to have the the RMSD differences in the dark-adapted structure between the wild-type and mutant, changes in the active site distances as well as in the protein activity.
Line 152. How many waters does the dark wild-type structure contain?
Line 178. Remind the reader that Lys 233 is the SB. Maybe just say the 'attached' Lys 233 side chain.
Line 244. Any non-canonical choices for residue protonation states in the MD should be noted. For example, if the SB is deprotonated following isomerization, which residue is now protonated. Information on line 584 is insufficient.

Fig 3. Not easy to see the differences between the structures. Not sure what to suggest: Add arrows or show the two structures overlaid?

Reviewer #5

(Remarks to the Author)
Morizumi et al reported cryo-EM structures of the dark-adapted and light-illuminated states of a potasium-selective channelrhodopsin (Hyphochytrium catenoides kalium channelrhodopsin 1: HcKCR1) and discussed the mechanism of the opening of the ion conduction pathway across the membrane. When I first read their manuscript, I felt that the main text was perfectly well written. This is probably because weak points in their structural analyses are not explicitly described in the main text and, instead, separately described in Supplementary Discussion. Judged from the last sentence in this Discussion, which indicates that ~40 % of the molecules could be in the closed or desensitized state in the laser-flash-illuminated sample,I guess that the retinal configuration in the open state might be estimated on the basis of the density map generated by cryo-EM symmetry relaxation analysis of laser-flash-illuminated HcKCR1_C110A trimer(Supplementary Fig. 3d). If this is the case, the following questions will arise.

1) Is it adequate to use symmetry relaxation method in cryo-SPRAC v4.4 ? This method would provide a useful structural information if all the trimers have the same shape. e.g., one protomer adopts a closed conformation while the other protomers adopt an open conformation. It seems difficult to exclude the ab-initio models of other possible shapes (e.g., all three protomers adopt an open conformation) before applying the symmetry relaxation method. If the authors succeeded in overcoming this difficulty, they are recommended to describe the detail of their structural analysis.

2) It appears that the density maps in Figure 1 and Supplementary Fig. 2 are obtained without applying the symmetry relaxation method. When a considerable fraction (~40%) of the molecules in the laser flash-illuminated state are a closed state(s), the density map of this illuminated state represents a weighted average of the closed and open states. This should not be regarded as a problem, provided that they observed a clear difference in the density map of an interesting region (e.g., around the retinal Schiff base) for the dark-adapted state and the laser-flash-illuminated state. They are recommended to add a figure showing enlarged density maps of the dark-adapted state and the laser-flash-illuminated state, by which light-induced structural changes can be convincingly discussed.

3) Supplementary Fig 1d indicates that the photocurrent action spectrum is largely red shifted as compared to the absorption spectrum of the dark-adapted spectrum. This result seems to suggest that a large content (possibly >50%) of the desensitized state co-exist in the dark-adapted state. Is this compatible with the result of HPLC analysis (Supplementary Fig.1g - 1h) ?

4) The term "the dark recovery" in Supplementary Fig. 1e is ambiguous.

Version 2:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The authors have addressed all my concerns and comments.

Reviewer #2

(Remarks to the Author)
The authors have addressed all of my questions, and to the best of my understanding the majority of issues raised by the other reviewers. They have performed requested additional experiments to reinforce their conclusions and have delivered a thoroughly revised manuscript, including more detailed descriptions of the experimental procedures, which will facilitate reproducibility. This has significantly increased the overall quality of the manuscript to a standard suitable for publication in Nature Communications.

Reviewer #3

(Remarks to the Author)
The authors answered all my major concerns. I just want to point out some minor points:

1) In the paragraph "Graph-based analyses of the protein-water H-bond networks" of the Methods section, please be consistent with units. Harmonic constraints are reported with units written in four different ways.

2) In Supplementary Fig. 7-9, the authors should clarify what is the meaning of the labels on the right side of the plots (i.e., 3,2,1,2-1,2-3,1-3). If the meaning is the same in all three figures, it will be enough to specify it in Supplementary Fig. 7.

3) In Supplementary Fig. 7 caption, it seems that "For clarity, the time series we show in Supplementary Figures 6-8" should be "For clarity, the time series we show in Supplementary Figures 7-9".

Reviewer #4

(Remarks to the Author)
The authors answered by questions and updated the manuscript to address my concerns.

Reviewer #5

(Remarks to the Author)
I am glad to find that the authors have constructively used my previous comments to improve their manuscript.

We thank all five Reviewers for their thorough reading of our manuscript and constructive criticisms, which helped us improve our submission. Our point-by-point answers are below in black, and the Reviewer's remarks are in blue. We highlighted the changes in the manuscript in yellow.

Reviewer #1 (Remarks to the Author):

The authors employed single-particle cryo-electron microscopy to determine structures of the slow-cycling HcKCR1 mutant C110A in the dark and upon illumination. The comparison of the structures revealed no major conformational changes, but a reorientation of the Schiff base NH bond from the extracellular to the cytoplasmic side and a series of side-chain reorientations, which led to the formation of a continuous intramolecular tunnel in the laser-flash-activated structure. MD simulations suggest that the intramolecular tunnel could be filled with water molecules and permeates potassium ions. Moreover, the authors further investigated the functional roles of putative pathway-forming residues by structure-guided mutagenesis and patch-clamp analysis. The manuscripts provides important findings, which may contribute to a comprehensive mechanistic description of light-gated potassium channels, and which may facilitate engineering of improved KCR variants for optogenetic applications.

Major comments:

1. The prerequisite for a precise description of light-induced changes in side-chain orientations are structures with sufficiently high resolutions. From Table 1 and the local resolution maps shown in Supplementary Fig. 2 and Supplementary Fig. 5, it however becomes apparent that the 7TM domains of the dark-adapted structure and the structure upon continuous illumination only reach a resolution of ~3 Å (compared to a resolution of up to ~2.6 Å for the 7TM domain of the structure upon laser-flash-illumination). The authors may discuss this limitation in their description of side-chain orientation changes upon illumination in the main text and/or provide further evidence for the validity of their results.

We thank the reviewer for pointing this out. We agree that the local resolution in the 7TM domain of the dark-adapted KCR and the continuous illuminated KCR is lower than that of the laser-flash-activated KCR. We reevaluated the local resolution of the maps and reploted the maps with adjusted resolution scale. The resolution of the 7TM domain of the maps are 2.8 Å (dark-adapted), 2.6 Å (laser-flash-illuminated), and 3.2 Å (continuous illumination). We updated the values in Table 1 and made the changes to Supplementary Figures 2 and 5.

The second sentence of the second paragraph in the results section now reads:

> "Single particle analysis allowed us to obtain density maps at 3.08 Å resolution for dark-adapted and 3.03 Å for laser-flash-illuminated *Hc*KCR1_C110A, respectively, and to determine high-resolution structures; the resolution of the maps within the 7TM domain reached 2.6 Å (laser-flash-illuminated *Hc*KCR1_C110A) and 2.8 Å (dark-adapted *Hc*KCR1_C110A) (Fig. 1, Table 1, Supplementary Fig. 2)."

While the resolution of dark-adapted KCR in the 7TM domain is 0.2 Å lower compared with the laser-flash-illuminated KCR, the difference does not result in major limitations when comparing the side chains in the two structures. The resolution of the continuous illuminated sample is lower but allowed us to determine the retinal configuration and the orientation of the Asp116 side chain, which was similar to the dark-adapted state and not like the laser-flash-illuminated state.

