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The CatSper channel controls chemosensation in sea urchin sperm

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

Editor: Anne Nielsen

1st Editorial Decision 22 July 2014

Thank you for submitting your manuscript for consideration by The EMBO Journal. It has now been seen by three referees whose comments are shown below.

As you will see from the reports, all referees express interest in the findings reported in your manuscript; however, at the same time they do raise a number of major and minor concerns that will have to be addressed before they can support publication of the manuscript.

You will notice that ref#1 in particular finds that further insight on the mechanism of sequential CatSper activation will have to be provided and that the relevance for the mammalian system should be established. This is partly mirrored in concerns raised by the other referees, especially ref#3 who finds that further experimental data is needed to substantiate the conclusiveness of using Ca2+ influx in sea urchin sperm as a surrogate for specific CatSper activity.

With regard to the question of broadening your findings to the mammalian system, we realize that this may not be a simple question given the broader functional differences between the two systems. However, since the techniques involved here are established in your lab it would greatly strengthen the current manuscript if you could also provide data on mammalian sperm. This would potentially also help address the request from ref #2 to discuss the role for membrane hyperpolarization in the context of internal fertilization.

To summarize, I would ask you to address the following aspects in a revised manuscript:
-> please address all points raised by ref#3 and provide some additional mechanistic insight on channel activation as outlined by ref#1
-> please address/discuss the minor points raised by ref #2
-> please address the minor points raised by ref #1 and incorporate some data for the generality of CatSper activation by hyperpolarization to the mammalian system (if possible within a reasonable time frame)

Given the rather extensive nature of these experiments, we would be happy to extend the deadline for the revised manuscript to offer you more time to expand and develop this study. I would also encourage you to contact me directly if you have any questions to the concerns raised by the referees or the experimental data required for the revised manuscript.

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

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

REFEREE COMMENTS

Referee #1:

This manuscript reports on the molecular and physiological identification of CatSper as the Ca2+ channel that controls the response to a chemotactants in sea urchin sperm. The authors then described the activation of the channel by pHi and the membrane potential and the relationship between the two modalities of channel activation.

General comments:

In principle the results can be of interest in further clarifying the mechanism of sperm activation. Unfortunately the studies fail short in two aspects and as such remain someone descriptive and thus at their present form are largely incremental. First, they stop short of providing a mechanism by which membrane hyperpolarization activates the Na+/H+ exchanger. This is an essential component of the model and once understood molecularly can be used to further test sperm activation. Second, the authors did not extend their findings to mammalian sperm to any extent. Mammalian sperm pH and Na+/H+ exchanger activity have been measured and thus similar technique should be used to extent the current finding to mammalian sperm to extent the significance of the studies if the manuscript should be considered further.

Additional comments:

1. Figure 1: the CatSper is depicted as a heteromultimer. This should be tested experimentally by Co-IP using the tagged proteins and, more important, with the native CatSper with antibodies against subunits for which antibodies are available.

2. a) Are the responses to depolarization by all K+ concentrations similarly inhibited by the CatSper inhibitors or do high K+ concentrations activate other channels? The sensitivity of the Ca2+ signals to the CatSper inhibitors at high and low K+ concentrations need to be compared.

b) how high K+ concentrations affected the pH clamp? If high K+ affect H+ permeability, it will change resting and clamped pH to change the ration shown in several figures between pH and Ca2+ and pH and the membrane potential.

3. Fig. 7 is referred to as Fig. 6d-f which is Fig. 7 in the manuscript and the results on sperm motility in the intended Fig. 7 are not included.

4. The imidazole conditions are far from being specific and there is no way to know if the effect is exclusively due to buffering of pHi. The non-specificity is reflected in inhibition of the change in membrane potential. The authors should use the physiological buffer HCO3- to ameliorate changes in pH without poisoning the sperm. The imidazole data are simply uninterruptable.

Referee #2:

This is a comprehensive analysis from a group that has made fundamental observations in the arena of chemotaxis. Additionally, in human cells they have identified the progesterone response via CatSper, observation of CatSper as a polymodal channel and regulation by a series of environmental chemicals.
In this study the focus is on sea urchin sperm specifically as related to the chemotactic response. This manuscript identifies the chronology and regulation of this response in particular the interplay between pHi, membrane potential and CatSper activation.
The authors initially identified the CatSper genes, localisation on sperm and used proteomics to identify the proteins in the flagellum (CatSper β and γ are missing). Following identification of CatSper and associated subunits, using techniques largely published before (e.g. stopped flow apparatus) the authors examine the regulation of CatSper. Initial experiments with inhibitors identified the calcium influx via CatSper with subsequent experiments defining the pHi sensitivity concluding that pHi was a sensitive switch for CatSper. Depolarisation invoked a calcium influx via CatSper (initially shown using inhibitors) and experiments using pHi clamp solutions show intimate inter relationship between pHi and membrane potential for activation of CatSper. The next steps were to examined the sensitivity of CatSper to pHi and membrane potential following activation via cGMP and resact (activators of chemotaxis). A strict chronology of events was identified with a change in membrane potential, pHi and then calcium influx. In chemotaxis experiments inhibition of CatSer (by mibefradil) inhibited chemotaxis.
The work represents a significant step forward in our understanding of the regulation of chemotaxis and identifies the key role (and modulation) of CatSper.
Whilst the analysis is comprehensive and detailed there are some minor points that require clarification/addressing:

