
Discrimination between cyclic nucleotides in a cyclic nucleotide-gated ion channel

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Discrimination between cyclic-nucleotides in a cyclic-nucleotide gated ion channel

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Supplementary Information

Table S1)

Method (DHS fit)	cN	$k_{off}(1/s)$	x_{β} (nm)	ΔG (k _B T)
20ms bond formation time	cAMP	1.2 ± 0.4	0.48 ± 0.08	10.4 ± 1.7
20ms bond formation time	cGMP	1.6 ± 0.3	0.69 ± 0.05	9.2 ± 1.3
1000ms bond formation time: State 1 bond	cAMP	1.1 ± 0.4	0.57 ± 0.07	9.7 ± 1.5
1000ms bond formation time: State 2 bond	cAMP	0.5 ± 0.2	0.48 ± 0.08	10.9 ± 2.0

Table S1) cN-CNBD binding kinetics fit parameter from using the DHS model.

Reporting Summary

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Statistics

For all statistical analyses, confirm that the following items are present in the figure legend, table legend, main text, or Methods section.

n/a Confirmed

- ☐ ☒ The exact sample size (n) for each experimental group/condition, given as a discrete number and unit of measurement
- ☐ ☒ A statement on whether measurements were taken from distinct samples or whether the same sample was measured repeatedly
- ☐ ☒ The statistical test(s) used AND whether they are one- or two-sided
Only common tests should be described solely by name; describe more complex techniques in the Methods section.
- ☒ ☐ A description of all covariates tested
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- ☐ ☒ A full description of the statistical parameters including central tendency (e.g. means) or other basic estimates (e.g. regression coefficient) AND variation (e.g. standard deviation) or associated estimates of uncertainty (e.g. confidence intervals)
- ☐ ☒ For null hypothesis testing, the test statistic (e.g. F , t , r) with confidence intervals, effect sizes, degrees of freedom and P value noted
Give P values as exact values whenever suitable.
- ☒ ☐ For Bayesian analysis, information on the choice of priors and Markov chain Monte Carlo settings
- ☒ ☐ For hierarchical and complex designs, identification of the appropriate level for tests and full reporting of outcomes
- ☒ ☐ Estimates of effect sizes (e.g. Cohen's d , Pearson's r), indicating how they were calculated

Our web collection on [statistics for biologists](#) contains articles on many of the points above.

Software and code

Policy information about [availability of computer code](#)

Data collection	All high-resolution images were collected using a HS-AFM (SS-NEX, Research Institute of Biomolecule Metrology Co.). The AFM-SMFS measurements were collected using a JPK Nanowizard 4. All MDS were performed using the GROMACS2020 simulation package using the Amber99sb-ildn force field. MST experiments were performed using a Monolith NT.115 Pico (NanoTemper Technologies, Germany) and Monolith NT.115 Premium capillaries (NanoTemper Technologies, Germany).
Data analysis	All AFM-SMFS data were analyzed by using JPK force curve processing software (7.0.72). All force histograms were fitted in Origin 2019b. The MST traces were analyzed using the software M.O. Affinity Analysis v2.3 (NanoTemper Technologies, Germany).

For manuscripts utilizing custom algorithms or software that are central to the research but not yet described in published literature, software must be made available to editors and reviewers. We strongly encourage code deposition in a community repository (e.g. GitHub). See the Nature Portfolio [guidelines for submitting code & software](#) for further information.

Data

Policy information about [availability of data](#)

All manuscripts must include a [data availability statement](#). This statement should provide the following information, where applicable:

- Accession codes, unique identifiers, or web links for publicly available datasets
- A description of any restrictions on data availability
- For clinical datasets or third party data, please ensure that the statement adheres to our [policy](#)

The source data files contain all data (force distribution histograms, H-bond distributions) necessary to interpret, verify and extend the presented work. In the absence of dedicated data repositories for raw data AFM force curves and MDS trajectories, and in light of the instructions needed to open these files in proprietary software (in the case of the AFM force curves) and the additional information (parameters and conditions) needed to understand and use the data, raw data AFM force curves and MDS trajectories can be received from Simon Scheuring (sis2019@med.cornell.edu) and Helmut Grubmüller (hgrubmu@gwdg.de), respectively, upon reasonable request.

Human research participants

Policy information about [studies involving human research participants and Sex and Gender in Research](#).

Reporting on sex and gender	N/A
Population characteristics	N/A
Recruitment	N/A
Ethics oversight	N/A

Note that full information on the approval of the study protocol must also be provided in the manuscript.

Field-specific reporting

Please select the one below that is the best fit for your research. If you are not sure, read the appropriate sections before making your selection.

☒ Life sciences ☐ Behavioural & social sciences ☐ Ecological, evolutionary & environmental sciences

For a reference copy of the document with all sections, see nature.com/documents/nr-reporting-summary-flat.pdf

Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

Sample size	No statistical methods were used to determine the sample size. Sample size was determined based on the amount that is sufficient for the histograms creating and Gaussian fitting applied in this study. For each pulling speed or different contact time, at least 38 events were used for the histogram creating and Gaussian fitting (in most cases above hundred events).
Data exclusions	No data exclusions were made.
Replication	All the experiments were replicated 2-3 times.
Randomization	For AFM-SMFS experiments, force distance curves at different pulling speed were collected from multiple random areas. For high resolution AFM imaging experiments, multiple areas were randomly acquired. As the CNBD molecules formed uniform 2D crystal in our experiments, we didn't allocate experimental groups.
Blinding	No blinding was required for single molecule biophysics research. We did quantitative analysis of our collected data. For the data analysis, we used the commercial JPK data processing software (7.0.72). All the data were analyzed in the same software and using the same criteria.