We modified the beginning and end of the paragraph on KCR upon continuous illumination. The paragraph begins now with:

> "In addition to laser-flash-illuminated *Hc*KCR1_C110A, we performed cryo-EM single particle analysis of *Hc*KCR1_C110A illuminated with continuous green light before cryo plunging (**Supplementary Fig. 6, Table 1**). The density map had a global resolution of 3.44 Å and up to 3.2 Å within the 7TM domain allowing us to model the isomerized retinal in the predominant 13-*cis*, 15-*syn* configuration."

And ends with:

> "The model obtained after continuous illumination gives structural insight into a desensitized KCR with no or weak conductance and might comprise a mixture of states as concluded earlier[23]. The presence of multiple states could also be an explanation for the lower resolution of the map obtained upon continuous illumination of *Hc*KCR1_C110A. Similarly, a complexity of protein conformations and retinal binding was observed in the cryo-EM structures of *Hc*KCR1 and *Hc*KCR1_C110T, respectively, prepared under continuous dim white light[16]."

The desensitized state is an interesting but complex state. This state is difficult to analyze due to its mixture of photointermediates and is the topic of a separate study.

2. The authors have shown in their own previous work on HcKCR1 that the reversal potential for the peak photocurrent differs from the reversal potential for the stationary photocurrent (https://doi.org/10.1038/s41593-022-01094-6), which indicates the likely formation of at least one, less potassium selective, additional open state upon absorption of a second photon. That important information is however missing in the authors´ structure-guided mutagenesis and patch-clamp analysis approach to assess the role of putative pathway-forming residues (Fig.5). It cannot be ruled out that the contribution of certain residues to channel selectivity differs in distinct open states. For better assessment of the role of certain residues in channel selectivity, the authors should show both, the reversal potentials for the peak photocurrents and the reversal potentials for the stationary photocurrents and discuss the results accordingly.

In the revision, we added the reversal potentials for the peak currents to Figure 5d and their amplitudes to Figure 5e. We updated the figure caption as follows:

> "In d and e, the symbols are individual-cell data recorded at the time of peak (empty circles) and the end of illumination (filled circles). <…> *, $p < 0.05$; **, $p < 0.001$; ***, $p <$ E-5 compared to the WT by the one-way ANOVA with the Tukey means comparison."

We also updated the corresponding paragraph of the text in the Results section as follows:

> **"Supplementary Fig. 25** shows the IV curves of the tested mutants, and **Fig. 5d,e** shows their reversal potentials (Vr) and photocurrent amplitudes at the time of the peak and the end of illumination. <…> Surprisingly, all tested substitutions of Met20 showed a more negative Vr than the wild type at the end of illumination and M20A, M20L, M20V, and M20G at the peak time, indicating an increased K+ selectivity. The Vr shift to more depolarized values is likely caused by the accumulation of a less K^+-selective photocycle intermediate upon prolonged illumination[7]. All Met20 mutants showed a reduced Vr shift compared to the WT by the two-tailed Mann-Whitney

test (**Source Data file**), suggesting that Met20 contributes to the formation of this intermediate in the wild-type. M20A, M20L, and M20V showed a significantly more negative Vr of the end current (one-way ANOVA followed by the Tukey test for means comparison; **Source Data file**) than the previously reported mutant H225F(KALI-1)[12], included in our study for comparison."

3. In lines 353-356 of the manuscript the authors claim: "We did not observe large photoinduced helical movements in HcKCR1_C110A and propose that subtle local changes are sufficient for light-gated K+ conductance in KCRs, as for active H+ translocation by haloarchaeal pumps48." Yet the extent of photoinduced conformational changes in haloarchaeal pumps is still subject of controversial discussions (e.g. https://doi.org/10.1016/j.sbi.2009.07.009). The authors should provide a more balanced view on literature.

To provide a more balanced view of the literature, we modified the sentence and added the reference suggested by the Reviewer:

"We did not observe large photoinduced helical movements in *Hc*KCR1_C110A and propose that subtle local changes are sufficient for light-gated K^+ conductance in KCRs, as for active H^+ translocation by a bacteriorhodopsin triple mutant[48], although the extent of global light-induced conformational changes in haloarchaeal proton pumps is still a subject of discussion[49]."

4. Fig. 5 e displays "Photocurrent amplitudes normalized to the WT recorded in the same experiment." to show the effect of mutations in putative pathway-forming residues on photocurrent size. The normalization procedure does not correct for the transfection trial dependent variability in the expression of the HcKCR1 variants. For proper assessment of the effect of the mutations on photocurrent size, the authors may compare HcKCR1 variant photocurrent densities from at least 3 different transfections instead.

We conducted additional patch clamp experiments to test each mutant upon three or more independent transfections. In the revision, we updated Figures 5d,e accordingly and updated the Statistics and Reproducibility section as follows:

"M20V, M20Y, and K84R were tested upon four independent transfections, and all other mutants upon three independent transfections on different experimental days; the data obtained were pooled together."

Minor comments:

a) When hypothesizing on the role of a putative H-bonded water chain in facilitating a H+ conductance in HcKCR1 the authors refer to an original work that inter alia describes a H+ conductance at very low pH values in HcKCR2 (lines 248-250, DOI: 10.1126/science.adj9696). The manuscript would benefit from the determination of the HcKCR1 proton permeability (e.g. PH+/PK+ ratio) by additional patch-clamp experiments.

We determined the relative pH/pK ratio ($<3 \times 10^4$) of *Hc*KCR1 in our earlier publication (Govorunova et al. 2022 Nature Neuroscience, DOI: 10.1038/s41593-022-01094-6).

We added this reference (Govorunova et al. 2022 Nat Neurosci) to the following sentence:

"In analogy to the role of the H-bonded water chain in BR proton transfer, we hypothesize that the H-bonded water chain that assembles in HcKCR1 could facilitate the H+ conductance of KCRs[7,14]."

b) Is Fig 2 c showing normalized values? The authors may provide this information in the figure caption or add a y-axis to the figure. Aside from that, the light intensity or pulse length seems to be not high or long enough to measure stationary HcKCR1 wt photocurrents, which compromises the comparison to the mutants.

The photocurrent traces in Figure 2c are indeed normalized at the peak value to facilitate comparison of their kinetics. In the revision, we added the phrase: "normalized at the peak value" to the Figure caption to clarify it. The light pulse duration in SyncroPatch experiments at 2.6 mW mm-2 (irradiance at the maximal LED current 900 mA) is limited to 200 ms by the LED duty cycle. We have already analyzed WT photocurrents evoked by longer light pulses in our earlier study conducted by manual patch clamping (Govorunova et al. 2022 Nature Neuroscience, DOI: 10.1038/s41593-022-01094-6). This study's automated patch clamp experiments aimed to probe the channels without premature activation by selecting fluorescent cells. This is a key measurement condition in the slow-cycling C110A and C110T mutants.

Reviewer #2 (Remarks to the Author):

Kalium channelrhodopsin 1 from Hyphochytrium catenoides (HcKCR1) is a light-gated potassium-selective channelrhodopsin that has emerged as a valuable tool for optogenetics. Morizumi et al. have solved the structures of a slow-cycling HcKCR1 mutant in a dark-adapted resting state and in illuminated states using single particle cryo-EM. These structures combined with molecular dynamics simulations and electrophyiological experiments using mutants of channel-forming residues provide molecular insights into the mechanism of K+ conductance, including gating and selectivity.