Minor comments:
Explanation of the difference in the dose-effect plots of MDL against Ca signals from Resact and from cGMP (fig 5C and 5D).
The authors should modify the Introduction/discussion as it refers to internal fertilisation at least to human work. There is reference to the oviduct. It would be better to use female reproductive tract as modulation of sperm when in cervical mucus, uterus etc. Additionally, prostaglandins will be in semen in humans etc. so not restricted to oviduct.
Comment on how the putative potential membrane hyperpolarisation with capacitation in mice fits with the internal fertilisation model? How does capacitation fit with Fig 8 (for humans?)
Potential error in the figs. Fig 6F and 6G as listed in fig legends are presented as Fig 7 and fig 7 as described in text and legends isn't there
Supplementary Fig 8 resolution relatively poor.

Referee #3:

General Comments: Sea urchin sperm have provided a major model system for investigation of sperm chemosensation. A major component of this process involves the ability of such sperm in open marine environments to swim to specific target and this is initiated by the ability of sperm to

respond to the presence of a small peptide termed "resact" which is released by eggs. The chemical sensing of this peptide initiates changes in sea urchin sperm membrane conductances and eventual an elevation of cytosolic Ca2+ that are critical for changes in motility that results in bulk movement towards the egg. This paper presents convincing evidence that, similar to mammalian sperm, activation of a Ca2+-permeant ion channel termed CatSper is the molecular entity that allows influx of Ca2+, as a consequence of the interplay of cytosolic alkalization and membrane depolarization. Furthermore, the paper provides valuable information regarding the timing and sequence of steps involving changes in cytosolic alkalization, membrane potential, and elevation in cytosolic Ca2+. Overall this is a solid and important contribution.
In general, the paper is clearly written and the results largely support the conclusions that are drawn. One small concern is that not infrequently the manuscript uses section headings and statements, which although likely to be true, cannot be considered to be definitely established by the results in that section. This could potentially be addressed with simple rewording. This arises in part because the authors are using changes in cytosolic Ca2+ as the surrogate of CatSper activity. Although this is likely to be, in general, appropriate in this case, changes in cytosolic Ca2+ can arise from a number of other factors. In the absence of direct recordings of ionic currents that show the hallmarks of CatSper (divalent cation selectivity, and strong monovalent ionic flux in the absence of Ca2+), most of the results that the authors use to argue for the key role of CatSper are actually indirect. Although there is no reason to doubt the conclusions of the authors, this reader would prefer that the paper were written with more circumspection regarding what the safe conclusion may be from any individual experiment. Although the work clearly establishes that cDNA for CatSper subunits are found in testis and that CatSper protein is present in sperm, that the Ca2+ elevations arise exclusively from CatSper are only supported indirectly by inhibition with certainly dirty pharmacological agents. With the rapid mixing experiments employed by the authors, would it be possible to at least confirm that the alkalization-activated Ca2+ elevations depend on the presence of extracellular Ca2+ in the mixing solution? Perhaps that is already known. But if one mixes a 30 mM NH4Cl solution that includes a Ca2+ buffer that would drop extracellular Ca2+ to below 100 μM, is there a Ca2+ signal? Is the pH signal unaffected in such circumstances? Given the rapidity of the mixing, I would presume that this could be done without any acute effects on the preexisting cytosolic Ca2+.
As the authors are no doubt aware, the submitted version was missing Figure 7 as described in the text, while the submitted Figure 6 legend (A-G) described both Fig 6 (A-D) and 7(A-C, which were really E-G of the legend.
Specific Comments:
1. Although the paper is clearly written, there are some cases where English editing would help.
p. 4. "since 100 years" would be better as: "for 100 years".

2. In Fig. 1D, the staining was not entirely clear in the copy I have. Was there any staining in the sperm head?
3. p. 7. The heading "CatSper in sea urchin sperm is controlled by pHi and membrane voltage" is an example of a statement which somewhat goes beyond what the results actually say. What is shown is that "Ca2+ elevations in sea urchin sperm ...". Then the question is, are those elevations due to influx through CatSper. That is never actually tested, although the two dirty inhibitors do block Ca2+ elevations. Given previous published work with a slow time course of inhibition by mibefradil, I am a bit surprised that the inhibition by mibefradil of the Ca2+ elevations can be considered to be at steady-state.

4. p. 8. Putting a number (2.5 s) on the time course of inhibition for these compounds seems unnecessary and probably not of much real physical meaning.
5. I gather that ΔR/R refers to the change in ratio of BCECF fluorescence, as opposed to the ΔF/F. This is somewhat explained in the methods, but perhaps some additional clarification could be provided to readers to explain the convention.
6. Figure 3 and text. Panel D. I believe the authors may have information that would relate the concentration-dependence of the NH4Cl signals to pH. It might be of interest and value to be able to know the pH ranges of the NH4Cl effects.