Reporting for specific materials, systems and methods

We require information from authors about some types of materials, experimental systems and methods used in many studies. Here, indicate whether each material, system or method listed is relevant to your study. If you are not sure if a list item applies to your research, read the appropriate section before selecting a response.

Materials & experimental systems

n/a	Involved in the study
<input checked="" type="checkbox"/>	<input type="checkbox"/> Antibodies
<input checked="" type="checkbox"/>	<input type="checkbox"/> Eukaryotic cell lines
<input checked="" type="checkbox"/>	<input type="checkbox"/> Palaeontology and archaeology
<input checked="" type="checkbox"/>	<input type="checkbox"/> Animals and other organisms
<input checked="" type="checkbox"/>	<input type="checkbox"/> Clinical data
<input checked="" type="checkbox"/>	<input type="checkbox"/> Dual use research of concern

Methods

n/a	Involved in the study
<input checked="" type="checkbox"/>	<input type="checkbox"/> ChIP-seq
<input checked="" type="checkbox"/>	<input type="checkbox"/> Flow cytometry
<input checked="" type="checkbox"/>	<input type="checkbox"/> MRI-based neuroimaging

Peer Review Information

Journal: Nature Structural & Molecular Biology

Manuscript Title: Discrimination between cyclic-nucleotides in a cyclic-nucleotide gated ion channel

Corresponding author name(s): Simon Scheuring

Reviewer Comments & Decisions:

Decision Letter, initial version:
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Message: 24th Oct 2022

Dear Simon,

Thank you again for submitting your manuscript "Discrimination between cyclic-nucleotides in a cyclic-nucleotide gated ion channel". I apologize for the delay in responding, which (as you know) resulted from the difficulty in obtaining suitable referee reports. Nevertheless, we now have comments (below) from the 2 reviewers who evaluated your paper. In light of those reports, we remain interested in your study and would like to see your response to the comments of the referees, in the form of a revised manuscript. Please be sure to address/respond to all concerns of the referees in full in a point-by-point response and highlight all changes in the revised manuscript text file.

We appreciate the requested revisions are potentially extensive. We thus expect to see your revised manuscript within 6 months. If you cannot send it within this time, please let us know. We will be happy to consider your revision as long as nothing similar has been accepted for publication at NSMB or published elsewhere. Should your manuscript be substantially delayed without notifying us in advance and your article is eventually published, the received date would be that of the revised, not the original, version.

We are committed to providing a fair and constructive peer-review process. Do not hesitate to contact us if there are specific requests from the reviewers that you believe are technically impossible or unlikely to yield a meaningful outcome.

As you already know, we put great emphasis on ensuring that the methods and statistics reported in our papers are correct and accurate. As such, if there are any changes that should be reported, please submit an updated version of the Reporting Summary along with your revision.

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Finally, please ensure that you retain unprocessed data and metadata files after publication, ideally archiving data in perpetuity, as these may be requested during the peer review and production process or after publication if any issues arise.

SOURCE DATA: we urge authors to provide, in tabular form, the data underlying the graphical representations used in figures. This is to further increase transparency in data reporting, as detailed in this editorial (<http://www.nature.com/nsmb/journal/v22/n10/full/nsmb.3110.html>). Spreadsheets can be submitted in excel format. Only one (1) file per figure is permitted; thus, for multi-paneled figures, the source data for each panel should be clearly labeled in the Excel file; alternately the data can be provided as multiple, clearly labeled sheets in an Excel file. When submitting files, the title field should indicate which figure the source data pertains to. We encourage our authors to provide source data at the revision stage, so that they are part of the peer-review process.

While we encourage the use of color in preparing figures, please note that this will incur a charge to partially defray the cost of printing. Information about color charges can be found at <http://www.nature.com/nsmb/authors/submit/index.html#costs>

We require deposition of coordinates (and, in the case of crystal structures, structure factors) into the Protein Data Bank with the designation of immediate release upon publication (HPUB). Electron microscopy-derived density maps and coordinate data must be deposited in EMDB and released upon publication. Deposition and immediate release of NMR chemical shift assignments are highly encouraged. Deposition of deep sequencing and microarray data is mandatory, and the datasets must be released prior to or upon publication. To avoid delays in publication, dataset accession numbers must be supplied with the final accepted manuscript and appropriate release dates must be indicated at the galley proof stage. Please find the complete NRG policies on data availability at <http://www.nature.com/authors/policies/availability.html>.

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We look forward to seeing the revised manuscript and thank you for the opportunity to review your work.

Kind regards,
Florian

Dr Florian Ullrich
Associate Editor, Nature
Consulting Editor, Nature Structural & Molecular Biology
ORCID 0000-0002-1153-2040

Referee expertise:

Referee #1: AFM

Referee #2: CNG channels, MD simulations

Reviewers' Comments:

Reviewer #1:

Remarks to the Author:

The manuscript utilizes AFM-based SMFS and MDS to identify that cAMP binds to SThK NBD stronger than cGMP and employs a deep binding state.

The authors developed a careful protocol for establishing well defined SThK 2-D crystals and used AFM tip chemistry from the literature to attach cAMP and cGMP to AFM tips through flexible linkers. SMFS carried out in their studies appears to be technically sound, although block experiments are required to proof specificity. If specific, injection of nucleotides or SthK should lead to abolishment of binding.