While the overall structure of HcKCR1 and the structural basis of ion selectivity have been described before, this work provides valuable insight into how photoisomerisation of the retinal cofactor triggers the opening of the K+-conductive channel. Capturing light-activated rhodopsins in an active state is crucial to understanding their mechanisms, but very challenging due to their short photocycles. The authors have managed to solve the structure of HcKCR1 in an open state by taking great care to thoroughly characterise the illuminated sample prior to data collection and through careful analysis of the structures. Molecular dynamics simulations and electrophysiology experiments clearly confirm the functional consequences of the conformational changes between the dark-adapted and light-activated states, i.e., opening of a continuous water-accessible channel and K+ conductance.

The paper is very clear and well-written, structural and functional experiments have been meticulously performed and analysed to a high standard. Description of experimental procedures are detailed and allow others to reproduce them. The results are robust and strongly support all of the authors' conclusions. This study provides significant insights into the mechanism of channelrhodopsins, which will be valuable for the broader microbial rhodopsin community but in particular for optogenetic applications involving HcKCR1. Based on the high quality of the research and the significance of the findings, I recommend publication of this paper in Nature Communications. I have some questions which I would like the authors to address and a few minor points which will improve the clarity of the paper.

1) It should be made clearer how the final models were obtained. While the methods specify that C3 symmetry was applied for the final refinement of the dark-adapted structure, imposed symmetries should also be indicated for the other two structures in the methods and results sections and in Supplementary Figures 2 and 5 illustrating the cryo-EM workflows. It should also be clearly mentioned if symmetry relaxation was used in the final refinements (see also the next point).

All final maps were refined with C3 symmetry imposed. The methods section as well as the figure legends of Supplementary Figures 2 and 5 has been updated. The information on imposed C3 symmetry was added where missing.

2) I appreciate the analysis of the symmetry relaxed structure of the laser-flash-illuminated state, demonstrating the presence of three different retinal forms, this is very valuable. However, why did the authors not deposit a model of this pseudo-symmetrical structure with the different retinal configurations? Furthermore, it appears that the deposited structures, based on the PDB validation report and the provided files, seem to be monomeric. While this is acceptable for crystallographic structures, a full trimeric model should be deposited for cryo-EM structures. Especially, if there are potential differences between the protomers.

The maps that we refined with symmetry relaxation had noticeably worse resolution and lacked the detail required to identify conformational changes. We therefore show the symmetry relaxation analysis (Supplementary Figure 3) only for the highest resolution map (laser-flash-illuminated *Hc*KCR1). The symmetry relaxed models have not been deposited. The primary use of symmetry relaxation was to observe differences in the individual protomers, specifically in the retinal chromophore configuration.

Deposited structures: A monomer model was deposited and in the assembly section it was defined as a trimer. This approach was done to be consistent with our previous deposition of the dark-adapted wild-type *Hc*KCR1 model. All individual protomers are identical, and the monomer model was refined with symmetry restraints. Deposition of a trimer model would yield identical results.

We realize that the model sent for review was a single monomer, and apologize for any confusion in that matter.

3) In addition to reference-based motion correction for the final refinement in Cryosparc, have the authors tried local and global CTF refinement (per-particle defocus, beam-tilt, trefoil, spherical aberration, tetrafoil) to improve the final resolution?

Yes, higher order ctf refinement has been performed. Local ctf refinement was also done, but did not improve the resolution.

4) Was a special cholesterol-producing Pichi pastoris strain used for protein expression? To my knowledge they primarily produce ergosterol, which makes the choice to model cholesterol rather than ergosterol questionable. If a special strain was used, this should be clearly stated in the methods.

We did not use a special cholesterol-producing Pichia strain but added cholesteryl hemisuccinate during protein purification to promote membrane protein stability. At the end of the second paragraph of the results we therefore noted in the original manuscript:

"Sterol lipids originating from the host membranes were modeled as cholesterol. Cholesteryl hemisuccinate (CHS) was added during purification, but the density of hemisuccinate was weak or missing for clear identification."

5) Is there a structural basis that could explain the observed increase in K+ selectivity of the M20 mutants?

Met20 is located near the aromatic cluster in the extracellular segment of the channel pore that plays a major role in K^+ selectivity. However, this residue is conserved in *Hc*CCR, a Na^+ channel, which suggests that Met20 has been selected for a different reason than K+ selectivity.

In the revision, we added the following sentences to the "Mutagenetic analysis of the K+ pathway" section:

> "Met20 is conserved in Na^+-selective *Hc*CCR, which suggests that this residue was evolutionarily selected for a channel property other than selectivity. However, Met20 is located at a 5.4 Å distance from Tyr222, the key determinant of K^+ selectivity (Fig. 4), and might influence its H-bonding network and/or hydrophobic interactions."

6) Could the presence of 11-cis retinal be related to a potential desensitised state? Has this been observed elsewhere and is anything known about the rate of decay compared to 13-cis retinal?

We hypothesize that our *Hc*KCR1_C110A structure obtained after continuous illumination might represent a desensitized state. However, the resolution of the map was insufficient to model the chromophore density with a mixture of different isomers, some of which make minor contributions. Nevertheless, an independent study identifying 11-*cis* isomers in wild-type *Hc*KCR1 (Ref. 12) confirms our HPLC results. A small amount of 11-*cis* retinal was also extracted from the light-adapted slow-cycling C128T mutant of *Chlamydomonas reinhardtii* ChR2 (*Cr*ChR2), and accumulation of this isomer was attributed to the photocycle side reactions (Ritter et al. 2013 JBC, DOI: 10.1074/jbc.M112.446427). Detailed analysis of these reactions in *Hc*KCR1 or *Hc*KCR1_C110A requires a separate study and is beyond the scope of this manuscript.

7) I am curious why the authors chose to solve the structures in 150 mM NaCl. Clearly this would be outside the scope of the paper, but would the presence of KCl be expected to make a difference and have the authors tried this?

We thank the Reviewer for pointing this out because, in fact, NaCl was a typo, a carry-over from our previous manuscript reporting structures of wild-type *Hc*KCR1 and *Hc*CCR obtained in NaCl. In this study, we purified the protein and reconstituted it in peptidiscs using KCl buffer. We corrected this typo in the revision. Please note that neither condition fully matches physiological, as in biological membranes, ChRs are exposed to K^+ from the inside and Na^+ from the outside of the membrane.

Minor points:

- Figure 2: WT dark-adapted structure not from this study, indicate PDB ID.

We have added the PDB code of the dark-adapted structure to the Figure 2 caption in the revision as follows:

> "Comparison of the *Hc*KCR1_C110A and wild-type *Hc*KCR1 (PDB: 8GI8) dark-adapted structures and functional characterization of C110 mutants."

- Table 1: Indicate map sharpening method, include model-to-map validation statistics such as CCmask/ligand, Q-score/EMRinger score, model-to-map FSC. I would also recommend including Ramachandran Z-scores for better assessment of model quality.

Table 1 has been updated to include sharpening method and additional validation statistics.

- Local resolutions in Supplementary Figs. 2 and 5 do not match with reported resolution range in Table 1 and the mentioned 2.6Å in the first results part (line 119).

We corrected this. We addressed this also in the response to comment 1 of reviewer 1:

We thank the reviewer for pointing this out. We agree that the local resolution in the 7TM domain of the dark-adapted KCR and the continuous illuminated KCR is lower than that of the laser-flash-activated KCR. We reevaluated the local resolution of the maps and reploted the maps with adjusted resolution scale. The resolution of the 7TM domain of the maps are 2.8 Å (dark-adapted), 2.6 Å (laser-flash-illuminated), and 3.2 Å (continuous illumination). We updated the values in Table 1 and made the changes to Supplementary Figures 2 and 5.

