

HCN4 channels sense temperature and determine heart rate responses to heat

Corresponding Author: Professor Mark Anderson

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

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

"HCN channels sense temperature and determine heart rate responses to heat" by Wu et al.

This interesting paper focusses on heat-activation mechanisms of HCN4 ion channels, that are critical in determining the beat rate of pacemaker cells in the heart. The main finding is that the effects of heat, and also of cAMP, in increasing the cardiac beat rate are abolished by a double mutation in the S4-S5 linker domain (M407Q/Y409F).

The authors claim that their results show that cAMP and heat both act via the M407/Y409 site ("the heat-sensing machinery within the S4-S5 linker"), but they do not completely rule out the possibility that the effect of heat may be to increase cAMP and that the mutation then removes the effect of cAMP on the HCN4 channel. The main evidence against this possibility is the finding that a mutation of the CNBD that removes the effect of cAMP on HCN4 does not remove the effect of heat (Fig. 5b, c). Direct measurements of the effect of heat on cAMP levels in isolated cardiomyocytes, with parallel measurements of cAMP increases in response to e.g. beta agonists, to prove that the technique works, should be carried out in addition.

A second major gap is that the paper shows an ignorance of developments in our understanding of the physiology of HCN ion channels over many years. This is seen (for instance) in the first line of the Abstract "HCN current increases due to cAMP binding..." which is true only for HCN2 and HCN4, while HCN1 and HCN3 are resistant to changes in cAMP, for reasons that have not been fully explained, even though both contain a CNBD.

Another example of lack of awareness of developments in the HCN ion channel field is that many groups have carried out voltage-clamp experiments which show that the current passing through HCN channels depends on two independent parameters – the current passing through the channel in its open state, and the degree of channel activation. These two can be separated by measuring tail currents, which give the degree of channel activation as a function of voltage, and from that extracting the open-channel current (see for example Bonzanni et al, 2018, Neurobiol Dis, Fig. 2, though many groups have developed a similar analysis). None of the graphs in the paper are separated out in this way, so it is difficult to see whether the effect of temperature is due to a change in channel gating or open-channel conductance, though it seems to be due to both. Typically ion channels have a relatively low Q10 for conductance, but gating can be much more thermally sensitive. This seems true also from their data for HCN4, because in Fig. 2b (transfected HEK cell) the 36/23 degree ratio for large voltage steps is around 1.5 for steps to -130mV, a voltage which spans the entire activation range for channel gating and therefore tells us about the channel Q10 in isolation, while for steps to -80mV the Q10 is more than 3 (RH panel in Fig. 2b) because of a significant effect on gating. There is a similar but smaller discrepancy in Fig. 2a (45/32 degree C, SAN cell) of 1.7 at -130mV and 2.0 at -80. Thus heat appears to have a dual effect: it increases the conductivity of the HCN4 ion channel, which is not a surprise as most/all ion channels show a similar effect, and it shifts the voltage activation range of HCN4 so as to generate more inward current in the critical range of pacemaker potentials. The statement throughout the paper that Q10 values are around 2 and are independent of voltage step size is therefore incorrect – the value depends on the amplitude of the voltage step, showing that there is an action of heat on both channel conductance and on gating. This needs to be sorted out by a proper separation and analysis of ion channel conductance and channel gating.

The G/Gmax values used in Fig. 3f and 5a do give a partial handle on the gating function, and do show that gating by cAMP is abolished by the M407/Y409 mutation, but a proper tail current analysis of the voltage dependence of the activation curve needs to be carried out.

Reviewer #2

(Remarks to the Author)

- The article presents a detailed and rigorous analysis of the activation of the HCN4 channel by cAMP, supported by quantitative and experimental data. This includes changes in the solvent-accessible area (SASA) and experiments with specific mutations to evaluate their impact. Multiple experimental techniques are used, including recordings of currents in transfected HEK 293 cells, mutation analysis, and structural and thermodynamic modeling. The study has direct implications for understanding thermal response and heart rate regulation, which is relevant to cardiac physiology and potentially for clinical applications.

- The article presents solid, detailed research with significant biological implications and potential medical applications.

- Major considerations:

- The article titled "HCN channels sense temperature and determine heart rate responses to heat" However, this effect is only demonstrated in the HCN-4 channel, so it is not possible to extrapolate and generalize the effect for the entire HCN family. Another suitable title could be "HCN-4 channel contributes to sensing temperature and determining heart rate responses to heat."