More details of the data must be presented to verify the deep binding state of cAMP. The AFM tip chemistry used does not strictly show only single molecular interactions. Dual, or even more, ruptures can occur, appearing simultaneously or sequentially. At the current stage of data presentation, it cannot be excluded that the high forces of cAMP arise from a double bond rupture, in particular as the binding probability of cAMP is generally higher than that of cGMP and increase of contact time increases the probability of multiple bond formation. To be more convincing, a statistical analysis of multiple bond ruptures in dependence of the contact time (at least for 0.02 and 1 s) both for both cAMP and cGMP must be shown. Another convincing argument is to verify that the higher forces of cAMP do not follow the Williams Markovian model for simultaneous dual bond rupture.

Other points:

The authors are not consistent in using the terms pulling velocity and loading rate. In fact, they vary z-velocity and calculate loading rate, for which error bars for fitting and

presentation should be included.

Another question is, whether the Bell-Evans is model a good model for MDS. In any case, it does not make sense to fit the SMFS and MDS data together with the Bell-Evans model. The loading rates are way too far apart and the fit results of the SMFS and MDS data alone are completely different.

Reviewer #2:

Remarks to the Author:

In this manuscript, the authors probe the differences in cAMP and cGMP binding to the cyclic-nucleotide-gated ion channels using single-molecule force spectroscopy. Electrophysiological studies show that saturating amounts of cAMP activates about 20 to 40% of SthK channels whereas cGMP activates only 0.01% of the channels. Thus, cGMP is an antagonist or an extremely weak partial agonist. X-ray structures of the isolated SthK CNBD in presence of cGMP and cAMP have show that the the central cavity is cAMP bound structure is more open compared to cGMP bound structure (Kesters et. al. PLOS One 10, e0116369, 2015). Using ligands tethered to AFM tip, the authors conduct force spectroscopy studies to probe mainly the cGMP and cAMP unbinding reaction on isolated SthK CNBD. They find that the cAMP unbinds from a deep pocket which is not accessible to cGMP. They suggest that the cAMP binding leads to conformational change which does not occur in the presence cGMP. Their experimental finding was further supported by the molecular dynamics simulation study, which not only enables access to force loading regime beyond the experimental range but also provides a structural view of the unbinding reaction.

Force spectroscopy has been previously used to study both covalent and non-covalent interactions like binding reactions. To the best of our knowledge, this is the first study to examine cAMP-CNBD binding reaction. The authors have wisely decided to focus on the isolated CNBD domain so as not to complicate things. The results are interesting but there are number of issues that need to be addressed.

Key concerns:

1. cAMP and cGMP can exist in both syn and anti configuration but the activated bound state prefers one or the other configuration. The distribution between the two states can be altered by substitutions in the purine ring. Given that the position of the attachment site for the tether is different for cGMP and cAMP, how can one rule out the observed differences in binding modes are not due to the differences in the attachment site.
2. The other question here is whether surface immobilization of the CNBD domain alters the affinity to the cAMP and cGMP. The authors estimate the affinity for cAMP is two fold more than for cGMP. How does this compare with binding affinity measurements in freely diffusing CNBD domains?
3. Is the binding of tethered ligand cGMP and cAMP specific? For instance, it is possible that some of the binding may be non-specific attachment. Can they show that the binding can be competed out in presence of ligands in solution?
4. In calculating the binding probability, the authors only considered the CNBD population density, but the density of the ligands on the AFM tip can also contribute directly to determining the bound probability. How do the authors make sure that the ligand density is the same when they switch from cAMP tip to cGMP tip?
5. Figure 5b shows a relatively broad distribution of the unbinding force. According to the authors, this indicates cAMP binds to the CNBDs in two different states, one in a loosely bound state and the other in a tightly bound state. How do the authors rule out the

unbinding of multi valence state, where multiple ligands bind to different CNBDs within the tetramer? To what extent the ligand population on the AFM tip was controlled to rule out such possibility?

6. In Figure 3b and 3f, the rupture force histograms for cGMP and cAMP appear similar within margin of error. This is further reflected in Figure 3i where the experimental cGMP and cAMP are not very different. The difference is between 5-10 pN. Considering the force constant of the cantilever is 100 pN/nm, how can the authors claim that this small change is significant?

7. In this study, the authors used NTA-Ni²⁺-His6 link to immobilize the CNBDs on the lipid bilayer. The unbinding force related to Tris NTA- Ni²⁺-His6 is about 60-80 pN (Koehler, Melanie, et al. Nano letters 20.5 (2020): 4038-4042) considering all the histidine is bound, the unbinding force is still relatively lower than typical tethering systems used in a force spectroscopy experiment i.e. biotin-streptavidin. Is it possible that the NTA-Ni²⁺-His6 bond breaks before the cAMP-CNBD linkage especially when the polyvalency is not established i.e. all six imidazoles are not bound to NTA?

8. Cyclic nucleotide binding to SthK channels has been investigated by multiple labs. Cryo-EM studies of the full length channels show that the cAMP and cGMP bound SthK structures are identical but X-ray structures of the isolated SthK CNBDs in presence of cAMP and cGMP shows clear differences between the two agonists. The authors dismissed this data by stating that (line 53, Pg. 2) the two structures are "quasi-identical" which is not correct. Please discuss your data in light of these published facts.