The second sentence of the second paragraph in the results section now reads:

> "Single particle analysis allowed us to obtain density maps at 3.08 Å resolution for dark-adapted and 3.03 Å for laser-flash-illuminated *Hc*KCR1_C110A, respectively, and to determine high-resolution structures; the resolution of the maps within the 7TM domain reached 2.6 Å (laser-flash-illuminated *Hc*KCR1_C110A) and 2.8 Å (dark-adapted *Hc*KCR1_C110A) (Fig. 1, Table 1, Supplementary Fig. 2)."

While the resolution of dark-adapted KCR in the 7TM domain is 0.2 Å lower compared with the laser-flash-illuminated KCR, the difference does not result in major limitations when comparing the side chains in the two structures. The resolution of the continuous illuminated sample is lower but allowed us to determine the retinal configuration and the orientation of the Asp116 side chain, which was similar to the dark-adapted state and not like the laser-flash-illuminated state.

We modified the beginning and end of the paragraph on KCR upon continuous illumination. The paragraph begins now with:

> "In addition to laser-flash-illuminated *Hc*KCR1_C110A, we performed cryo-EM single particle analysis of *Hc*KCR1_C110A illuminated with continuous green light before cryo plunging (**Supplementary Fig. 6, Table 1**). The density map had a global resolution of 3.44 Å and up to 3.2 Å within the 7TM domain allowing us to model the isomerized retinal in the predominant 13-*cis*, 15-*syn* configuration."

And ends with:

> "The model obtained after continuous illumination gives structural insight into a desensitized KCR with no or weak conductance and might comprise a mixture of states as concluded earlier[23]. The presence of multiple states could also be an explanation for the lower resolution of the map obtained upon continuous illumination of *Hc*KCR1_C110A. Similarly, a complexity of protein conformations and retinal binding was observed in the cryo-EM structures of *Hc*KCR1 and *Hc*KCR1_C110T, respectively, prepared under continuous dim white light[16]."

The desensitized state is an interesting but complex state. This state is difficult to analyze due to its mixture of photointermediates and is the topic of a separate study.

- Supplementary Figure 2: Add corrected curve in FSC plots

The figure has been updated to show the corrected FSC curve.

- Consider illustrating H-bonds in Figure 4 for easier interpretability, in particular those that change between dark and illuminated states (e.g., D116-R244).

We changed Figure 4 and added additional side chains and labels to residues in the right column (computational electrophysiology) to make it easier comparable to the cryo-EM structures in the other two columns.

- There seems to be a mixture of 3 and 1 letter codes used to describe amino acid residues in the manuscript, which can be confusing at times.

We used the 3-letter code for individual residues mentioned in the text and the 1-letter code for the mutants. For visual clarity, we used the 1-letter code for individual residues in the figures.

- Potentially flesh out what specific properties could be engineered for optogenetic applications and how the new structural insights would facilitate this.

To explain this, we added the following paragraph to the Discussion in the revision:

> "The advent of all-optical electrophysiology[11] requires spectrally separated optogenetic tools, which are rarely found in nature. Our structural analysis of photoinduced changes in a ChR molecule will facilitate the molecular engineering of color-tuned variants with intact channel function. A better understanding of channel gating and selectivity mechanisms will eventually enable the design of optogenetic tools with a desired combination of biophysical properties."

Reviewer #3 (Remarks to the Author):

This manuscript by Morizumi and colleagues investigates the light-gating of a potassium-selective channelrhodopsin. The authors used single-particle cryo-electron microscopy (cryo-EM) to solve the Kalium channelrhodopsins (KCR1) structures, in dark-adapted and laser-flash-activated states, from Hyphochytrium catenoides. Classical molecular dynamics (MD) and computational electrophysiology simulations were applied to study the hydrogen bond networks and the potassium ion translocation mechanism. The conclusions drawn from simulations are interesting, but poorly supported.

1. The authors performed classical MD simulations of the dark-adapted and laser-illuminated trimers, obtained from the cryo-EM experiments, to then analyze the H-bond networks. The relative method section "Graph-based analyses of the protein-water H-bond networks" lacks critical details for the simulations reproducibility. It is unclear what equilibration protocol was used, the simulations time length, and how many replicates were performed.

The equilibration protocol used weak harmonic constraints placed on hetero-atoms; these constraints were released in 6 steps, then all harmonic constraints were switched off and simulations proceeded to the production runs. As noted in section 'Statistics and reproducibility', for both the dark and laser-

illuminated trimers we computed the H-bond network separately for each monomer, and chose the average H-bond occupancy that gave similar H-bond networks in all monomers of the same simulation. For the laser-illuminated trimer, in which the cytoplasmic H-bond of the retinal Schiff base is more dynamic than the extracellular network of the all-trans retinal Schiff base of the dark trimer, we performed an independent simulation and verified that a water-mediated water chain is sampled at the cytoplasmic side of the retinal Schiff base. The missing info on the simulation length was an oversight.

To address the questions from the Reviewer, the following information was added to the methods section "Graph-based analyses of the protein-water H-bond networks" of the revised manuscript.

> "Equilibration protocol: The simulation systems were geometry optimized and heated to 300K, followed by equilibration in six steps, 1 ns each, in which harmonic constraints placed on selected hetero-atoms were progressively released. Heating and equilibration step #1 used harmonic constraints of 4 kcal/mol·Å^2 on the backbone, 2 kcal/mol on the side chains, retinal hetero-atoms, and the oxygen atoms of the crystallographic water molecules, 1 kcal/mol.Å on ions, bulk water oxygen atoms, and hetero-atoms of the lipid headgroups, and 0.5 kcal/mol on the hetero-atoms of the lipid acyl chains; equilibration step #2, constraints on the lipid acyl chains were switched off; step #3, harmonic constraints on the backbone were halved, to 2 kcal/molÅ; step 4, all remaining constraints were halved; step #5, harmonic constraints on the lipid headgroups were removed, and all remaining constraints reduced to 0.5 kcal/mol Å. This completed the equilibration, all harmonic constraints were removed, and simulations proceeded with production runs. Replicates performed: For the dark-adapted trimer, as shown in the Supplementary Figures, the H-bond networks of the RSB are very similar in the three protomers; the H-bonds of the Schiff base cluster have high occupancies, and similar values, in each of the three monomers. Consequently, only one simulation of the dark trimer was performed. For the laser-flash-illuminated trimer, for which the cytoplasmic Schiff base cluster is more dynamic, and there is more variability of the H-bond cluster than in the dark counterpart, we performed one independent simulation. Length of the simulations: The simulations we prolonged to 484.0 ns for the dark-adapted trimer, 424.0 ns for the main 13-*cis*-retinal-bound trimer, and 457.3 ns for the repeat 13-*cis*-retinal-bound trimer."

Regarding the results section "Formation of a transient water chain", it is difficult for the reader to interpret the results having two long supplementary tables. It might be useful to have a histogram graph (with relative error bars), or a time series of distances, to compare the most important hydrogen bonds discussed in the main manuscript. Moreover, the node labels in Supplementary Figure 6b, c and d are too small and hard to read.

We have replaced the long supplementary tables with Supporting Information Figures that show, for each simulation and each trimer, the average H-bond occupancy and the average water bridge length for each of the H-bond connection of the graph. To ensure that the node labels can be read, we separated the former Suplementary Figure 6 into separate figures, such that in total we have 12 multi-panel figures with H-bond graphs. Furthermore, we now include separate figures with the graphs of each protomer, displaying the H-bond occupancies and average water bridge length. The time series of the number of H-bonds sampled during each simulation are presented in the new Supplementary Figures 7-9.