7. Figure 3. Panel E. Would it be possible to elaborate in the figure legend about how the traces in panel E are generated?

8. p. 9. "The -26.3 mV" membrane potential with 190 mM K+ required some thought. Perhaps the readers could be reminded that cytosolic K+ in sea urchin sperm is likely to be around 500 mM (or whatever it is), given the 1000 mOs of the cytosol.
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p. 32. Figure 1 Legend. "Structural features of ". I do not consider topological maps of expected transmembrane segments as "Structural features". This also applies to Supplementary Figure 1, where the legend mentions "Primary Structure". Amino acid sequence is not structure.

p. 33. The title to the legend may be a little strong unless there is reason to think that MDL 12330A is an absolutely selective inhibitor of CatSper.

Figure 5, when compared to blocker effects on Ca2+ in other figures, might be taken to suggest that the blockers may have some effects unrelated to actions exclusively on CatSper, given the various in EC50s and slopes of inhibitory effects.

Figure 8. The sea urchin model as presented implies a very direct link between elevation of cytosolic Ca2+ and chemotaxis. Isn't it really the whole cascade of events that leads to a change in flagellar beating that is chemotaxis? I would think the arrow leading away from [Ca2+] would go to something having to do with a change in motility. Chemotaxis is an emergent property of the whole system that requires repeated activation of this sequence depending on the resact gradients.

1st Revision - authors' response 13 October 2014

General response to all referees.

We thank all referees for their valuable and constructive criticism. We revised the manuscript accordingly. The changes are detailed in the responses to the respective referee.

Response to referee #1:

In principle the results can be of interest in further clarifying the mechanism of sperm activation. Unfortunately the studies fail short in two aspects and as such remain someone descriptive and thus at their present form are largely incremental.

We would like to take issue with this general comment. For the first time, we (1) demonstrate the functional expression of CatSper in sperm of a non-mammalian species; (2) show that CatSper controls sperm chemotaxis; (3) characterize in intact sperm, in quantitative terms, the activation mechanism of CatSper by a cooperative interplay of pHi and Vm. These findings provide important

insights into the physiology of CatSper in general and, in particular, into its role during chemosensation.

First, they stop short of providing a mechanism by which membrane hyperpolarization activates the Na+/H+ exchanger. This is an essential component of the model and once understood molecularly can be used to further test sperm activation.

It has been known for 30 years that the Na+/H+ exchanger is activated by hyperpolarization, which produces an alkalization (*Lee, J. Biol. Chem. 1984a, Lee, J. Biol. Chem. 1984b, Lee, J. Biol. Chem. 1985, Lee & Garbers, J. Biol. Chem. 1986*). Moreover, the sperm-specific Na+/H+ exchanger (termed sNHE) carries a classical S1-S4 voltage-sensor domain (*Wang et al., Nat. Cell Biol. 2003, Nomura & Vacquier, Cell Motil. Cytoskeleton 2006*), which is homologous to that of voltage-gated ion channels; it has been proposed to control the exchanger activity. The focus of this manuscript is on CatSper. Certainly, this interesting sNHE deserves to be studied on its own. However, this goes beyond the scope of this manuscript. We reworked the introduction and the discussion to highlight the function of sNHE more clearly.

Second, the authors did not extend their findings to mammalian sperm to any extent. Mammalian sperm pH and Na+/H+ exchanger activity have been measured and thus similar technique should be used to extent the current finding to mammalian sperm to extent the significance of the studies if the manuscript should be considered further

Unfortunately, Na+/H+ exchange has not been studied in mammalian sperm in detail, and the mechanism(s) controlling pHi of mammalian sperm are unclear. Moreover, the voltage control of the mammalian sNHE has not been elucidated; not least, because the exchanger - similar to CatSper - is not functional in heterologous systems. Altogether, in contrast to sea urchin sperm, the physiology of this exchanger in mammalian sperm has been a long-standing mystery. The role and voltage control of Na+/H+ exchange and pHi homeostasis and its significance to control CatSper in mammalian sperm deserves to be studied on its own. However, such studies require years of experimentation and go far beyond the scope of the present manuscript. A recent paper by the group of Cecilia Santi (*Chavez et al. 2014, JBC*) suggests that a hyperpolarization-induced alkalization, perhaps mediated by sNHE, is involved in activation of CatSper in mouse sperm. In an attempt to address the referee´s concerns, we now discuss the generality of our findings and the knowledge about mammalian sperm in more detail.

1. Figure 1: the CatSper is depicted as a heteromultimer. This should be tested experimentally by Co-IP using the tagged proteins and, more important, with the native CatSper with antibodies against subunits for which antibodies are available.