Additional comments:

1. The resolution of Figure 1b & 1c could be improved.
2. Figure 1d should include the dashed line along which the cross section 1 is plotted.
3. In the Figure 2b the contact time should be labeled from the beginning of 2 (red line) until the end of 4 (blue line). Black line 3 is shorter than the actual contact time.
4. The authors used the popular Bell-Evans model to fit the dynamic force spectroscopy data. Bell-Evans fitting gives the ability to calculate off rate constant (k_{off}), distance to the transition state ($x\beta$) as well as TS barrier height (ΔG). The authors may want to report the ΔG for both unbinding experiments (cAMP vs cGMP) and a comparison of the ΔG values can be given for a better understanding to readers.
5. Figure 3e should also include an inset showing the zoomed-in region of the unbinding force curve.

Author Rebuttal to Initial comments

Reviewers' Comments:

REVIEWER #1

Remarks to the Author:

Comment 1: The manuscript utilizes AFM-based SMFS and MDS to identify that cAMP binds to SThK NBD stronger than cGMP and employs a deep binding state.

The authors developed a careful protocol for establishing well defined SThK 2-D crystals and used AFM tip chemistry from the literature to attach cAMP and cGMP to AFM tips through flexible linkers. SMFS carried out in their studies appears to be technically sound, although block experiments are required to proof specificity. If specific, injection of nucleotides or SthK should lead to abolishment of binding.

Response 1: We thank for the reviewer for their overall positive assessment of our work.

Regarding the blocking experiments: We of course agree with the reviewer. Indeed, we did perform block experiments by injecting saturating concentrations of either cAMP or cGMP (2mM, Philipp A.M. Schmidpeter et al., J Gen Physiol. 2018, 150, 821-834) to the fluid cell and performed the same type of SMFS experiments. The frequency of unbinding events decreased dramatically in these control experiments, indicating that the analyzed unbinding events were indeed specific. Accordingly, we amended the revised version of our manuscript, lines 120-124, lines 145 – 147, and novel figure panel Figure 2c.

Comment 2: More details of the data must be presented to verify the deep binding state of cAMP. The AFM tip chemistry used does not strictly show only single molecular interactions. Dual, or even more, ruptures can occur, appearing simultaneously or sequentially. At the current stage of data presentation, it cannot be excluded that the high forces of cAMP arise from a double bond rupture, in particular as the binding probability of cAMP is generally higher than that of cGMP and increase of contact time increases the probability of multiple bond formation. To be more convincing, a statistical analysis of multiple bond ruptures in dependence of the contact time (at least for 0.02 and 1 s) both for both cAMP and cGMP must be shown. Another convincing argument is to verify that the higher forces of cAMP do not follow the Williams Markovian model for simultaneous dual bond rupture.

Response 2: We thank the reviewer for their excellent and constructive suggestions. Indeed, we find in our data curves with multiple rupture events. As the reviewer points out, multiple rupture events increased with increasing contact time for both cNs, as expected. In revision, we provide a new Figure S1 reporting about the force-distance curves that revealed multiple rupture bonds.

However, as the reviewer points out, multiple bonds could also rupture simultaneously.

Regarding this, we note that only cAMP revealed a higher force peak, and not cGMP. As we find a similar frequency of occurrence of sequential multiple bonds for both cNs, it seems highly unlikely that cAMP would only produce simultaneous ruptures. Therefore, the higher force unbinding event of cAMP should indeed arise from a different binding mode. Further, if the higher forces of cAMP would arise from simultaneous multiple bond ruptures, the frequency of simultaneous multiple bond rupture events should increase with increasing loading rate (Boris B. Akhremitchev et al., Biophys. J. 2008, 95, 3964–3976). This is not the case (Figure S3b).

Finally, we followed the reviewer's suggestion and applied the Williams Markovian model to test whether the higher forces could be due to simultaneous double bond ruptures or not. We

found that the experimental dynamic force spectrum of cAMP did not agree well with the Williams Markovian model for simultaneous dual bond rupture (Figure S5). All these analyses are in favor of an interpretation that the higher force rupture events indeed arose from a second, deeper binding mode and not from simultaneous multiple bond ruptures. Along with new Figures S1 and S5, we revised our manuscript in lines 291–302, lines 504–508.

Other points:

Comment 3: The authors are not consistent in using the terms pulling velocity and loading rate. In fact, they vary z-velocity and calculate loading rate, for which error bars for fitting and presentation should be included.

Response 3: We thank the reviewer for this suggestion. In the revision, we added the error bars of the loading rate for fitting (Figure 3i).

Comment 4: Another question is, whether the Bell-Evans is model a good model for MDS. In any case, it does not make sense to fit the SMFS and MDS data together with the Bell-Evans model. The loading rates are way too far apart and the fit results of the SMFS and MDS data alone are completely different.

Response 4: We agree with the reviewer that the unbinding rates k_{off} , obtained from the Bell-Evans fit results to the AFM and MDS data individually differ, as reported in Table 1, by 1-2 orders of magnitude. Note, however, that to determine k_{off} values from the fits to the MDS rupture forces alone implies as quite bold extrapolation over 6 orders of magnitude, as also reflected by the large error bars in Table 1, such that we do not think this seemingly large difference is actually a matter of concern. Note also that the obtained rupture lengths, which do not require such extrapolation, agree very well within experimental error. Importantly, our claim that the MD simulations pass the test against the AFM experiment does not at all rest on whether or not a Bell-Evans fit to the combined rupture forces 'makes sense' - but solely rests on the individual measured and/or computed rupture forces in Figure 3i, which show the same trend for both cGMP and cAMP and, hence, reflect the difference the MD simulations seek to explain. The three different Bell-Evans fits for each case are shown in this Figure 3i to guide the eye and enable to better judge this agreement. Finally, looking at Figure 3i, we would tend to say that such a simple fit seems to describe the data surprisingly well for the system at hand, which is why we think, as described in the text, that a fit to the merged data can provide a more accurate estimate for the off-rates. None of our conclusions rest on that notion, though. Interestingly, comparison to our previous study of streptavidin/biotin unbinding (Rico et al, *PNAS*, 2019, 116, 6594-6601) shows that such a log-linear behavior does not always shows up, such that, in contrast to here, a more advanced fit was required (essentially showing that MDS are also capable of producing more complex behavior of more complex unbinding energy landscapes). We added in revision an according remark in the caption of Figure 3.