The sentence "the RSB could transiently connect to the H-bonded Ser70/Asp116 cluster in the intracellular segment via a chain of three H-bonded water molecules" (at line 246-247) is not clear to me. As far as I

understand, Lys233 is establishing a water-mediated h-bond with Ser70 only in one protomer of the laser-illuminated trimer with a 54% occupancy. I do not think this is statistically significant.

We have now prolonged the simulation of the 13-*cis*-retinal-bound structure, and include an independent simulation of the 13-*cis*-retinal-bound structure. In both of these simulations we observe transient formation of the water-mediated bridge between K233 and S70 in two of the monomers. That is, of the 6 monomers, 4 have the transient formation of the water-mediated bridge between K233 and S70. This water bridge is mediated by about 3 H-bonded waters in all monomers where it is present, and has average occupancies of 68% (Protomer 2, Supplementary Figure 16), 51% (Protomer 3, Supplementary Figure 17), 91% (Protomer 2, Supplementary Figure 20), and 45% (Protomer 3, Supplementary Figure 21). We think that, with the two prolonged simulations of 13-*cis*-retinal-bound-trimers, the finding that the water-mediated bridge between K233 and S70 is statistically relevant.

The results section "Rearrangement of the aromatic cluster" describes a perturbation of a cluster of aromatic residues, but it is not clear whether this claim is also supported by simulations.

The computational electrophysiology simulations show that Trp102 orients itself in the direction of the RSB. It binds the potassium ion via cation-π interaction and directs its movement towards the aspartate residues (Asp105 and Asp229). On the other hand, the permeating ion binds to the polar Tyr222 residue via the hydroxyl group instead of cation-π interaction, thereby directing the ion into the channel.

To highlight this, we have added the following text in the section on "The K^+ flow through the channel":

> "In addition, we observe rearrangement of the Trp102 residue at the extracellular site, wherein it orients itself towards RSB. It binds the permeating ion via a cation-π interaction and directs it towards the aspartate residues (Asp105 and Asp229) during inward permeation."

We also analyzed the H-bond networks and added at the end of the paragraph 'Rearrangement of the aromatic cluster':

> "The MD simulations and graph-based analyses confirm that the dynamics of the aromatic cluster is markedly different in the dark-adapted vs. laser-flash-illuminated *Hc*KCR1_C110A trimers. In the dark, Tyr81, Trp102, Tyr106, and Tyr222 are part of the H-bond cluster of the all-*trans* RSB; the shortest-distance connection between Tyr106 and Tyr222 is via 2-3 H-bonded water molecules, or via water, Tyr81, and Lys84 (**Supplementary Figs. 11d, 12d, 13d**). By contrast, in the laser-illuminated trimer, the shortest-distance H-bond connection between Tyr106 and Tyr222 are no longer simple water bridges, but pass via water-mediated bridges, and side chains other than in the dark can be recruited to mediate this connection - including, in one monomer, His225 (**Supplementary Figs. 11-13, 15-17**). "

The authors used the computational electrophysiology technique to investigate the potassium ion flow through the channel. However, reading the relative method section "Computational electrophysiology", it is not clear if they simulated two copies of the whole trimer or two copies of a protomer. Then, they should clarify which barostat and pressure coupling were used for the equilibration and production phases. Lastly, a description of the procedure for the transmembrane voltage calculation is also needed.

For the computational electrophysiology calculations, we simulated a protomer for both the dark-adapted as well as the laser-flash-illuminated structures. Berendsen and Parrinello-Rahman pressure coupling schemes were employed to maintain a pressure of 1 bar throughout the simulation during the equilibration and production phases, respectively. To generate the transmembrane voltage, an ion imbalance was created between the two compartments of the double-membrane box. This was done by varying the concentration of the potassium ions where compartment A had 6 K^+ ions lower than compartment B while the chloride ion concentration was maintained the same in both.

To reflect these, we have revised the Computational Electrophysiology section in the manuscript and added 6 more references as follows (modifications in grey):

> "Molecular dynamics (MD) simulations were performed to probe the potassium ion flow through the protein in both dark-adapted and laser-flash-illuminated structures. The 15-*anti* retinylidene Schiff base was modelled in its protonated form in both the isomers: all-*trans* configuration in the former and 13-*cis* form in the latter. The simulations were performed through the computational electrophysiology (CompEL) protocol[35,36] using one protomer for each structure. In this approach, the separation of two aqueous compartments is obtained by duplicating the membrane patch in the direction of the membrane normal. The transmembrane voltage results from a constant ion concentration difference between the two compartments.
>
> The respective ion channel is embedded in a lipid bilayer of POPC (1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine) and solvated in water with 600 mM of K+ and Cl- ions on both sides of the bilayer. The embedding was done with CHARMM-GUI[75-77]. The protonation states of the residues were modeled at pH 7. The single-membrane system was first energy minimized to stabilize the system, thereby avoiding steric clashes. This is followed by equilibration in a stepwise manner through seven NPT cycles where the number of particles (N), pressure (P) and temperature (T) were kept constant. During the equilibration steps, positional restraints are applied on the heavy atoms to first equilibrate the solvent and ions around the lipids and protein using the LINCS algorithm[78]. The positional and torsional restraints are then gradually relaxed to equilibrate the whole system. The pressure was maintained at 1 bar using the Berendsen coupling scheme[79] while the temperature was kept constant at 303 K by utilizing the velocity-rescaling thermostat[80]. The equilibrated single-membrane setup is then duplicated and stacked parallel to form the double-membrane configuration (Extended Data Fig. 7). The advantage of using the double-membrane setup in parallel orientation is that it allows simultaneous sampling of the ion movement under the effect of both positive and negative transmembrane voltages.
>
> Periodic boundary conditions were applied, forming two distinct compartments (A and B). To generate transmembrane voltages, an ion imbalance was created between the two compartments. This was achieved by varying the concentration of potassium ions where compartment A had 6 K+ ions less than compartment B while the chloride ion concentration was maintained the same in both compartments. The GROMACS tool *gmx potential* was used to quantify the transmembrane voltages during the simulation. To keep the number of ions constant in the compartments throughout the simulation time, after ion permeation, ion/water exchanges are carried out. The ion count is checked at every 100 time steps, with the threshold set to 1 and coupl-steps set to 10.
>
> The double-membrane setup dimensions for the dark-adapted and laser-flash-illuminated models were 72 × 72 × 192 $Å^3$ and 72 × 72 × 186 $Å^3$, respectively. Each setup consisted of ~90,000 atoms (~16,000 water molecules, 250 lipid molecules and ~160 potassium and chloride ions). Five

replicates of the setup were generated for each model and production runs were simulated independently for 500 ns. For these runs, the Parrinello-Rahman pressure coupling scheme was employed to maintain the pressure at 1 bar[82]. All simulations were ran using GROMACS (v. 2023.3)[82,83]. Forcefields for the retinal were taken from references[71,72,84-87] and used in CHARMM format. TIP3P waters[62] and CHARMM36m forcefields were used[68,69]. The particle-mesh Ewald method (PME) was used for long-range electrostatics interactions[88] with a cutoff of 1.2 nm for Coulomb and Lennard-Jones interactions."