- A major point is how the Q10 is being calculated, only considering 2 temperatures. It must be demonstrated that the Arrhenius plot is linear in the temperature range used, making it necessary to include at least 5 different temperatures. The Q10 reported in this study is calculated by paired R2, R1 and T2, T1 from the same experimental sample, be it an isolated cell, heart, or mouse.

- The Q10 value is calculated based on the steady-state current density change by temperature from the same cell; this comprises the effect of temperature on channel gating and unitary conductance. However, the Q10 for the time constant of activation and deactivation at an activation voltage in SAN cells and Hek cells transfected with HCN4 must be calculated.

- minor considerations

- Please discuss why no homozygous knockin mice were born from Hcn4+/QF x Hcn4+/QF crosses, considering that the HCN4 M407/Y409 mutant showed the same biophysical properties as WT.

- Please explain the rationale behind designing the mutants M407Q, Y409F, F540Y, K562M, and F613Y.

- In Addition to the HCN4 isoform, other HCN channel isoforms that contribute to If current in sinoatrial node (SAN) cardiomyocytes, including HCN1 and HCN2, have been reported by different authors (Robinson and Siegelbaum, Ludwig, Moroni, and others). These studies have used techniques such as electrophysiology, molecular biology, and knockout models in mice to characterize the properties and function of these isoforms in regulating heart rate. How can it explain that these isoforms are irrelevant despite retaining the residues that give them temperature sensitivity? Please discuss these discrepancies regarding the contribution of HCN isoforms to the If current.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

Point 1:

The authors here advance a number of indirect arguments that the main effect of heat is not caused by a heat-induced elevation in cAMP. They then agree with the criticism as follows: "we agree that our studies do not absolutely rule out the formal possibility that heat contributes to increase If, in part, by enhancing cAMP" and they decline to carry out a conclusive measurement of cAMP levels at different levels of heat on the grounds that "The measurement of cAMP in isolated SAN cells is technically challenging and could not be completed in a 'reasonable' time".

The possibility that heat-induced increases in cAMP might be responsible for part of their effect goes right to the heart of the claims made in this paper. Measurement of cAMP in isolated cells is in fact straightforward, using ELISA. In my view the authors should have carried out this simple experiment, which they concede is justified and important.

Point 2:

Textual change made which is adequate.

Point 3:

The authors agree that the criticism raised is reasonable, and they have made textual alterations to concede that the effect of temperature is to affect both ion channel conductance and channel gating. However the referee request that "a proper tail current analysis of the voltage dependence of the activation curve needs to be carried out," in order to separate out the effects of temperature on ion channel conductance and gating, has been ignored. The only excuse given for not doing this is that "we are focusing here on the effect of temperature on channel function at physiologically relevant voltages". Unfortunately it is at exactly these voltages (-60mV to -90mV) that ion channel current will be determined by both channel gating and conductance. Are the effects of their M407/Y409 mutation on ion channel conductance, on gating or on both? This could have been easily determined with voltage-clamp experiments, using techniques available within the lab, and using voltage-clamp protocols to which references were given in the first referee report. In my view it is unacceptable that no further experiments have been carried out in response to this point, which goes right to the heart of the mechanism that the authors are proposing.

I note also that only textual changes have been made in response to Referee 2.

Reviewer #2

(Remarks to the Author)

The article "HCN4 channels sense temperature and determine heart rate responses to heat" presents a novel and comprehensive analysis of the activation of the HCN4 channel by cAMP, supported by robust quantitative and experimental data. This includes changes in the solvent-accessible surface area (SASA) and experiments with specific mutations to evaluate their impact. The study employs various experimental techniques, such as current recordings in transfected HEK 293 cells, mutation analysis, and structural and thermodynamic modeling. The findings have direct implications for understanding thermal response and heart rate regulation, which is crucial in the field of cardiac physiology. The article presents solid, detailed research with significant biological implications and potential medical applications. The authors have demonstrated an exceptional level of thoroughness in addressing all my concerns, further reinforcing the validity and scientific rigor of the study.

Version 2:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

The authors have made adequate responses to the two major objections that were raised.