REVIEWER #2

Remarks to the Author:

In this manuscript, the authors probe the differences in cAMP and cGMP binding to the cyclic-nucleotide-gated ion channels using single-molecule force spectroscopy. Electrophysiological studies show that saturating amounts of cAMP activates about 20 to 40% of SthK channels whereas cGMP activates only 0.01% of the channels. Thus, cGMP is an antagonist or an extremely weak partial agonist. X-ray structures of the isolated SthK CNBD in presence of cGMP and cAMP have show that the the central cavity is cAMP bound structure is more open compared to cGMP bound structure (Kesters et. al. PLOS One 10, e0116369, 2015). Using ligands tethered to AFM tip, the authors conduct force spectroscopy studies to probe mainly the cGMP and cAMP unbinding reaction on isolated SthK CNBD. They find that the cAMP unbinds from a deep pocket which is not accessible to cGMP. They suggest that the cAMP binding leads to conformational change which does not occur in the presence cGMP. Their experimental finding was further supported by the molecular dynamics simulation study, which not only enables access to force loading regime beyond the experimental range but also provides a structural view of the unbinding reaction.

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Response: We thank the reviewer for their positive general reception of our work. We have amended our manuscript according to reviewer's recommendations and hope to address all the reviewer's issues satisfactorily.

Key concerns:

Comment 1: cAMP and cGMP can exist in both syn and anti configuration but the activated bound state prefers one or the other configuration. The distribution between the two states can be altered by substitutions in the purine ring. Given that the position of the attachment site for the tether is different for cGMP and cAMP, how can one rule out the observed differences in binding modes are not due to the differences in the attachment site.

Response: In the X-ray structures SthK CNBDs bind both cAMP and cGMP in anti-configuration (Kesters, et al., PLOS One, 2015, 10, e0116369). The anti-configuration is also the more extended form of the cN, and since the linker is in both cases attached to the amine group of the purine rings, and the phosphate of cAMP and cGMP are deep inside the binding pocket, the attachment should not significantly influence the conformer, nor should pulling on the purine ring bias it towards the syn-configuration. In the MD simulations we have taken care to make sure the experimental linker attachment is also accurately reflected. Triggered by the reviewer's

comment, we have now analyzed our simulations in detail with respect to syn-/anti-conformations. Indeed, during the pulling simulations, up to 0.5 nm center of mass distance (which is where the h-bonds most markedly distinguish between cAMP and cGMP), no syn conformation is seen; for larger distances, further down the unbinding path, syn conformations appear in only 12% of all frames. Of course, we cannot rule out that the linker attachment somehow contributes to the observed difference in unbinding forces. However, cAMP and cGMP show readily different binding energetics (h-bond strengths) within the binding pocket, where the attachment mode should play a minor role. We have changed the text in the discussion such as to make this point clearer and now also mention that the attachment modes may also play a role (lines 349-357).

Comment 2: The other question here is whether surface immobilization of the CNBD domain alters the affinity to the cAMP and cGMP. The authors estimate the affinity for cAMP is two fold more than for cGMP. How does this compare with binding affinity measurements in freely diffusing CNBD domains?

Response 2: The reviewer is concerned about the effect of the CNBD immobilization on the binding affinity. In our work, the immobilization of the CNBDs is actually very soft, *i.e.* we use a His6-tag to immobilize a His6-C-linker-CNBD construct. This His6-tag should readily provide some degree of flexibility, and the presence of the C-linker further distances the CNBD from the binding-site. In addition, we used a ‘soft surface’, *i.e.* a lipid bilayer to mimic the native environment and orientation of the C-linker-CNBD (see Figure 1c). Thus, overall, we think that our immobilization method is quasi ideal and might represent a more native situation for a ion channel CNBD as compared to floating in bulk. Furthermore, we address here a comparison between cAMP and cGMP binding, and the immobilization conditions are consistent throughout our entire experiments with both cNs. Overall, we conclude that our method is beneficial for investigating and comparing the kinetics of cAMP-CNBD and cGMP-CNBD. Anyway, for comparison, we measured the binding kinetics between the CNBD and cNs in bulk by performing microscale thermophoresis (MST) experiments. Two binding curves were detected for cAMP-CNBD measurements. The first curve had a K_D value of $0.4 \pm 0.5 \mu\text{M}$, and the second curve had a K_D value of $1.6 \pm 1.1 \mu\text{M}$ (new Figure S2a,b). The K_D value of cGMP-CNBD is $3.3 \pm 1.8 \mu\text{M}$ (new Figure S2c,d). These results are in line with the affinity of the full length channel has been determined as $0.6 \mu\text{M}$ for cAMP and $2.7 \mu\text{M}$ for cGMP (Schmidpeter *et al.* JGP 2018, 150, 821-834.). However, these measurements were performed in bulk. Thus, while the tendency is the same as in our experiments, it is difficult to quantitatively compare these results (lines 201-208).

Comment 3: Is the binding of tethered ligand cGMP and cAMP specific? For instance, it is possible that some of the binding may be non-specific attachment. Can they show that the binding can be competed out in presence of ligands in solution?