We thank the reviewer for this nice suggestion. Of note, analysis of the *A. punctulata* testis transcriptome identified mRNAs encoding the accessory subunits CatSper β and CatSper γ (now shown in Figure 1 and Supplementary Figure S1), both of which were not identified in the analysis of the genome. In purified flagella, we identified proteotypic peptides of the predicted accessory subunits (now shown in Supplementary Table S1), indicating that *Ap*CatSper β and γ are expressed in sperm and are located in the flagellum.

We performed the requested immunoprecipitations, using both the anti-*Ap*CatSper 2 and anti-*Ap*CatSper 3 antibodies. Analysis of the co-immunoprecipitates by Western blotting and protein mass spectrometry indicates that *Ap*CatSper 1-4, δ, β, and γ interact to form a protein complex. The results are shown in Figure 1E and F, and in the Supplementary Table 2.

2. a) Are the responses to depolarization by all K+ concentrations similarly inhibited by the CatSper inhibitors or do high K+ concentrations activate other channels? The sensitivity of the Ca2+ signals to the CatSper inhibitors at high and low K+ concentrations need to be compared.

We thank the reviewer for this valuable suggestion. We now show in Figure 4B-D and in Supplementary Figure S2 that Ca2+ responses evoked by 80 and 160 mM K+ are suppressed by the drugs.

b) how high K+ concentrations affected the pH clamp? If high K+ affect H+ permeability, it will

change resting and clamped pH to change the ration shown in several figures between pH and Ca2+ and pH and the membrane potential.

High extracellular K+ does not affect the pHi clamp, which is independent of the ionic composition of the medium. We performed only a single set of pHi-clamp experiment in the presence of elevated extracellular K+ (190 mM) to determine the pHthr for CatSper activation at depolarized Vrest (Figure 3E, F, Supplementary Figure 3C, D). Furthermore, we show that pHrest was more acidic at depolarized Vrest (Figure 3F). All other experiments were performed at "normal" extracellular K+ concentration (9 mM). We hope that this explanation answers the referee´s concern.

3. Fig. 7 is referred to as Fig. 6d-f which is Fig. 7 in the manuscript and the results on sperm motility in the intended Fig. 7 are not included.

We apologize and corrected for this mistake. In addition, we now include two supplementary movies that also illustrate that the CatSper inhibitors abolish sperm accumulation in a resact gradient.

4. The imidazole conditions are far from being specific and there is no way to know if the effect is exclusively due to buffering of pHi. The non-specificity is reflected in inhibition of the change in membrane potential. The authors should use the physiological buffer HCO3- to ameliorate changes in pH without poisoning the sperm. The imidazole data are simply uninterruptable.

We are aware of the pitfalls of imidazole – a commonly used membrane-permeant pH buffer. Therefore, we carefully performed the required controls (shown in Supplementary Figure 8), demonstrating that imidazole is not suited to study signaling in sea urchin sperm. We clearly state this in the text. Nevertheless, in order to demonstrate the fallacy of this tool, we included the data in the manuscript.
We thank the reviewer for his suggestion to use HCO3- instead of imidazole. Unfortunately, the pHi responses were similar in the absence and presence of 10-30 mM HCO3-, demonstrating that HCO3- does not buffer the pHi response. The data are now shown in Supplementary Figure 8E.

Response to referee #2:

Explanation of the difference in the dose-effect plots of MDL against Ca signals from Resact and from cGMP (fig 5C and 5D).

We now include a paragraph explaining potential reasons for these differences. However, we caution against rash interpretations, because the blocking mechanism(s) of MDL and mibefradil is unknown (e.g., closed channel versus open channel block). Depending on the mechanism, the potency depends on the stimulus strength. The stimulus strength during extracellular and intracellular uncaging of resact and cGMP, respectively, is difficult to compare. However, cGMP evoked much larger signals than resact, suggesting that the cGMP stimulus strength was larger. Thus, the observation that mibefradil, but not MDL, inhibited the resact and cGMP responses with different potencies might indicate different blocking mechanisms.

The authors should modify the Introduction/discussion as it refers to internal fertilisation at least to human work. There is reference to the oviduct. It would be better to use female reproductive tract as modulation of sperm when in cervical mucus, uterus etc. Additionally, prostaglandins will be in semen in humans etc. so not restricted to oviduct.

We agree and amended the introduction accordingly.

Comment on how the putative potential membrane hyperpolarisation with capacitation in mice fits with the internal fertilisation model? How does capacitation fit with Fig 8 (for humans?)

We thank the referee for this suggestion. In fact, a recent paper on mouse sperm by the group of Cecilia Santi (*Chavez et al. 2014, JBC*) suggests that a hyperpolarization-induced alkalization during capacitation, perhaps mediated by sNHE, is involved in activation of CatSper also in mouse sperm. Taking this into account, we reworked the discussion and included a paragraph on the cellular events during sperm capacitation. Although these capacitation events, at first sight, seem to

be reminiscent of the hyperpolarization-induced events during chemotaxis in sea urchin sperm, we caution against rash interpretation: mammalian sperm capacitation proceeds in minutes to hours not sub-seconds, and the underlying mechanisms, in particular the role of CatSper, are ill-defined. In Figure 8, we focus on models of chemosensation in sea urchin and human sperm, which we state now more clearly.