The results reported in the "The K+ flow through the channel" section are missing statistical quantifications. These data are only qualitatively described and cannot support any claim. For instance, the authors could quantify ion permeation and conductance (similar to what has been done in Figure 2 and Supplementary Figure 1 in Zhuang Y et al., 2022 https://doi.org/10.1073/pnas.2208081119). They should also quantify the contacts established between the permeating ions and the protein residues, showing that the reported interactions are statistically meaningful. This could support the claim that the described residues are involved in the channel gating.

We agree with the reviewer that the computational electrophysiology results are qualitative rather than quantitative. We performed 5 independent runs of 500 ns which is too short to observe a significant number of ion permeations to perform a qualitative analysis of the conductance. Given this fact, we still observe around 10 ions permeating through the laser-flash-illuminated structure in both inward as well as outward directions. These results show qualitatively that *Hc*KCR1 is capable of forming a channel upon illumination. Our simulations also show that the aspartate residues across TM3 and TM7 (ASP116, ASP229 and ASP105) play an important role during potassium ion permeation.

To obtain quantitative insights into the conductance, we would need to carry out more runs and for a longer time period. Such analysis is beyond the scope of the present study and will be performed in the future.

To highlight this, we have added the following text at the end of the section on "The K^+ flow through the channel":

"These results qualitatively show that *Hc*KCR1_C110A is conductive for K^+ upon photo-illumination, with the movement through the channel guided by the aspartate residues. Due to the small number of ions permeating over the limited simulation period, a quantitative analysis of the conductance cannot be performed. A longer simulation time revealing significantly higher permeation events for deriving statistically meaningful single-channel conductance will be the scope of future studies."

We also modified the conclusion:

"Our results reveal molecular mechanisms of light-gated K^+ and H^+ conductance and selectivity of *Hc*KCR1. The structures showed subtle but informative rearrangements of *Hc*KCR1 upon laser-flash-illumination, which are sufficient for ion permeation as confirmed by MD simulations. These insights will facilitate KCR engineering for optogenetic needs. However, further studies using MD simulations of the potassium channel gating as well as time-resolved vibrational spectroscopy and serial crystallography are needed to further elucidate the chemistry of KCRs' unique function."

Such studies will be very informative but are beyond the scope of the present study.

As a general remark, the authors should dedicate a couple of supplementary figures to show the stability of the systems in both computational settings.

The Cα RSMD profiles computed for each monomer of the trimer simulations are presented in Supplementary Figures 7-9. These profiles show structural stability for the TM helical regions of each monomer, with RMSD values <2Å.

We have also added the following Supplementary Figure:

Supplementary Figure 23 | Protein stability during electrophysiology simulations. Root mean square deviations (RMSD) of the protein backbone atoms across the simulation time for channels 1 and 2 for dark-adapted and laser-flash-illuminated *Hc*KCR1_C110A structures. The transmembrane voltage across each channel is also provided.

This figure shows that the protein structures for both dark-adapted and laser-flash-illuminated models do not show drastic structural changes and remain stable throughout the simulation time in both channels, with a maximum deviation of the backbone atoms of ~3 Å.

We have modified the section "The K^+ flow through the channel" in the manuscript as follows:

> "To accelerate the rate of K^+ passage through the channel during the limited simulation time (500 ns), we chose a high transmembrane voltage of 400 to 900 mV (Supplementary Fig. 22b,c). We sampled five independent trajectories for the dark-adapted and laser-flash-illuminated structures each. We monitored the root mean square deviations (RMSD) of the protein backbone atoms and observed that the structures remain stable throughout the simulation time (Supplementary Fig. 23). Across the trajectories, we observed altogether 10 K+ ions permeating through the channels in the laser-flash-illuminated structure whereas no cation permeation was observed in the case of the dark-adapted structure. The low simulated conduction rate aligns well with the generally low conductance characteristic of *Hc*KCR1. The 10 permeating events in the illuminated structure were equally distributed among inward and outward permeations under negative and positive transmembrane voltages, respectively (Supplementary Videos 1 and 2). This was facilitated by the pore opening between TM1-3 and TM7 and was largely assisted by the aspartate residues in TM3 and TM7 (Fig. 4, right)."

Finally, I have to point out that no simulation input or output data have been shared, significantly reducing the reported study's reproducibility.

To improve both the reliability and reproducibility of the present study, I suggest following the "Reliability and reproducibility checklist for molecular dynamics simulations" (https://www.nature.com/articles/s42003-023-04653-0) included in the Nature Communication Computational tools reporting guidelines https://www.nature.com/ncomms/submit/resources.

We have added the input files and representative output trajectory files in the Figshare repository to allow reproducibility of the simulations. These files can be accessed at https://doi.org/10.6084/m9.figshare.27292005. For the purpose of peer-review, the data files can be viewed at https://figshare.com/s/d075460024da2b099374 (private link).

Reviewer #4 (Remarks to the Author):

This is a nice, clearly written paper reporting the structure and structural changes of a molecule that can be used in ontogenetic studies. For publication in Nature Communications a bit more effort should be made to highlight why knowing the structure of this protein can be of wider interest. There are connections in the paper to the wavelength of excitation and the rate of the channel opening and closing. Can having the detailed open and closed structure help make changes to the protein that would make it more useful for different uses.

We added a paragraph at the end of the discussion, which is also the response to the last comment of reviewer #2:

> "The advent of all-optical electrophysiology[11] requires spectrally separated optogenetic tools, which are rarely found in nature. Our structural analysis of photoinduced changes in a ChR molecule will facilitate the molecular engineering of color-tuned variants with intact channel function. A better understanding of channel gating and selectivity mechanisms will eventually enable the design of optogenetic tools with a desired combination of biophysical properties."

The resolution is at the border of high resolution (≈2.7-3.1Å). The main conclusion is that side chain motions are most important. It would be useful to discuss the positional uncertainty at this resolution.

We addressed this in the response to comment 1 of reviewer 1:

We thank the reviewer for pointing this out. We agree that the local resolution in the 7TM domain of the dark-adapted KCR and the continuous illuminated KCR is lower than that of the laser-flash-activated KCR. We reevaluated the local resolution of the maps and reploted the maps with adjusted resolution scale. The resolution of the 7TM domain of the maps are 2.8 Å (dark-adapted), 2.6 Å (laser-flash-illuminated), and 3.2 Å (continuous illumination). We updated the values in Table 1 and made the changes to Supplementary Figures 2 and 5.

The second sentence of the second paragraph in the results section now reads:

> "Single particle analysis allowed us to obtain density maps at 3.08 Å resolution for dark-adapted and 3.03 Å for laser-flash-illuminated *Hc*KCR1_C110A, respectively, and to determine high-resolution structures; the resolution of the maps within the 7TM domain reached 2.6 Å (laser-flash-illuminated *Hc*KCR1_C110A) and 2.8 Å (dark-adapted *Hc*KCR1_C110A) (Fig. 1, Table 1, Supplementary Fig. 2)."

While the resolution of dark-adapted KCR in the 7TM domain is 0.2 Å lower compared with the laser-flash-illuminated KCR, the difference does not result in major limitations when comparing the side chains in the two structures. The resolution of the continuous illuminated sample is lower but allowed us to determine the retinal configuration and the orientation of the Asp116 side chain, which was similar to the dark-adapted state and not like the laser-flash-illuminated state.

We modified the beginning and end of the paragraph on KCR upon continuous illumination. The paragraph begins now with:

> "In addition to laser-flash-illuminated *Hc*KCR1_C110A, we performed cryo-EM single particle analysis of *Hc*KCR1_C110A illuminated with continuous green light before cryo plunging (**Supplementary Fig. 6, Table 1**). The density map had a global resolution of 3.44 Å and up to 3.2 Å within the 7TM domain allowing us to model the isomerized retinal in the predominant 13-*cis*, 15-*syn* configuration."