Response 3: We thank the reviewer for pointing out the missing report of this standard control. The correctly oriented membrane-binding of the CNBDs in our experimental setup should

significantly reduce unspecific interactions between the ligand and protein and/or surface. In addition, we now present control experiments proving the specificity of binding by injecting saturating concentrations of cAMP or cGMP (2mM, Philipp A.M. Schmidpeter et al., J Gen Physiol. 2018, 150, 821-834) into the AFM fluid cell during AFM-SMFS experiments. We found that the frequency of unbinding events decreased by ~85% in these control experiments, new figure panel Figure 2c. We describe these controls in the revised version of our manuscript in lines 120 – 124.

Comment 4: In calculating the binding probability, the authors only considered the CNBD population density, but the density of the ligands on the AFM tip can also contribute directly to determining the bound probability. How do the authors make sure that the ligand density is the same when they switch from cAMP tip to cGMP tip?

Response 4: We thank the reviewer for this constructive comment. We agree with the reviewer that the density of the ligands on the AFM tip is important, it is integrated as one of the fit parameters in *equation 3*. In our previous fitting, $m_l A_c$ was set to 1 based on the SMFS measurements at 20 ms contact time that did not reveal dual-unbinding force curves. Upon further reflection (and in response to a comment by reviewer 1), we found that we could make a more precise estimate of the ligand exposure at the tip based on a statistical analysis of multiple bond ruptures as function of the contact time for both, cAMP and cGMP, see new supplementary Figure S1. While we mainly detected single rupture events, upon prolonged tip exposure, we found ~15% double bond rupture events (triple bond rupture events were negligible with relative frequencies <1%). Thus, we set $m_l A_c$ to 2 and fitted all our data again in an amended Table 1. However, such differences only account for changes in the affinity by a factor <2, and certainly are unrelated to the ~1000-fold difference in the effect of cAMP and cGMP on channel gating. On the other hand, the main finding of our work is that cAMP can enter the deep bound state that is inaccessible to cGMP.

Comment 5: Figure 5b shows a relatively broad distribution of the unbinding force. According to the authors, this indicates cAMP binds to the CNBDs in two different states, one in a loosely bound state and the other in a tightly bound state. How do the authors rule out the unbinding of multi valence state, where multiple ligands bind to different CNBDs within the tetramer? To what extent the ligand population on the AFM tip was controlled to rule out such possibility?

Response 5: We apologize that we didn't discuss this point thoroughly in the original submission. In our data analysis, we only focused on the single unbinding events. This allowed us to rule out possible sequential unbinding events of multi bond ruptures. However, there is the possibility that multiple bonds rupture happened exactly simultaneously, as the reviewer suggests. We analyzed the occurrence of multiple rupture events as a function of ligand exposure time and found that the multiple rupture events increased with increasing contact time for both cNs (new Figure S1). However, we only found higher force peaks for cAMP, and not for cGMP, though both cNs revealed the same percentage of multiple bindings upon extended exposure. Second, if the higher forces of cAMP would arise from double bond rupture, the frequency of

simultaneous multiple bond rupture should increase with increasing loading rate (Boris B. Akhremitchev et al., Biophysical Journal 2008, 95, 3964–3976). However, this is not the case in our data (Figure S3b). Finally, third, we applied a Markovian model (*eq. 5*), to test whether the higher forces analyzed could originate from double bond ruptures. We found that the measured dynamic force spectra of cAMP did not agree with the Williams Markovian model for simultaneous dual bond rupture (new Figure S5). Considering all these data analyses, we interpret the higher forces to arise from a second, deeper binding mode. Accordingly, we revised our manuscript in lines 291–298, lines 504–508 (along with new figures S1 and S5).

The rupture of multi bonds was tested using the Markovian model:

$$r = k_{off} \frac{k_B T}{x_\beta} \left[\sum_{n=1}^N \frac{1}{n^2} \exp \left(-\frac{F^* x_\beta}{n k_B T} \right) \right]^{-1} \quad (eq. 5)$$

where r is the loading rate, k_{off} and x_β are the parameters derived from the low force Bell-Evans model fit (Figure 5b, gray line), N is the number of bonds, and F^* is the most probable unbinding force.

Comment 6: In Figure 3b and 3f, the rupture force histograms for cGMP and cAMP appear similar within margin of error. This is further reflected in Figure 3i where the experimental cGMP and cAMP are not very different. The difference is between 5-10 pN. Considering the force constant of the cantilever is 100 pN/nm, how can the authors claim that this small change is significant?

Response 6: Of course, the essential part of the force histograms (Figure 3b and f) lies in the repetition of the unbinding experiment over thousands of force-distance cycles, which should result in significant ensemble distributions. Next, the Bell-Evans model is used to fit all the distributions, thus integrating the tendency of all data. Finally, we agree with the reviewer, and it is one of the key findings, that the overall fitting of the experimental data for cAMP and cGMP revealed very similar kinetics, with a k_{off} of cAMP-CNBD that is only two-fold lower than that of cGMP-CNBD. However, electrophysiology studies showed that cAMP evoked a ~3 orders of magnitude increased activation of SthK as compared to cGMP. Thus, at the end of this paragraph, we put a sentence: ‘But can these rather minor differences alone explain the different action of the two cNs on channel function?’ (lines 172-173). And indeed, we do not think so. We agree that these unbinding characteristics are very similar. This is why we extended our experiments to the force distance cycle with prolonged ligand exposure time (all the data in Figure 3b,f,i represents unbinding forces after 20ms bond formation time). Only in the latter case of extended ligand exposure, we started to see real differences in the behavior of the two cNs.

