The Journal of Physiology

Bile acids regulate the epithelial Na+ channel in native tissues through direct binding at multiple sites

Xue-Ping Wang, Viktor Tomlin, Andrew J Nickerson, Runze Tian, Merve Ertem, Abagail McKernan, Xiaoguang Lei, Oleh Pochynyuk, and Ossama Kashlan
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The referees have opted to remain anonymous.

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Reviewing Editor: Morag Mansley

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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. Depending on transfer agreements, referee reports obtained elsewhere may or may not be included in this compilation. Referee reports are anonymous unless the Referee chooses to sign their reports.)

1st Editorial Decision **01-Jul-2022**

Dear Professor Kashlan,

Re: JP-RP-2022-283318 "Bile acids regulate the epithelial Na+ channel in native tissues through direct binding at multiple sites" by Xue-Ping Wang, Viktor Tomlin, Andrew Nickerson, Runze Tian, Merve Ertem, Abagail McKernan, Xiaoguang Lei, Oleh Pochynyuk, and Ossama Kashlan

Thank you for submitting your manuscript to The Journal of Physiology. It has been assessed by a Reviewing Editor and by 2 expert Referees and I am pleased to tell you that it is considered to be acceptable for publication following satisfactory revision.

Please advise your co-authors of this decision as soon as possible.

The reports are copied at the end of this email. Please address all of the points and incorporate all requested revisions, or explain in your Response to Referees why a change has not been made.

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- Statistical Summary Document

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I look forward to receiving your revised submission.

If you have any queries please reply to this email and staff will be happy to assist.

Yours sincerely,

Dr Peying Fong
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- You must start the Methods section with a paragraph headed Ethical Approval. A detailed explanation of journal policy and regulations on animal experimentation is given in Principles and standards for reporting animal experiments in The Journal of Physiology and Experimental Physiology by David Grundy J Physiol, 593: 2547-2549. doi:10.1113/JP270818.). A checklist outlining these requirements and detailing the information that must be provided in the paper can be found at: https://physoc.onlinelibrary.wiley.com/hub/animal-experiments. Authors should confirm in their Methods section that their experiments were carried out according to the guidelines laid down by their institution's animal welfare committee, and conform to the principles and regulations as described in the Editorial by Grundy (2015). The Methods section must contain details of the anaesthetic regime: anaesthetic used, dose and route of administration and method of killing the experimental animals.

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- If n {less than or equal to} 30, all data points must be plotted in the figure in a way that reveals their range and distribution. A bar graph with data points overlaid, a box and whisker plot or a violin plot (preferably with data points included) are acceptable formats.

- If n > 30, then the entire raw dataset must be made available either as supporting information, or hosted on a not-for-profit repository e.g. FigShare, with access details provided in the manuscript.

- 'n' clearly defined (e.g. x cells from y slices in z animals) in the Methods. Authors should be mindful of pseudoreplication.

- All relevant 'n' values must be clearly stated in the main text, figures and tables, and the Statistical Summary Document (required upon revision).

- The most appropriate summary statistic (e.g. mean or median and standard deviation) must be used. Standard Error of the Mean (SEM) alone is not permitted.

- Exact p values must be stated. Authors must not use 'greater than' or 'less than'. Exact p values must be stated to three significant figures even when 'no statistical significance' is claimed.

- Statistics Summary Document completed appropriately upon revision.

- Please include an Abstract Figure. The Abstract Figure is a piece of artwork designed to give readers an immediate understanding of the research and should summarise the main conclusions. If possible, the image should be easily 'readable' from left to right or top to bottom. It should show the physiological relevance of the manuscript so readers can assess the importance and content of its findings. Abstract Figures should not merely recapitulate other figures in the manuscript. Please try to keep the diagram as simple as possible and without superfluous information that may distract from the main conclusion(s). Abstract Figures must be provided by authors no later than the revised manuscript stage and should be uploaded as a separate file during online submission labelled as File Type 'Abstract Figure'. Please ensure that you include the figure legend in the main article file. All Abstract Figures should be created using BioRender. Authors should use The Journal's premium BioRender account to export high-resolution images. Details on how to use and access the premium account are included as part of this email.