1) They have performed a "tail analysis" of I_f currents in order to separate the effects on channel conductance and on channel activation. This shows clearly that there is a strong effect of temperature, particularly on the voltage-dependence of HCN4 activation (Fig. 2C, Fig. 3F, 3G) and that the main effect of the HCN4-407/409 mutation seems to be to abolish the action of elevated temperature on activation (Fig. 3G). This is an interesting conclusion which is now clearly separated from the effects of temperature on ion channel conductance.

2) Given that the main effect of temperature is to modulate ion channel gating, the possibility was raised that cAMP levels might be modulated by temperature, and that this might be the cause of the modulation of HCN4 voltage dependence.

However, the authors have now ruled this out by showing that temperature seems to have no effect on cAMP levels.

I think that the changes made to the MS have produced a much stronger paper, and I am happy to approve it for publication.

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We thank the reviewers for their careful reading and insightful comments. We believe the revised manuscript is improved because of your input. A detailed, point-by-point set of responses follow.

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

“HCN channels sense temperature and determine heart rate responses to heat” by Wu et al.

This interesting paper focusses on heat-activation mechanisms of HCN4 ion channels, that are critical in determining the beat rate of pacemaker cells in the heart. The main finding is that the effects of heat, and also of cAMP, in increasing the cardiac beat rate are abolished by a double mutation in the S4-S5 linker domain (M407Q/Y409F).

1. The authors claim that their results show that cAMP and heat both act via the M407/Y409 site (“the heat-sensing machinery within the S4-S5 linker”), but they do not completely rule out the possibility that the effect of heat may be to increase cAMP and that the mutation then removes the effect of cAMP on the HCN4 channel. The main evidence against this possibility is the finding that a mutation of the CNBD that removes the effect of cAMP on HCN4 does not remove the effect of heat (Fig. 5b, c). Direct measurements of the effect of heat on cAMP levels in isolated cardiomyocytes, with parallel measurements of cAMP increases in response to e.g. beta agonists, to prove that the technique works, should be carried out in addition.

Thank you for raising this interesting point. We completely agree that our finding that the EA mutation, which disrupts binding of cAMP to the channel, does not prevent heat-induced increases in I_f speaks against enhanced cAMP production being the main driver for thermally responsive I_f and heart rate changes. The results of these experiments strongly suggest that increases in cAMP are not required for heat-induced increases in I_f . Notable, also, is our finding that SAN cells are resistant to heat-induced rate increases after exposure to I_f antagonists, yet sustain a normal rate increase to isoproterenol (i.e., an agonist operating the cAMP pathway) in the presence of these antagonists (Fig S2a). We interpret these findings to suggest that if heat-induced increases in cAMP transpire in native cells that they are likely modest and considerably lower than cAMP increases induced by β -adrenergic receptor agonist activation. Thus, should heat enhance cAMP production it is almost certainly not the predominant pathway for heat to augment I_f and heart rate. Finally, heat increases I_f even in the presence of isoproterenol (Fig 1f), a condition where any thermally-induced contribution to cAMP production should be small compared to β -adrenergic agonist stimulation. Taken together, these findings strongly suggest that heat actions on HCN4 are independent of the cAMP pathway. Nevertheless, we agree that our studies do not absolutely rule out the formal possibility that heat contributes to increase I_f , in part, by enhancing cAMP. The measurement of cAMP in isolated SAN cells is technically challenging and could not be completed in a ‘reasonable’ time. Thus, we respectfully request to forego additional studies for this purpose and to add a brief comment in the Discussion section (page 9 and page 10, yellow highlighted) to include the possibility that EA mutations disturb cAMP production as a caveat to our findings and an untested dimension to the biology of heat in heart rate and I_f .

2. A second major gap is that the paper shows an ignorance of developments in our understanding of the physiology of HCN ion channels over many years. This is seen (for instance) in the first line of the Abstract “HCN current increases due to cAMP binding...” which is true only for HCN2 and HCN4, while HCN1 and HCN3 are resistant to changes in cAMP, for reasons that have not been fully explained, even though both contain a CNBD.

Thank you for these points. We agree that our sentence is not adequately precise. We edited the sentence to emphasize our focus on HCN4 (see Title and Abstract).