Comment 7: In this study, the authors used NTA-Ni2+-His6 link to immobilize the CNBDs on the lipid bilayer. The unbinding force related to Tris NTA- Ni2+-His6 is about 60-80 pN (Koehler, Melanie, et al. Nano letters 20.5 (2020): 4038-4042) considering all the histidine is bound, the unbinding force is still relatively lower than typical tethering systems used in a force spectroscopy experiment i.e. biotin-streptavidin. Is it possible that the NTA-Ni2+-His6 bond

breaks before the cAMP-CNBD linkage especially when the polyvalency is not established i.e. all six imidazoles are not bound to NTA?

Response 7: We regret that this part was not well explained. The C-linker - CNBD domain is a stable tetramer (see Figure 1), and each C-linker - CNBD domain has thus 4 His6. The unit cell dimension of the C-linker - CNBD tetramer is, $a = b = 11 \text{ nm}$, $\gamma = 90^\circ$ (thus an area of 121 nm^2) (Figure 1g). The area occupied by one lipid molecule in a supported lipid bilayer is $\sim 0.25 \text{ nm}^2$ (Takeshi Fukuma et al., ACS nano 2012,6, 9013–9020). This suggest that there are ~ 480 lipid molecules (in each leaflet) under each C-linker – CNBD tetramer. In this work, the membrane contains 20% DGS-NTA- Ni^{2+} , *i.e.* there are ~ 100 DGS-NTA- Ni^{2+} lipids under each C-linker – CNBD tetramer, which provides largely sufficient Ni^{2+} to allow all the His of all tags to form NTA- Ni^{2+} bonds. Considering the unbinding force of NTA- Ni^{2+} -His6 is 60-80 pN, 4 NTA- Ni^{2+} -His6 bonds should immobilize the CNBD domain sufficiently for our measurements (in addition, effects of avidity will emerge from the multiple interactions). We discuss this part more precisely in the revised version of our manuscript lines 455 – 464.

Comment 8: Cyclic nucleotide binding to SthK channels has been investigated by multiple labs. Cryo-EM studies of the full length channels show that the cAMP and cGMP bound SthK structures are identical but X-ray structures of the isolated SthK CNBDs in presence of cAMP and cGMP shows clear differences between the two agonists. The authors dismissed this data by stating that (line 53, Pg. 2) the two structures are “quasi-identical” which is not correct. Please discuss your data in light of these published facts.

Response 8: We thank the reviewer for the suggestion and apologize for the confusing statement. Indeed, to the best of our understanding, in the X-ray structures the C-helices and for that the CNBDs are very similar (Figure R1), and the major conformational changes occurred in the C-linker. The RMSD of the isolated SthK CNBDs between cAMP-bound (4D7T) and cGMP-bound (4D7S) conformations is 0.979 \AA (with the main contribution to this RMSD from the C-linker). Accordingly, we amended the text: “The X-ray structures of the cAMP- and cGMP-bound CNBD-C-linker protomers revealed that the CNBDs were very similar, while conformational changes in the C-linker helices occurred. These changes related to the binding of cAMP and cGMP resulted in clear differences in the tetramer assembly, where the cAMP-bound CNBD-C-linker tetramer was in an activated conformation while the cGMP-bound CNBD-C-linker tetramer was in a resting conformation¹³”, lines 52-57.

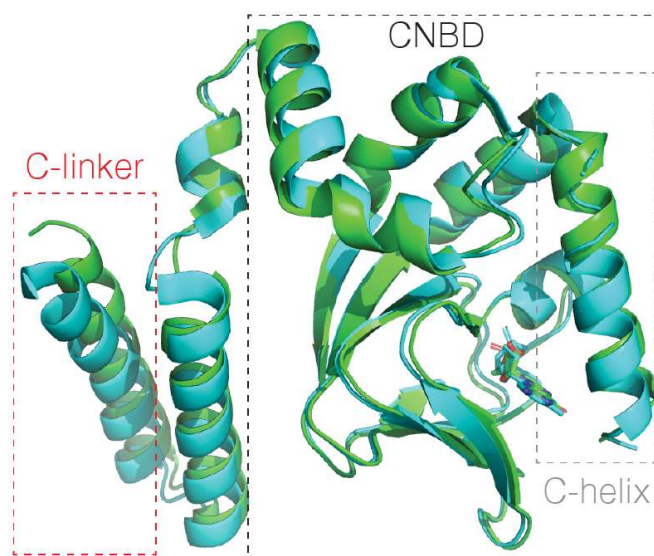


Figure R1 |. Comparison of isolated SthK CNBDs: cAMP-bound (4D7T, cyan) and cGMP-bound (4D7S, green).

Additional comments:

Comment 1: The resolution of Figure 1b & 1c could be improved.

Response 1: We thank for the reviewer's suggestions. The resolution of Figure 1b & 1c was improved. It is possible that the PDF conversion played bad tricks on our figures...

Comment 2: Figure 1d should include the dashed line along which the cross section 1 is plotted.

Response 2: We thank the reviewer for pointing this. We amended this.

Comment 3: In the Figure 2b the contact time should be labeled from the beginning of 2 (red line) until the end of 4 (blue line). Black line 3 is shorter than the actual contact time.

Response 3: We thank the reviewer for pointing this. We amended it.