EDITOR COMMENTS

Reviewing Editor:

The review of the manuscript, "Bile acids regulate the epithelial Na+ channel in native tissues through direct binding at multiple sites" that was submitted to The Journal of Physiology is complete, having been assessed by 2 referees as well as the reviewing and senior editors.

The Editors have carefully read your manuscript and considered the points raised by the referees. We are happy to inform you that we recommend to Provisionally Accept this manuscript for publication. The points raised by both referees should be addressed and the additional requirements for the Journal, including more detail on the animals used, as well as completion of a statistical summary document, should be met.

Methods requirements:

- Strain of mice used for split open tubule recordings is missing, as is age/sex.

- Whilst strain of mice used for Isc measurements in distal colon is stated in a figure legend, it should appear in the methods section.

- Details of access to food and water for mice are also missing.

Senior Editor:

Your manuscript has been evaluated in detail by two expert Referees and a Reviewing Editor. While all concur on the potential influence of your study, you will see from the attached critiques that all raise points that must be addressed. I encourage you to address all fully.

In need of greatest attention are 1) consideration of physiological context, 2) rationale for using different mouse models to compare CCD versus colonic effects, and 3) testing whether lack of t-CA effect on ENaC in distal colonic can be attributed to pre-activation. This is a speculation that, as suggested by Ref. 2, can be performed by entailing pretreatment with a protease inhibitor such as aprotin.

Please note that methods should appear in the Methods section rather than within figure legends. The strain of mice used for Isc measurements in distal colon is stated in a figure legend, it should appear in the methods section. Methods also appear in figure legends pertaining to UV crosslinking experiments.

In addition, please include the strain, age, and sex of mice used for split open tubule recordings.

Also please do ensure that details of access to food and water for mice are included.

REFEREE COMMENTS

Referee #1:

In this study the authors set out to investigate how bile acids exert their actions on ENaC function. The manuscript is well conceived and presented and potentially provides new insights into how bile acids regulate fluid and electrolyte transport across epithelial cells from different organs.

Specific Comments

- On what basis were the bile acids, TCA and THDCA, and their concentrations chosen for study? THDCA is found in porcine bile but are its levels in mouse or human bile likely to be high enough to affect ENaC under any circumstances. Indeed, the concentrations of bile acids employed in this study are very high (1 mM). While such concentrations might occur in the colon during conditions of bile acid malabsorption, is there any evidence to suggest that BAs in the CCD can reach such high levels. Otherwise, the effects reported here may be simply artifactual.

- Figure 2: Data is presented showing that, in contrast to its effects in CCD, T-CA does not induce ENaC activity in mouse colon. However, is this not likely to be due to the differing conditions employed in each experiment? In CCD, TCA was tested on tissues from mice on a normal diet, while in colon the effects of the bile acid were tested on mice on a low salt diet. This would presumably increase basal ENaC activity, thereby masking the effects of TCA in the colon. These experiments would need to be repeated in mice on a normal diet in order to be able to make comparisons between effects of TCA in CCD and colon.

- In general, the text of the Results section contains a lot of methodological information that could be omitted as it is already covered in the Methods section.

Referee #2:

This paper is an interesting paper describing the effects of bile acids on epithelial sodium channels (ENaC) in colon, kidney distal nephron, and several heterologous expression systems. It is well written and the rationale for experiments is clear. There are a few minor problems with the paper.

(1) In the discussion of Fig. 3 the authors speculate that the absence of an effect of t-CA might be due to an effect of prior ENaC activation by endogenous proteases. This does seem likely, but why not test the hypothesis by pretreating the tissue with aprotinin?

(2) Also in Fig.3B the magnitude of the amiloride response appears the same whether t-HDCA is present or not. Is this just the particular tissue sample?

(3) The model is complicated, but the addition of the extra parameters does improve the fit. An F test is appropriate to determine if the extra parameters are justified, but a slightly better description of the implementation of the test would be appreciated.

(4) In equation (1), I believe you mean to have a greek delta (zFδnV).

(5) The lack of binding to ENaC alpha seems inconsistent with the effect of t-CA on alpha expressed alone in oocytes. Have I misunderstood?