And ends with:

> "The model obtained after continuous illumination gives structural insight into a desensitized KCR with no or weak conductance and might comprise a mixture of states as concluded earlier[23]. The presence of multiple states could also be an explanation for the lower resolution of the map obtained upon continuous illumination of *Hc*KCR1_C110A. Similarly, a complexity of protein conformations and retinal binding was observed in the cryo-EM structures of *Hc*KCR1 and *Hc*KCR1_C110T, respectively, prepared under continuous dim white light[16]."

The desensitized state is an interesting but complex state. This state is difficult to analyze due to its mixture of photointermediates and is the topic of a separate study.

Line 153. It would seem to be worth the effort to make a stab at identifying and differentiating K+ and water. In cryoEM my understanding ions scatter differently depending on their charge. Also a census of hydrogen bonds or nearby charges might help identify ion binding. In addition, the MD calculations could find which sites, if any, prefer K+.

MD simulations show that the positively charged potassium ions are largely transiently bound by the negatively charged aspartate residues (Asp105, Asp229 and Asp116) present along the channel. Apart from these, other binding sites that direct the movement of the ion are the polar hydroxyl residues such as serine (Ser70), threonine (Thr120) and tyrosine (Tyr222).

To address this, we add the following text in the revised manuscript in section "The K+ flow through the channel":

"Apart from the negatively charged aspartate residues, the permeating ion also binds favorably to the polar residues containing hydroxyl groups. Ser70, Thr120 and Tyr222 acts as active binding sites for the permeating ion and direct its motion through the channel (**Supplementary Video 3**)."

While assigning potassium ions in the density map is an option, we opted to err on the side of caution as we could not confidently model any potassium ions within the channel. There are few densities which are a bit larger but confidently modeling K^+ would require higher resolution.

We also refer to a publication by Tajima et al. 2023 Cell (PMID 37652010) on KCR structures who could not conclusively identify K^+ ions in their high resolution dark state structures. Supported by Attenuated Total Reflection Fourier Transform InfraRed [ATR-FTIR] spectroscopy [Figures S3G and S3H], UV-Vis absorption spectra measurement [Figure S3I], Fluorescence-detection Size-Exclusion Chromatography-based ThermoStability assay [FSEC-TS] [Figure S3J], and MicroScale Thermophoresis [MST] experiments [Figure S3K]), they concluded that K^+ ions do not bind stably to KCRs in any specific site. We added in the paragraph "Formation of a K^+ conduction pathway":

"It is worth the notion that stable K^+ binding could not be found for dark state *Hc*KCRs[12]."

It seems clear that this protein differs from standard channel rhodopsins in that it does not undergo large conformational changes to open a channel. Is there a better correspondence with halorhodopsin which is a Cl pump or is this protein functioning in a mode different from ChR or halorhodopsin?

KCRs belong to the family of bacteriorhodopsin-like ChRs (BCCRs), as they share some structural and functional properties with haloarchaeal proton pumps. In particular, the residues corresponding to Asp85 (the Schiff base proton acceptor in bacteriorhodopsin) and Asp96 (the proton donor) are conserved in KCRs and, as we show in this manuscript, play an important role in channel gating. On the contrary, in halorhodopsins, Asp85 is replaced with Thr and Asp96 with Ala. Pairwise sequence alignment of *Hc*KCR1 and *Np*HR (used as an optogenetic tool) shows only 18.6% identity and 32.6% similarity, so there are no reasons to believe in any correspondence between KCRs and halorhodopsins. Please also note that the concepts of "halorhodopsin" (singular) and "ChR" (singular) are outdated, as ~7,000 different halorhodopsins and ~1,000 different ChRs have been identified from various organisms.

Dehydration of an ion can be difficult. Is the K+ dehydrated through its full path?

The potassium ion is indeed not completely dehydrated during its movement through the channel in the laser-flash-illuminated KCR structure. To understand this, we track the surrounding environment of the permeating K^+ ion for water molecules within a threshold of 4 Å. As can be observed in the new Supplementary Figure 24, the K^+ ions were only partially dehydrated and have at least one coordinated water molecule during its transport through the channel in both inward and outward directions.

To highlight this, we have added the following text in the section on "The K^+ flow through the channel":

> "As the potassium ions are seldom free and have a coordinated hydration sphere around it, we also monitored the hydration changes. We observed that during permeation in both inward and outward directions, there is at least one coordinated water molecule, even when the potassium ion interacts with the aspartates (**Supplementary Fig. 24**)."

Figure legend:

Supplementary Figure 24 | Snapshots of computational electrophysiology simulations. Snapshots showing potassium ion (green spheres) binding to the aspartate residues during permeation through the channel in outward (**a**) and inward (**b**) direction. Water molecules surrounding the permeating ion (within 4 Å) are also shown as red spheres.

The above figure shows that the permeating ion is not completely dehydrated. This would mean that the tunnel would open wide enough to pass partially hydrated K^+.

Figure 5 shows serious constrictions in the channel. Can a figure from the MD be added that shows the channel width in individual snapshots when a K is transiting through each constriction. Figure 5 would also be more useful if key amino acids were marked along the path.

MD simulation snapshots showing a potassium ion passing the tunnel constrictions have already been shown in Figure 4 of the original submission. In the revision, we added the side chains of the constriction-forming residues to this figure. We also added a cartoon showing the positions of the mutated amino acid residues along the tunnel (ion conduction path) to Figure 5a.

Line 57: Halorhodopsin is an ion channel which has similar generic structure so this is protein is not that unique. Unclear what is the job of the protein in its native state. E.g. line 66 should give the conductance of the natively used ion.

As explained above, halorhodopsins and KCRs do not have a "similar generic structure". Moreover, halorhodopsins are not ion channels at all, as they actively transport chloride across the membrane (i.e., independently of the electrochemical gradient). In our previous publication on *Hc*KCR1 (Ref. 7), we convincingly demonstrated that "the job of the protein in its native state" is passive K^+ conductance. The unitary conductance values of other ChRs we refer to (Refs. 4 and 5) have been determined for the natively conducted ions (Na^+ for *Cr*ChR2 and Cl^- for *Gt*ACRs).

Line 85: It is important that the ion goes through the monomer rather than the pore being formed in the center of sever monomers. But with the monomer channel you have here it's not so clear the importance of the protein assembling as dimers, trimers, or tetramers. More important that the multimer level is the same in the cell and in the nonodisk.

The functional role of ChR oligomeric assembly is unknown and can be nonexistent. Currently, there are no experimental procedures for controlling the oligomeric state of KCRs, making evaluating its possible functional role very difficult. To the best of our knowledge, only one study (Bestsennaia et al. 2024 Angew Chem Int Ed Engl, DOI: 10.1002/anie.202307555) probed ChR oligomeric assembly in biological membranes by quantitative photoactivated localization microscopy and confirmed that *Cr*ChR2 maintains its dimeric state found in detergent-purified or lipid-reconstituted protein.

The C110A structure is used, which seems like a fine choice. It would be useful to know what region of the protein this residue is found in when the mutation is first mentioned. It's good to have the the RMSD differences in the dark-adapted structure between the wild-type and mutant, changes in the active site distances as well as in the protein activity.

To specify the position of Cys110 in the protein when this residue is first mentioned in the text, we updated the corresponding sentence in the revision as follows:

> "Replacement of the highly conserved Cys110 in the middle of TM3 with alanine or threonine results in a >1,000-fold decrease in the channel closing rate[22]."