3. Another example of lack of awareness of developments in the HCN ion channel field is that many groups have carried out voltage-clamp experiments which show that the current passing through HCN channels depends on two independent parameters – the current passing through the channel in its open state, and the degree of channel activation. These two can be separated by measuring tail currents, which give the degree of channel activation as a function of voltage, and from that extracting the open-channel current (see for example Bonzanni et al, 2018, Neurobiol Dis, Fig. 2, though many groups have developed a similar analysis). None of the graphs in the paper are separated out in this way, so it is difficult to see whether the effect of temperature is due to a change in channel gating or open-channel conductance, though it seems to be due to both. Typically ion channels have a relatively low Q10 for conductance, but gating can be much more thermally sensitive. This seems true also from their data for HCN4, because in Fig. 2b (transfected HEK cell) the 36/23 degree ratio for large voltage steps is around 1.5 for steps to -130mV, a voltage which spans the entire activation range for channel gating and therefore tells us about the channel Q10 in isolation, while for steps to -80mV the Q10 is more than 3 (RH panel in Fig. 2b) because of a significant effect on gating. There is a similar but smaller discrepancy in Fig. 2a (45/32 degree C, SAN cell) of 1.7 at -130mV and 2.0 at -80. Thus heat appears to have a dual effect: it increases the conductivity of the HCN4 ion channel, which is not a surprise as most/all ion channels show a similar effect, and it shifts the voltage activation range of HCN4 so as to generate more inward current in the critical range of pacemaker potentials. The statement throughout the paper that Q10 values are around 2 and are independent of voltage step size is therefore incorrect – the value depends on the amplitude of the voltage step, showing that there is an action of heat on both channel conductance and on gating. This needs to be sorted out by a proper separation and analysis of ion channel conductance and channel gating.

The G/Gmax values used in Fig. 3f and 5a do give a partial handle on the gating function, and do show that gating by cAMP is abolished by the M407/Y409 mutation, but a proper tail current analysis of the voltage dependence of the activation curve needs to be carried out.

We thank the Reviewer for pointing out this inconsistency, we fully agree with the view that temperature is affecting two components of the current, voltage-dependent open probability as well as the conductance. We have considered this in the new version of the manuscript, where we estimated Q10 values at – 130 mV, e.g. the voltage at which open probability is approaching saturation and the heat increase in current is only a function of the increase in conductance. We compared this to the Q10 value at our reference voltage – 60 mV, a condition in which channel function is determined by both

effects of T on conductance and on gating. As properly predicted by the reviewer, the Q₁₀ value exhibits a significant voltage dependency that is higher at moderate voltages where both conductance and gating contribute to heat sensitivity.

This is now stated in the text, page 3 and 4, yellow highlighted, and in the redesigned Figure 2 with revised panels c and d.

We further underline, in the context of the double mutant, that the mutations eliminated the heat sensitivity of both, conductance and voltage dependency, by introducing the following sentence in page 6, (yellow highlighted):

“This mutant is insensitive to heat and shows no increase in the maximal current nor a shift of the activation curve in response to heat; as a consequence, the Q₁₀ value at -60 mV is close to 1 (Fig 3d).”

The finding that the double mutation eliminates both effects of temperature on conductance and voltage dependency, prevented us from further separating the impact of temperature on the two biophysical components of channel function. Another reason not to proceed further in our biophysical characterization was that such a separation is not crucial, in the context of the paper, as much as we are focusing here on the effect of temperature on channel function at physiologically relevant voltages (- 60 mV), a condition in which both components of channel function are increased by temperature.

Reviewer #2 (Remarks to the Author):

- The article presents a detailed and rigorous analysis of the activation of the HCN4 channel by cAMP, supported by quantitative and experimental data. This includes changes in the solvent-accessible area (SASA) and experiments with specific mutations to evaluate their impact. Multiple experimental techniques are used, including recordings of currents in transfected HEK 293 cells, mutation analysis, and structural and thermodynamic modeling. The study has direct implications for understanding thermal response and heart rate regulation, which is relevant to cardiac physiology and potentially for clinical applications.
- The article presents solid, detailed research with significant biological implications and potential medical applications.

Thank you very much for your comments!

- Major considerations:

1. - The article titled “HCN channels sense temperature and determine heart rate responses to heat” However, this effect is only demonstrated in the HCN-4 channel, so it is not possible to extrapolate and generalize the effect for the entire HCN family. Another suitable title could be “HCN-4 channel contributes to sensing temperature and determining heart rate responses to heat.”