(6) Amiloride is not competitive, but that does not seem to preclude t-CA from associating with the ion permeability pathway at a site distinct from the amiloride binding site.

(7) In the section on curve fitting the voltage-dependent data, on the second line I believe you mean "... as a function of voltage ..."

(8) Although not critical, I have been trying to imagine scenarios in which the two bile acids with opposing effects on ENaC in the colon might work in a physiological context. Any speculation on the question?

END OF COMMENTS

Confidential Review **17-May-2022**

Introduction to the response to reviewer critiques

The reviewers agreed that our manuscript presents novel insights into how bile acids regulate fluid and electrolyte transport across epithelia in several systems, but also raised several concerns. Below, we provide a point-by-point response to each of the reviewers' concerns, and outline revisions to the manuscript.

While revising the manuscript, we caught and corrected two errors related to reporting statistics. Neither error affect the conclusions of the work. The first is a reporting error on Figure 4B: the P-value should be 0.0006 for the alpha-beta group, not 0.006 as reported previously. We believe this to be a transcription error. The second error regards Figure 9. Although the statistical test we reported performing is the appropriate test, i.e. ordinary two-way ANOVA with Sidak's post hoc test, the p-values in the original submission came from a repeated measures two-way ANOVA with Sidak's post hoc test, which is not the appropriate test. No differences were detected using either test. We believe this comes from a failure to update the figure during editing. We have updated both figures accordingly. We also caught an error in Figure 7. During figure editing, the aspect ratio of the blot images were inadvertently stretched vertically, making bands appear fuzzier. We have updated the figure to present the affected images with the correct aspect ratio.

Each response is indented and colored blue below the concern it addresses. Times font is used to identify revised manuscript text. Page numbers are given for the starting location of each revision. References within the responses are given as PubMed IDs in parentheses.

Reviewing editor:

Methods requirements:

- Strain of mice used for split open tubule recordings is missing, as is age/sex.

> Response: We have revised Methods subsection *Isolation of split-open renal collecting ducts* (page 5) accordingly.

- Whilst strain of mice used for Isc measurements in distal colon is stated in a figure legend, it should appear in the methods section.

> Response: We have revised accordingly.

- Details of access to food and water for mice are also missing.

> Response: We revised the *Ethical Approval* subsection of Methods (page 5) to note that: All animals had free access to food and water.

Senior editor:

Your manuscript has been evaluated in detail by two expert Referees and a Reviewing Editor. While all concur on the potential influence of your study, you will see from the attached critiques that all raise points that must be addressed. I encourage you to address all fully.

In need of greatest attention are 1) consideration of physiological context, 2) rationale for using different mouse models to compare CCD versus colonic effects, and 3) testing whether lack of t-CA effect on ENaC in distal colonic can be attributed to pre-activation. This is a speculation that,

as suggested by Ref. 2, can be performed by entailing pretreatment with a protease inhibitor such as aprotin.

> Response: We have revised the manuscript to further discuss physiological context. We also performed new experiments to address concerns about the different mouse models used in CCD vs colon experiments. We think that the speculation that low salt diet/aldosterone increased ENaC cleavage in the colon is worthy of a stand-alone detailed investigation. Each response is detailed below.

Please note that methods should appear in the Methods section rather than within figure legends. The strain of mice used for Isc measurements in distal colon is stated in a figure legend, it should appear in the methods section. Methods also appear in figure legends pertaining to UV crosslinking experiments.

> Response: We have revised legends for Figures 3-4, and 7-9 to remove methods, as advised. In response to reviewer 1, we also revised the Results describing crosslinking experiments to remove methods descriptions.

In addition, please include the strain, age, and sex of mice used for split open tubule recordings.

> Response: We revised the Methods subsection *Isolation of split-open renal collecting ducts* (page 5) accordingly.

Also please do ensure that details of access to food and water for mice are included.

> Response: We revised the Methods subsection *Ethical Approval* (page 5) accordingly.

Referee #1:

- On what basis were the bile acids, TCA and THDCA, and their concentrations chosen for study? THDCA is found in porcine bile but are its levels in mouse or human bile likely to be high enough to affect ENaC under any circumstances. Indeed, the concentrations of bile acids employed in this study are very high (1 mM). While such concentrations might occur in the colon during conditions of bile acid malabsorption, is there any evidence to suggest that BAs in the CCD can reach such high levels. Otherwise, the effects reported here may be simply artifactual.