Potential error in the figs. Fig 6F and 6G as listed in fig legends are presented as Fig 7 and fig 7 as described in text and legends isn't there

We apologize and corrected for this mistake. In addition, we now include two supplementary movies that also illustrate that the CatSper inhibitors abolish sperm accumulation in a resact gradient.

Supplementary Fig 8 resolution relatively poor.

We now include Supplementary Figure 8 at higher resolution.

Response to the referee #3

One small concern is that not infrequently the manuscript uses section headings and statements, which although likely to be true, cannot be considered to be definitely established by the results in that section. This could potentially be addressed with simple rewording. This arises in part because the authors are using changes in cytosolic Ca2+ as the surrogate of CatSper activity. Although this is likely to be, in general, appropriate in this case, changes in cytosolic Ca2+ can arise from a number of other factors. In the absence of direct recordings of ionic currents that show the hallmarks of CatSper (divalent cation selectivity, and strong monovalent ionic flux in the absence of Ca2+), most of the results that the authors use to argue for the key role of CatSper are actually indirect. Although there is no reason to doubt the conclusions of the authors, this reader would prefer that the paper were written with more circumspection regarding what the safe conclusion may be from any individual experiment. Although the work clearly establishes that cDNA for CatSper subunits are found in testis and that CatSper protein is present in sperm, that the Ca2+ elevations arise exclusively from CatSper are only supported indirectly by inhibition with certainly dirty pharmacological agents.

We thank the referee for this well-taken remark. We don´t want to make unwarranted claims. Therefore, we carefully rephrased several paragraphs according to the referee´s suggestions. Moreover, we included a cautionary paragraph concerning the non-specific action of the drugs.

With the rapid mixing experiments employed by the authors, would it be possible to at least confirm that the alkalization-activated Ca2+ elevations depend on the presence of extracellular Ca2+ in the mixing solution? Perhaps that is already known. But if one mixes a 30 mM NH4Cl solution that includes a Ca2+ buffer that would drop extracellular Ca2+ to below 100 µM, is there a Ca2+ signal? Is the pH signal unaffected in such circumstances? Given the rapidity of the mixing, I would presume that this could be done without any acute effects on the preexisting cytosolic Ca2+.

In Supplementary Figure 2, we now show that the Ca2+ response, but not the pHi response, is abolished when sperm are mixed with both NH4Cl and EGTA; thereby, extracellular Ca2+ was lowered to $\lesssim$ 400 nM at the time of stimulation. This experiment demonstrates that alkalization stimulates a Ca2+ influx.

As the authors are no doubt aware, the submitted version was missing Figure 7 as described in the text, while the submitted Figure 6 legend (A-G) described both Fig 6 (A-D) and 7(A-C, which were really E-G of the legend.

We apologize and corrected for this mistake. In addition, we now include two supplementary movies that also illustrate that the CatSper inhibitors abolish sperm accumulation in a resact gradient.

1. Although the paper is clearly written, there are some cases where English editing would help. p. 4. "since 100 years" would be better as: "for 100 years".

Corrected.

2. In Fig. 1D, the staining was not entirely clear in the copy I have. Was there any staining in the sperm head?

The antibody did not stain the head. Sometimes, we observed staining in the “neck” region that harbors the single mitochondrion. We also scrutinized the flagellar localization of CatSper by protein mass spectrometry.

3. p. 7. The heading "CatSper in sea urchin sperm is controlled by pHi and membrane voltage" is an example of a statement which somewhat goes beyond what the results actually say. What is shown is that "Ca2+ elevations in sea urchin sperm ...". Then the question is, are those elevations due to influx through CatSper. That is never actually tested, although the two dirty inhibitors do block Ca2+ elevations. Given previous published work with a slow time course of inhibition by mibefradil, I am a bit surprised that the inhibition by mibefradil of the Ca2+ elevations can be considered to be at steady-state.

We changed the heading and text accordingly. In fact, we did not test whether the drug action reached steady-state within the recording time; this is now stated in the text.

4. p. 8. Putting a number (2.5 s) on the time course of inhibition for these compounds seems unnecessary and probably not of much real physical meaning.

We agree and deleted this phrase.

5. I gather that ΔR/R refers to the change in ratio of BCECF fluorescence, as opposed to the ΔF/F. This is somewhat explained in the methods, but perhaps some additional clarification could be provided to readers to explain the convention.

We now define ΔF/F (%) and ΔR/R (%) in the legend of Figure 1.

6. Figure 3 and text. Panel D. I believe the authors may have information that would relate the concentration-dependence of the NH4Cl signals to pH. It might be of interest and value to be able to know the pH ranges of the NH4Cl effects.

We did not calibrate the NH4Cl-evoked changes of ΔR/R into absolute pH values. Unfortunately, it is impossible to calibrate the responses in retrospect, because ΔR/R (%) x ΔpH-1 varies considerably among preparations, which requires calibration for each individual experiment. Nevertheless, comparing NH4Cl responses with those evoked by the pHi-null solutions suggests that 10 mM NH4Cl increase pHi to $\lesssim$ 7.6. However, because this value is rather ill-defined, we prefer not to give numbers.