Thank you for raising this point. We did study HCN1 and HCN2 in addition to HCN4. Similar to the experience of other labs, we were unable to express HCN3. Importantly HCN1, 2 and HCN4 share a thermal response phenotype that is blocked by the M407Q/Y409F (homologous) mutation (please see Fig S5a). In the revised manuscript we emphasized these findings to make our general conclusion clearer (see page 2 and page 9, yellow highlighted). However, because we only demonstrated the effect of heat on HCN4 for determining heart rate, we also edited the title as you suggest.

2. - A major point is how the Q10 is being calculated, only considering 2 temperatures. It must be demonstrated that the Arrhenius plot is linear in the temperature range used, making it necessary to include at least 5 different temperatures. The Q10 reported in this study is calculated by paired R2, R1 and T2, T1 from the same experimental sample, be it an isolated cell, heart, or mouse.

To address reviewer's concerns, we added the Arrhenius plot of action potential rates recorded from SAN cell in the temperature range used in this study to demonstrate that the Arrhenius plot is linear in the temperature range (Fig S1). A similar treatment for I_f and HCN4 current is presented in Fig S3. The Arrhenius plots are also linear in the temperature range. Given the linearity of these Arrhenius plots and because the use of the Q_{10} calculation as applied in our manuscript matches the work of others in this and related fields, we think the Q_{10} should reflect the response of action potential rates and HCN currents to heat [1-9].

3. - The Q10 value is calculated based on the steady-state current density change by temperature from the same cell; this comprises the effect of temperature on channel gating and unitary conductance. However, the Q10 for the time constant of activation and deactivation at an activation voltage in SAN cells and Hek cells transfected with HCN4 must be calculated.

This criticism on the dual effect of temperature on gating and conductance is correct and reinforces the criticism raised by Reviewer 1 (point 3 from Reviewer1, above). We have addressed this point in our answer to Reviewer 1 (see response to Reviewer1 point 3). We further followed your suggestion and have analyzed the impact of heat on channel activation at -130 mV. This new analysis confirmed that heat is greatly accelerating channel activation (Fig 2d), which is compatible with the positive shift in $V_{1/2}$.

As for calculation of Q10 for the time constant of activation and deactivation, please see the last paragraph of our response to Reviewer1 point 3 and also DiFrancesco's comments on Q10 calculation for the time constant of I_f current: "However, values of Q10 for the time constant have to be considered a poor indication of the actual value of the activation energy for the physical process underlying the current change during a voltage clamp. This is particularly true if (as is likely to occur), the activation energy itself has a temperature dependence, even if small (S. Kroll and D. Noble, personal communication)."[6]

- minor considerations

- Please discuss why no homozygous knockin mice were born from Hcn4+/QF x Hcn4+/QF crosses,

considering that the HCN4 M407/Y409 mutant showed the same biophysical properties as WT.

This is an interesting point. Our hypothesis is that lethality of the homozygous M407/Y409 mutation follows from its lack of response to cAMP. In agreement with this concept, homozygous mice with a point mutation in the CNBD (HCN4 R669Q), which abolishes cAMP binding, also die during embryonic development [10]. Based on these findings we suspect that cAMP-dependent activation of HCN4 is required for proper development. We added this discussion in page 10 (yellow highlighted).

- Please explain the rationale behind designing the mutants M407Q, Y409F, F540Y, K562M, and F613Y.

Thank you for this question. We did discuss this rationale in the original manuscript and have attempted to make it more clear in the revision (see pages 5 and 6). The modified two paragraphs on page 5 and 6, with the corrections yellow highlighted, now reads:

Figure 3c shows the average change in SASA ($\langle\Delta\text{SASA}\rangle$) of the whole human HCN4 channel during channel activation by cAMP. The average value is 1.3 \AA^2 with a standard deviation ($\sigma(\text{SASA})$) of 16.0 \AA^2 . We analyzed residues showing absolute values of ΔSASA greater than 3 times $\sigma(\text{SASA})$ ($> 48 \text{ \AA}^2$) between apo and holo (activated) states of the human channel (Table S1). The residues with the most significant increases in the apolar area exposed during its activation are Met 407 (104 \AA^2), Phe 540 (54.9 \AA^2), and Phe 613 (44.4 \AA^2), and the most significant decreases, due to polar area sequestration, are Tyr 409 (-141.6 \AA^2), Arg 668 (-57.8 \AA^2), and Glu 695 (-77.8 \AA^2). The hydration of polar and apolar areas of these residues have opposing contributions to the energetics and, as a consequence, to the temperature dependence of processes in which conformational changes are involved.