The original manuscript already contains the RMSD difference between the dark-adapted structures of the wild type and mutant and the description of changes in the photoactive site distances caused by the mutation:

> "The backbone RMSD between the HcKCR1_C110A and the wild-type HcKCR1 dark-adapted state structures was 0.318 Å (Fig. 2a). <…> The replacement of cysteine with alanine allows the RSB^+ to move farther away from its counterion Asp229 (from 3.4 to 4.6 Å), the homolog of Asp212 in bacteriorhodopsin (BR)."

Likewise, "changes in the protein activity" caused by the C110A/T mutants are reported in Figure 2c-f of the original manuscript.

Line 152. How many waters does the dark wild-type structure contain?

To address this question in the revision, we updated the corresponding sentence as follows:

> "The dark-adapted *Hc*KCR1_C110A structure contains 20 water molecules per protomer (compared to 15 water molecules and a sodium ion in the wild-type structure[15]), whereas the illuminated-state structure contains 27 water molecules."

Line 178. Remind the reader that Lys 233 is the SB. Maybe just say the 'attached' Lys 233 side chain.

In the revision, the corresponding sentence was modified as follows:

> "The pronounced change of the RSB position upon the photoinduced all-*trans* to 13-*cis* isomerization altered the conformation of the Lys233 side chain to which the retinal is attached (Supplementary Fig. 3f,g), merging the separated cavities into a continuous K^+ conduction pathway (Fig. 4)."

Line 244. Any non-canonical choices for residue protonation states in the MD should be noted. For example, if the SB is deprotonated following isomerization, which residue is now protonated. Information on line 584 is insufficient.

In the trimer simulations all protein residues had standard protonation states, i.e., all Asp/Glu were negatively charged, all Arg/Lys, positively charged, and all His residues, neutral; the RSB was considered protonated in all trimer simulations.

We also added this information at the beginning of the computational electrophysiology methods section. Please see also reply to comments of reviewer #3. The modified passage reads now:

> "The 15-*anti* retinylidene Schiff base was modelled in its protonated form in both the isomers: all-*trans* configuration in the former and 13-*cis* form in the latter."

Fig 3. Not easy to see the differences between the structures. Not sure what to suggest: Add arrows or show the two structures overlaid?

We thank the reviewer for making us aware of the problem and revised Figure 3. We framed the regions where major changes occur between the dark and laser-flash-illuminated structures to guide the eye.

Reviewer #5 (Remarks to the Author):

Morizumi et al reported cryo-EM structures of the dark-adapted and light-illuminated states of a potasium-selective channelrhodopsin (Hyphochytrium catenoides kalium channelrhodopsin 1: HcKCR1) and discussed the mechanism of the opening of the ion conduction pathway across the membrane. When I first read their manuscript, I felt that the main text was perfectly well written. This is probably because weak points in their structural analyses are not explicitly described in the main text and, instead, separately described in Supplementary Discussion. Judged from the last sentence in this Discussion, which indicates that ~40 % of the molecules could be in the closed or desensitized state in the laser-flash-illuminated sample,I guess that the retinal configuration in the open state might be estimated on the basis of the density map generated by cryo-EM symmetry relaxation analysis of laser-flash-illuminated HcKCR1_C110A trimer(Supplementary Fig. 3d). If this is the case, the following questions will arise.

1) Is it adequate to use symmetry relaxation method in cryo-SPRAC v4.4 ? This method would provide a useful structural information if all the trimers have the same shape. e.g., one protomer adopts a closed

conformation while the other protomers adopt an open conformation. It seems difficult to exclude the ab-initio models of other possible shapes (e.g., all three protomers adopt an open conformation) before applying the symmetry relaxation method. If the authors succeeded in overcoming this difficulty, they are recommended to describe the detail of their structural analysis.

Maps were initially refined with no symmetry relaxation. Symmetry relaxation was only performed after the final refined map was obtained. There is a possibility that particles containing different conformations between the protomers were excluded during ab-intio, however given how similar the dark and laser-flash-illuminated structures are, the differences would be too small to be separated during ab-intio or 3D classification.

With symmetry relaxation individual protomers in the laser-flash-illuminated KCR1 map did appear to have different densities for the retinal, however there was also a significant reduction in overall resolution that limited interpretation and model building. A reason might be that the trimers may have slightly different shape depending on how many protomers adopt an open/closed conformation. Since the overall conformation doesn't change much between open/closed conformations, we were glad to see some changes for the retinal, which we could use to interpret opening of the channel by movement of the retinal Schiff base–Lys233 side chain region.

In Figure 4 we wanted to achieve the highest resolution and detail in the maps to see differences between dark-adapted and laser-flash-illuminated states and therefore did not use symmetry relaxation.

2) It appears that the density maps in Figure 1 and Supplementary Fig. 2 are obtained without applying the symmetry relaxation method. When a considerable fraction (~40%) of the molecules in the laser flash-illuminated state are a closed state(s), the density map of this illuminated state represents a weighted average of the closed and open states. This should not be regarded as a problem, provided that they observed a clear difference in the density map of an interesting region (e.g., around the retinal Schiff base) for the dark-adapted state and the laser-flash-illuminated state. They are recommended to add a figure showing enlarged density maps of the dark-adapted state and the laser-flash-illuminated state, by which light-induced structural changes can be convincingly discussed.

We thank the reviewer for this recommendation. We added two panels (f and g) to Supplementary Figure 3 where we highlight the changes of the retinal Schiff base–Lys233 side chain region between dark-adapted and laser-flash-illuminated KCR.

We added to the figure caption:

“f-g, Enlarged views of the conformational changes of Trp199 and Lys233 side chains accompanying retinal isomerization, displayed in the C3 symmetry-imposed map densities. Water molecules are shown as cyan spheres. Orange discs emphasize Lys233 side chain movement.”

In the text we emphasized this by modifying a sentence in the paragraph "Photoisomerization unblocks the central gate":

> “The pronounced change of the RSB position upon the photoinduced all-*trans* to 13-*cis* isomerization altered the conformation of the Lys233 side chain to which the retinal is attached (Supplementary Fig. 3f,g), merging the separated cavities into a continuous K^+ conduction pathway (Fig. 4).”

3) Supplementary Fig 1d indicates that the photocurrent action spectrum is largely red shifted as compared to the absorption spectrum of the dark-adapted spectrum. This result seems to suggest that a large content (possibly >50%) of the desensitized state co-exist in the dark-adapted state. Is this compatible with the result of HPLC analysis (Supplementary Fig.1g - 1h) ?

By definition, the desensitized state is formed after prolonged illumination, so it cannot be present in the dark-adapted sample. The large redshift of the action spectrum compared to the absorption spectrum indicates the presence of a large non-electrogenic fraction in the purified protein sample. We hypothesize that this fraction is 13-cis-retinal-bound because the binding of 13-cis-retinal results in more blue-shifted rhodopsin than the binding of all-trans-retinal, as it has been observed in bacteriorhodopsin, Anabaena sensory rhodopsin and other proteins. Our HPLC results (Suppl. Figure 1g,h) showing the presence of 13-cis isomers even in the dark-adapted protein sample confirm this hypothesis.

4) The term "the dark recovery" in Supplementary Fig. 1e is ambiguous.

In the revision, we replaced the corresponding sentence in the Figure 1 caption with the following one:

> “e, The time course of the unphotolyzed state recovery monitored at 529 nm upon incubation in the darkness.”