7. Figure 3. Panel E. Would it be possible to elaborate in the figure legend about how the traces in panel E are generated?

We now explain in more detail the content of Figure 3E.

8. p. 9. "The -26.3 mV" membrane potential with 190 mM K+ required some thought. Perhaps the readers could be reminded that cytosolic K+ in sea urchin sperm is likely to be around 500 mM (or whatever it is), given the 1000 mOs of the cytosol.

We now explain in more detail the calibration procedure in the methods section, to which we refer in the results part.

9. p. 10. The statement recovering the recovery-from-inactivation mechanism of T-type Ca2+ channels seems superfluous and not particularly relevant.

We agree and deleted this phrase.

10. p. 11. The slope of the relationship between ΔVthr and ΔpH is fit yielding a value of 75 mV. Is that supposed to have some physical meaning? If not, I think the number is superfluous.

We now put this number into perspective: In patch-clamp recordings from mouse sperm, a change of pHi from 6 to 7 shifts the voltage-dependence of CatSper activation by about -70 mV (Kirichok et al, 2006), indicating that the pHi sensitivity of mammalian and sea urchin CatSper is similar.

11. p. 11. "Chemotactic Ca2+ influx". I realize that the authors are using the phrase as a jargon to signify something about the Ca2+ influx being a key component of the ability of the sperm to undergo chemotaxis, but technically I do not think that Ca2+ influx per se can be chemotactic. It sounds peculiar.

We agree and amended the text accordingly.

12. p. 11-12. There is no figure that is concerned with chemotactic steering in my copy of the manuscript, but the current legend to figure 6 seems to describe both Figure 6 and 7.

We apologize and corrected for this mistake. In addition, we now include two supplementary movies that also illustrate that the CatSper inhibitors abolish sperm accumulation in a resact gradient.

p. 14. The final paragraph of the discussion uses exact phrases from sentences two paragraphs earlier. This sounds a bit too redundant.

We agree and changed the discussion accordingly.

p 19. In the sentence providing the equation for the pHi-null point solutions, perhaps a colon should precede the equation.

Corrected.

p. 19. Although a previous paper is mentioned, putting in a sentence about how Vm measurements are calibrated would be helpful.

We now describe the calibration procedure in more detail.

p.20. In regards to preparation of the TMA BA solutions, a comment regarding the osmolarity changes might be useful. It seems that these should be minor, but confirming that might be good.

We agree and indicate that the change in osmolarity was $\lesssim$ 13%.

p. 21. "Gaussian profile of 112 microns...." Is this the description of the Resact gradient or something else? Please clarify.

This describes how the resact gradient in Figure 7 and Supplementary Movie 1 and 2 was established. We now show the gradient profile in the figure. For a detailed description, we refer to earlier work.

p. 32. Figure 1 Legend. "Structural features of ". I do not consider topological maps of expected transmembrane segments as "Structural features". This also applies to Supplementary Figure 1, where the legend mentions "Primary Structure". Amino acid sequence is not structure.

We changed the title of Figure 1 to “Features of ApCatSper 1-4…”. The amino-acid sequence of proteins is often referred to as “primary structure”. Therefore, we did not change the legend of Supplementary Figure 1.

p. 33. The title to the legend may be a little strong unless there is reason to think that MDL 12330A is an absolutely selective inhibitor of CatSper.

We agree and changed the legend accordingly.

Figure 5, when compared to blocker effects on Ca2+ in other figures, might be taken to suggest that the blockers may have some effects unrelated to actions exclusively on CatSper, given the various in EC50s

We now include a cautionary remark concerning the non-specific action of the inhibitors.

Figure 8. The sea urchin model as presented implies a very direct link between elevation of cytosolic Ca2+ and chemotaxis. Isn't it really the whole cascade of events that leads to a change in flagellar beating that is chemotaxis? I would think the arrow leading away from [Ca2+] would go to something having to do with a change in motility. Chemotaxis is an emergent property of the whole system that requires repeated activation of this sequence depending on the resact gradients.

We agree and changed the term to “motility response”.

2nd Editorial Decision 04 November 2014

Thank you for submitting a revised version of your manuscript to The EMBO Journal and my apologies for the slight confusion with the status depicted in our online system. Your manuscript was sent back to one of the original referees and this person has now provided us with a set of comments listed below.

As you will see the referee finds that the main criticisms from the original manuscript have been addressed; however, this person does still ask for a number of revisions and clarifications in the text before your study can be officially accepted for publication. I would therefore ask you to incorporate these changes in a final revision of the manuscript text with a particular focus on toning down potential overstatements and on discussing remaining technical caveats (eg the use of imidazole treatment and the conclusiveness of fig 8C)

In addition, I would need you to address the following editorial points:

-> Please ensure that the number of replicas used for calculating statistics as well as the nature of the error bars is indicated in the relevant figure legends.