Comment 4: The authors used the popular Bell-Evans model to fit the dynamic force spectroscopy data. Bell-Evans fitting gives the ability to calculate off rate constant (k_{off}), distance to the transition state ($x\beta$) as well as TS barrier height (ΔG). The authors may want to report the ΔG for both unbinding experiments (cAMP vs cGMP) and a comparison of the ΔG values can be given for a better understanding to readers.

Response 4: We thank the reviewer for this suggestion. The dynamic force spectra for cAMP-CNBD and cGMP-CNBD unbinding were fitted using the Bell-Evans model (*eq. 1*):

$$F = \left(\frac{k_B T}{x_\beta} \right) \ln \frac{r x_\beta}{k_{\text{off}} k_B T} \quad (\text{eq. 1})$$

where F is the rupture force, r is the loading rate, k_B is the Boltzmann constant, T is the absolute temperature, k_{off} is the dissociation constant at zero force, and x_β is the distance to the unbinding barrier from the bound-state free energy minimum. We can calculate the ΔG using an extended version of the Bell-Evans model, by fitting our data using the DHS model (O. K. Dudko, et al., Phys. Rev. Lett. 2006, 96, 108101)

$$F \cong \frac{\Delta G}{v x_\beta} \left[1 - \left(\frac{k_B T}{\Delta G} \ln \frac{k_{\text{off}} k_B T e^{\frac{\Delta G}{k_B T} + 0.577}}{x_\beta r} \right)^v \right]$$

ΔG , k_{off} , x_β can be derived from the fitting (cusp potential). We incorporated this part in the revised version of our manuscript lines 381 – 385, and new panel Figure 6b and Table S1.

Method (DHS fit)	cN	$k_{\text{off}}(1/\text{s})$	x_β (nm)	ΔG (k _B T)
20ms bond formation time	cAMP	1.2 ± 0.4	0.48 ± 0.08	10.4 ± 1.7
20ms bond formation time	cGMP	1.6 ± 0.3	0.69 ± 0.05	9.2 ± 1.3
1000ms bond formation time: State 1 bond	cAMP	1.1 ± 0.4	0.57 ± 0.07	9.7 ± 1.5
1000ms bond formation time: State 2 bond	cAMP	0.5 ± 0.2	0.48 ± 0.08	10.9 ± 2.0

Table S1) cN-CNBD binding kinetics fitted by using DHS model.

Comment 5: Figure 3e should also include an inset showing the zoomed-in region of the unbinding force curve.

Response 5: We added an inset in Figure 3e.

Decision Letter, first revision:

Message: Our ref: NSMB-A46642A

19th Jan 2023

Dear Professor Scheuring,

Thank you again for submitting your manuscript "Discrimination between cyclic-nucleotides in a cyclic-nucleotide gated ion channel". The reports of the referees are below, and based on these comments, we are happy to accept your paper, in principle, for publication as an Article in Nature Structural & Molecular Biology, on the condition that you revise your manuscript in response to the comments of the referees and our editorial requirements.

You will see that reviewer #1 requests clarification of pulling velocity and loading rate.

The text and figures require revisions. Note that, within a few days, we will send you detailed instructions for the final revision, along with information on editorial and formatting requirements. We recommend that you do not start revising the manuscript until you receive this additional information.

To facilitate our work at this stage, we would appreciate if you could send us the main text as a word file. Please make sure to copy the NSMB account (cc'ed above).

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In recognition of the time and expertise our reviewers provide to Nature Structural & Molecular Biology's editorial process, we would like to formally acknowledge their contribution to the external peer review of your manuscript entitled "Discrimination between cyclic-nucleotides in a cyclic-nucleotide gated ion channel". For those reviewers who give their assent, we will be publishing their names alongside the published article.

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Sincerely,
Katarzyna Ciazynska
(she/her)
Associate Editor
Nature Structural & Molecular Biology
<https://orcid.org/0000-0002-9899-2428>

Reviewer #1 (Remarks to the Author):

The authors have significantly improved their manuscript and convincingly supported the notion of the deep binding state. Not fully cleaned up, however, is the confusion between pulling velocity and loading rate. Pulling speed is $\mu\text{m/s}$, loading rate is pN/s . What is experimentally varied is pulling speed, not loading rate.

Reviewer #2 (Remarks to the Author):

I am satisfied with the revisions. I have no additional suggestions.

Author Rebuttal, first revision:

Reviewers' Comments:

REVIEWER #1

Remarks to the Author:

The authors have significantly improved their manuscript and convincingly supported the notion of the deep binding state. Not fully cleaned up, however, is the confusion between pulling velocity and loading rate. Pulling speed is $\mu\text{m/s}$, loading rate is pN/s . What is experimentally varied is pulling speed, not loading rate

We thank the reviewer for their initial comments and constructive suggestions that helped us improve our work.

The reviewer is right that the only parameter that is varied in experiments is the pulling speed. The loading rates is extracted from the slope of the adhesive peak before rupture. Of course, the reviewer and we know that increasing the pulling speed results through the stochasticity of the unbinding process, is a means to increase the loading rate. We regret that we missed to clean the text and clarify this entirely during the revision process and have now done so. (Lines: 114-117, 261, 271, 382, 385)

Final Decision Letter:

Message 24th Feb 2023

:

Dear Dr. Scheuring,

We are now happy to accept your revised paper "Discrimination between cyclic-nucleotides in a cyclic-nucleotide gated ion channel" for publication as a Article in Nature Structural & Molecular Biology.

Acceptance is conditional on the manuscript's not being published elsewhere and on there being no announcement of this work to the newspapers, magazines, radio or television until the publication date in Nature Structural & Molecular Biology.

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