Met 407 and Tyr 409 are part of the S4-S5 intracellular linker that exhibits a large conformational change after activation (Fig 3b and c, and Supp. Movie1). We excluded residues in disordered regions (e.g., at the C-terminus of the cyclic nucleotide binding domain) from the analysis, focusing on those observed in the structures. To avoid making mutations affecting the activation mechanism, we did not consider residues coordinating intracellular Mg^{2+} (His 406, Asp 410, Glu 556 and His 552, Fig S2b)²¹, nor those participating in cAMP binding, such as Ile 661 or Arg 587, and ignored amino acids forming salt bridges stabilizing one particular state of the channel, such as Arg 668 and Glu 695. The above considerations led to a final list of five candidates with the potential to modify the temperature dependence of I_f : Met 407, Tyr 409, Phe 540, Lys 562, and Phe 613 (see Supplementary Materials). Based on our structural analysis and modeling, and the predicted energy contributions due to its solvation or lack thereof between the closed and cAMP-active HCN4, we designed a set of mutations M407Q, Y409F, F540Y, K562M, and F613Y chosen to minimize residue size variations to limit the structural changes in the mutated protein (see Supplementary Notes). These mutations are predicted to reduce the activation energy barrier or produce positive contributions to the overall $\Delta\Delta C_p^{\text{mut-wt}}$, thereby reducing its temperature dependence (lowering $Q_{10}^{\text{mut}}/Q_{10}^{\text{WT}}$, equation 5, see Supplementary Materials). Consistent with our predictions, HCN4 channel current recorded from the 5x mutant HCN4 channel was not responsive to temperature between 27-38°C (Fig S4c-e).

- In Addition to the HCN4 isoform, other HCN channel isoforms that contribute to I_f current in sinoatrial node (SAN) cardiomyocytes, including HCN1 and HCN2, have been reported by different authors (Robinson and Siegelbaum, Ludwig, Moroni, and others). These studies have used techniques such as electrophysiology, molecular biology, and knockout models in mice to characterize the properties and function of these isoforms in regulating heart rate. How can it explain that these isoforms are irrelevant despite retaining the residues that give them temperature sensitivity? Please discuss these discrepancies regarding the contribution of HCN isoforms to the I_f current.

Thank you for making this point. The major isoform underlying I_f in sinoatrial cells is undoubtedly HCN4. Based on various knockout models, HCN4 is responsible for about 75 % of the current in SAN cells [11-13]. The rest of I_f is mainly provided by HCN1 and, to a lesser extent, HCN2 channels [14, 15]. These findings are also consistent with studies on the expression level of the three isoforms in the sinoatrial node [16]. Thus, our data suggest that the effect of heat on the HCN1 and HCN2 isoforms is not strong enough to increase I_f in a manner relevant to increasing heart rate. Another possibility is that M407/Y409-mutated HCN4 channels form heteromers with HCN1 or HCN2, thereby impairing the heat sensitivity of these isoforms.

We have briefly discussed these points in the revised manuscript (page 9, yellow highlighted).

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9. Kiyosue, T., et al., *Ionic mechanisms of action potential prolongation at low temperature in guinea-pig ventricular myocytes*. *The Journal of Physiology*, 1993. **468**(1): p. 85-106.
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11. Herrmann, S., et al., *HCN4 provides a 'depolarization reserve' and is not required for heart rate acceleration in mice*. *The EMBO Journal*, 2007. **26**(21): p. 4423-4432.
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13. Baruscotti, M., et al., *Deep bradycardia and heart block caused by inducible cardiac-specific knockout of the pacemaker channel gene *Hcn4**. Proceedings of the National Academy of Sciences, 2011. **108**(4): p. 1705-1710.
14. Fenske, S., et al., *Sick Sinus Syndrome in HCN1-Deficient Mice*. Circulation, 2013. **128**(24): p. 2585-2594.
15. Ludwig, A., et al., *Absence epilepsy and sinus dysrhythmia in mice lacking the pacemaker channel HCN2*. The EMBO Journal, 2003. **22**(2): p. 216-224.
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REVIEWER COMMENTS

Thank you for carefully reviewing our manuscript. In response to your comments, we performed new measurements of cAMP in HEK 293 and SAN cells expressing WT and M407Q/Y409F mutant *Hcn4* and performed new analyses of tail currents (see below). Changes to the revised manuscript are marked by yellow highlighting.