Thank you again for giving us the chance to consider your manuscript for The EMBO Journal, I look forward to your final manuscript revision.

REFEREE COMMENTS

Referee #3:

General comments: Overall, the revised version of this manuscript largely addresses all my concerns. The overall sets of experiments appear solidly grounded and lead to new understanding of the events that follow chemotactic signaling in sea urchin sperm.

There remain a few sentences which I feel may overstate exactly what the results indicate. Some of these are listed below, should the authors want to consider modifications.

p. 5. "final target of the chemotactic signaling pathway in sperm". I doubt this is what the data really show. What even constitutes a final target? The Ca2+ elevation itself is part of the pathway and has additional consequences. The phrase seems like unnecessary hyperbole.

p. 9. "alkalization serves as a sensitive switch" ... Biologically, what defines a switch? This issue is raises again. The signal is not off and on, but involves a continuum. That doesn't seem to be a switch.

p. 9. "We wondered whether also depolarization" would be better as "We also wondered whether ...."

p. 11. "we tested the hypothesis that the pHi increase shifts the voltage-dependence of CatSper". Wasn't this idea already established in Fig. 4E,F. Perhaps here the paper "further tests the hypothesis".

p. 11. "the notion that the resact-induced alkalization primes CatSper to open upon depolarization". Along with "switch", I think the idea of "priming" is also mechanistically inexact and unnecessary. Based on current understanding of most allosteric regulation of proteins - any protein!!!-, it is more likely to think that membrane potential and pH are acting in concert to allosterically regulate CatSper Po.

p. 12 first use of "rash interpretations" I think there are many statements in this manuscript that might be considered "rash interpretations" , so deciding to not make another seems odd! But seriously, I think better wording might be appropriate here.

p. 12. The imidazole and HCO3- results essentially do not allow any conclusions, but yet the last sentence draws a conclusion and actually a very strong one. I think a statement that the imidazole and HCO3 tests allow no conclusions would be better.

p. 13. "We unravel in quantitative terms the synergy between pHi and Vm to control CatSper" I guess I missed the part where the CatSper Po was defined as a function of pH and Vm. Synergy? What is that in terms of physical mechanism? If it can't be defined, it certainly can't be unraveled in quantitative terms. I guess this would be an "inflamed and infected rash interpretation". The example of Slo family channels which are dually regulated by independent allosteric regulatory paths, voltage and ligand, is perhaps pertinent to thinking about how pH and voltage may be regulating CatSper.

p. 14. Again, much of p. 14 uses oversimplified terminology which ignores the likely biophysical underpinnings of what is most likely really going on with CatSper regulation. This pertains to "switch" and "priming". In the description of priming given here, do the authors really mean that hyperpolarization per se is changing something in the CatSper channel, so that with subsequent depolarization at a given pH the channels will be more active? That seems to be implicit in the sentence discussing hyperpolarization by Slo3 in mouse and human sperm. I don't see any data in the paper that support anything other than the idea that CatSper Po will be definable by whatever the current Vm and pH may be.

Regarding Fig. 8C, I find this panel problematic. The fact that each subsequent flash shows a weaker DR/R can arise from a number of reasons. The first pulse itself may already be near the limit of how high pH can go. Furthermore, since the pH elevation evoked by release of caged cGMP involves several intermediate steps, saturation of cGMP-dependent steps may have occurred or there may be a limit in the NHE effect or ... If one flashes the sperm with single pulse of 4 times the energy to produce a 4-fold higher cGMP elevation, does the DR/R signal go to 4-fold higher? That seems unlikely. In any case, I am unable to see that Fig. 8C really allows any conclusion to be drawn without additional information regarding the linearity of responses to quantitatively different amounts of uncaging. Even then, there are too many steps that can contribute to saturation.

2nd Revision - authors' response 12 November 2014

Response to referee #3:

We thank the referee for the constructive criticism. We revised the manuscript accordingly. Moreover, we identified a splice variant of CatSper δ, which we now indicate in a modified version of Supplementary Figure S1.

There remain a few sentences which I feel may overstate exactly what the results indicate. Some of these are listed below, should the authors want to consider modifications.

p. 5. "final target of the chemotactic signaling pathway in sperm". I doubt this is what the data really show. What even constitutes a final target? The Ca^{2+} elevation itself is part of the pathway and has additional consequences. The phrase seems like unnecessary hyperbole.

Our wording might indicate oversimplification, but certainly not hyperbole. We amended this paragraph accordingly.

p. 9. "alkalization serves as a sensitive switch" ... Biologically, what defines a switch? This issue is raises again. The signal is not off and on, but involves a continuum. That doesn't seem to be a switch.

It is unequivocal that modulation of channels by any modality involves a transition from one state to the other. We used the term "switch", because it is commonly used in the literature to indicate extremely steep "dose" dependencies. We rephrased several paragraphs to indicate more clearly that ΔpH_i allosterically controls the voltage dependence of CatSper activation.

p. 9. "We wondered whether also depolarization" would be better as "We also wondered whether ...."

Changed.

p. 11. "we tested the hypothesis that the pHi increase shifts the voltage-dependence of CatSper". Wasn't this idea already established in Fig. 4E,F. Perhaps here the paper "further tests the hypothesis"

Changed to "we scrutinized the hypothesis…"

p. 11. "the notion that the resact-induced alkalization primes CatSper to open upon depolarization". Along with "switch", I think the idea of "priming" is also mechanistically inexact and unnecessary. Based on current understanding of most allosteric regulation of proteins - any protein!!!-, it is more likely to think that membrane potential and pH are acting in concert to allosterically regulate CatSper Po.

See comment above. We rephrased several paragraphs to indicate more clearly that ΔpH_i allosterically controls the voltage dependence of CatSper activation

p. 12 first use of "rash interpretations" I think there are many statements in this manuscript that might be considered "rash interpretations" , so deciding to not make another seems odd! But seriously, I think better wording might be appropriate here.

We would like to take issue with the referee´s comment that many of our statements are actually based on rash interpretations - we assume that this comment was meant in jest. Nevertheless, we amended this paragraph accordingly.

p. 12. The imidazole and HCO3- results essentially do not allow any conclusions, but yet the last sentence draws a conclusion and actually a very strong one. I think a statement that the imidazole and HCO3 tests allow no conclusions would be better.

We rephrased the paragraph.

p. 13. "We unravel in quantitative terms the synergy between pHi and Vm to control CatSper" I guess I missed the part where the CatSper Po was defined as a function of pH and Vm. Synergy? What is that in terms of physical mechanism? If it can't be defined, it certainly can't be unraveled in quantitative terms. I guess this would be an "inflamed and infected rash interpretation". The example of Slo family channels which are dually regulated by independent allosteric regulatory paths, voltage and ligand, is perhaps pertinent to thinking about how pH and voltage may be regulating CatSper.

We rephrased the wording, but we maintain the claim that we unraveled in quantitative terms the "interplay" between pH_i and V_m to control "Ca^{2+} influx" via CatSper in intact sperm. By quantitative

rather than descriptive experimentation, we determined pairs of voltage threshold (V_{thr}) and pH_i threshold (pH_{thr}) for the Ca^{2+} influx, which yielded the $\Delta V_{thr}/\Delta pH$ ratio.

p. 14. Again, much of p. 14 uses oversimplified terminology which ignores the likely biophysical underpinnings of what is most likely really going on with CatSper regulation. This pertains to "switch" and "priming". In the description of priming given here, do the authors really mean that hyperpolarization per se is changing something in the CatSper channel, so that with subsequent depolarization at a given pH the channels will be more active? That seems to be implicit in the sentence discussing hyperpolarization by Slo3 in mouse and human sperm. I don't see any data in the paper that support anything other than the idea that CatSper Po will be definable by whatever the current Vm and pH may be.

We rephrased the paragraph accordingly.

Regarding Fig. 8C, I find this panel problematic. The fact that each subsequent flash shows a weaker ΔR/R can arise from a number of reasons. The first pulse itself may already be near the limit of how high pH can go. Furthermore, since the pH elevation evoked by release of caged cGMP involves several intermediate steps, saturation of cGMP-dependent steps may have occurred or there may be a limit in the NHE effect or ... If one flashes the sperm with single pulse of 4 times the energy to produce a 4-fold higher cGMP elevation, does the •R/R signal go to 4-fold higher? That seems unlikely. In any case, I am unable to see that Fig. 8C really allows any conclusion to be drawn without additional information regarding the linearity of responses to quantitatively different amounts of uncaging. Even then, there are too many steps that can contribute to saturation.

We can exclude that saturation of the pH indicator accounts for saturation of ΔpH_i; the cGMP-evoked pH_i response is much smaller than that evoked by saturating NH_4Cl concentrations. Kashikar et al. 2012 (*Journal of Cell Biology*) shows that repetitive cGMP photorelease (at 1Hz) evokes repetitive Ca^{2+} and V_m responses, indicating that cGMP is rapidly degraded in between the flashes. Moreover, each flash is photolysing only a few percent of the caged cGMP (e.g. Bönigk et al. 2009, *Science Signalling*) and each of at least ten flashes in a row produces a similar Ca^{2+} and V_m response (Kashikar et al. 2012 *Journal of Cell Biology*). This argues against the referee´s concern that ΔpH_i saturates due to cGMP accumulation, a nonlinear dose-response relation, ceasing of cGMP release, or a combination thereof. Therefore, we maintain our conclusion that ΔpH_i shifts the voltage dependence of CatSper to a permissive voltage range and, thereby, enables sperm to transduce periodic V_m changes into periodic Ca^{2+} changes. This is required for sperm navigation on periodic paths in a chemoattractant gradient. We slightly reworked the respective paragraph, which we hope addresses the referee´s concern. On a final note, we agree that the molecular mechanism underlying the saturation of ΔpH_i upon repetitive stimulation requires future studies.