Reviewer #1 (Remarks to the Author):

Point 1:

The authors here advance a number of indirect arguments that the main effect of heat is not caused by a heat-induced elevation in cAMP. They then agree with the criticism as follows: "we agree that our studies do not absolutely rule out the formal possibility that heat contributes to increase I_f , in part, by enhancing cAMP" and they decline to carry out a conclusive measurement of cAMP levels at different levels of heat on the grounds that "The measurement of cAMP in isolated SAN cells is technically challenging and could not be completed in a 'reasonable' time".

The possibility that heat-induced increases in cAMP might be responsible for part of their effect goes right to the heart of the claims made in this paper. Measurement of cAMP in isolated cells is in fact straightforward, using ELISA. In my view the authors should have carried out this simple experiment, which they concede is justified and important.

Thank you for your comments and suggestions. As suggested, we carried out experiments to measure cAMP levels in isolated SAN cells and HEK-293 cells under low temperature (23 ± 1 °C) and higher temperature (38 ± 1 °C) using ELISA. We used isoproterenol or forskolin as positive controls. The Methods (page 11-12) and Results (page 8) are presented in the revised manuscript, as indicated, and in Supplementary Figure 8. The new measurements do not show increased cAMP levels at high compared to low temperatures but do confirm anticipated cAMP increases to isoproterenol (in SAN cells experiments) or forskolin (in HEK-293 cells experiments).

Point 2:

Textual change made which is adequate.

Thank you!

Point 3:

The authors agree that the criticism raised is reasonable, and they have made textual alterations to concede that the effect of temperature is to affect both ion channel conductance and channel gating. However the referee request that "a proper tail current analysis of the voltage dependence of the activation curve needs to be carried out," in order to separate out the effects of temperature on ion channel conductance and gating, has been ignored. The only excuse given for not doing this is that "we are focusing here on the effect of temperature on channel function at physiologically relevant voltages". Unfortunately it is at exactly these voltages (-60 mV to -90 mV) that ion channel current will be determined by both channel gating and conductance. Are the effects of their M407/Y409 mutation on ion channel conductance, on gating or on both? This could have been easily determined with voltage-clamp experiments, using techniques available within the lab, and using voltage-clamp

protocols to which references were given in the first referee report. In my view it is unacceptable that no further experiments have been carried out in response to this point, which goes right to the heart of the mechanism that the authors are proposing.

I note also that only textual changes have been made in response to Referee 2.

Thank you for your comments and suggestions. We re-analyzed the absolute values of the tail current data. This allows us to decompose the impact of temperature into the two components - namely voltage dependent gating and conductance. The analysis provides a detailed picture on how elevated temperature augments channel conductance and causes a positive shift in the voltage dependent activation of the channel. We highlighted in the revised text that the temperature induced shift in voltage dependent gating is in agreement with an accelerated activation kinetics of the channel by elevated temperature. The voltage dependency of the Q_{10} value suggests that effects on gating and conductance contribute to the elevated HCN4 currents at the free running membrane voltage. We have changed, accordingly, the text in the methods (page 12-13) and results (page 4 and page 6) and we have modified Figure 2c and 3d.

Reviewer #2 (Remarks to the Author):

The article "HCN4 channels sense temperature and determine heart rate responses to heat" presents a novel and comprehensive analysis of the activation of the HCN4 channel by cAMP, supported by robust quantitative and experimental data. This includes changes in the solvent-accessible surface area (SASA) and experiments with specific mutations to evaluate their impact. The study employs various experimental techniques, such as current recordings in transfected HEK 293 cells, mutation analysis, and structural and thermodynamic modeling. The findings have direct implications for understanding thermal response and heart rate regulation, which is crucial in the field of cardiac physiology. The article presents solid, detailed research with significant biological implications and potential medical applications. The authors have demonstrated an exceptional level of thoroughness in addressing all my concerns, further reinforcing the validity and scientific rigor of the study.

Thank you for your comments!