> Response: One objective of our study was to determine whether regulation observed in other systems reflected function in mouse tissues. TCA and THDCA were chosen because they had opposing effects on mouse ENaC in our previous study (31092599), and seemed a good test of the hypothesis. While 1 mM concentrations were used to facilitate detection in inherently noisier native tissue preparations, we observed effect-magnitudes in the CCD similar to those in oocytes (compare Fig. 2 to Figs. 4-6). Oocyte data show that 200-300 μM gives about half of the effect of 1 mM of the same compound.
>
> In humans and mice, TCA and other bile acids are likely high in the biliary tree, where ENaC is present (26475057). We are unaware of direct measurements in the CCD, but blood and urine levels in liver disease patients suggest high levels. Reported serum values are ~140 μM for alcoholic hepatitis patients, and similar for hepatitis C patients with cirrhosis (29654817, 21348908). There is a strong log-log correlation between

urinary bile acid levels normalized to creatinine and serum bile acid levels (21348908). Based on the blood-urine correlation, serum levels reported in alcoholic hepatitis and hepatitis C patients, and low urine volume in cirrhotic patients (<0.5 L/day), we expect urinary concentrations for many of the sickest patients to approach the millimolar range. These data could mean high CCD bile acid concentrations in liver disease, but we cannot be certain.

We have revised the second paragraph of the Discussion (page 17) to note DCA as a secondary bile acid, which is more relevant in humans, and to include some of the points above:

Given the correlation between blood and urine bile acid levels and the significant reduction in urine volumes observed in cirrhotic patients (24772051, 21348908), urine bile acid levels likely fall into the 0.1 – 1 mM range for the sickest patients. Whether high urinary concentrations correspond to high concentrations in the CCD is uncertain. If so, bile acids may aberrantly regulate ENaC in the kidney.

- Figure 2: Data is presented showing that, in contrast to its effects in CCD, T-CA does not induce ENaC activity in mouse colon. However, is this not likely to be due to the differing conditions employed in each experiment? In CCD, TCA was tested on tissues from mice on a normal diet, while in colon the effects of the bile acid were tested on mice on a low salt diet. This would presumably increase basal ENaC activity, thereby masking the effects of TCA in the colon. These experiments would need to be repeated in mice on a normal diet in order to be able to make comparisons between effects of TCA in CCD and colon.

Response: We agree that the different conditions employed likely contributed to the different t-CA results between the CCD and colon. We therefore performed new experiments to test t-CA on colons from mice on a normal diet (page 12 and Figure 3A, B). Indeed, t-CA increased ENaC-mediated currents, consistent with results in the CCD and heterologous systems.

- In general, the text of the Results section contains a lot of methodological information that could be omitted as it is already covered in the Methods section.

Response: We have revised the Results describing crosslinking experiments and legends for Figs. 3-4, and 7-9 to remove methods already covered in the Methods section.

Referee #2:

This paper is an interesting paper describing the effects of bile acids on epithelial sodium channels (ENaC) in colon, kidney distal nephron, and several heterologous expression systems. It is well written and the rationale for experiments is clear. There are a few minor problems with the paper.

(1) In the discussion of Fig. 3 the authors speculate that the absence of an effect of t-CA might be due to an effect of prior ENaC activation by endogenous proteases. This does seem likely, but why not test the hypothesis by pretreating the tissue with aprotinin?

Response: We now include experiments testing the effect of t-CA on colons harvested form mice fed a normal salt diet (page 12, Figure 3A, B). These experiments show that t-CA increased ENaC-mediated currents in the colon under these conditions, consistent with results in the CCD, cultured cells, and oocytes. We think our speculation that low salt diet/aldosterone increased ENaC cleavage in the colon is likely true, but also believe it is a question worthy of a stand-alone detailed investigation.

(2) Also in Fig.3B the magnitude of the amiloride response appears the same whether t-HDCA is present or not. Is this just the particular tissue sample?

Response: We did not detect differences in the amiloride response after t-HDCA treatment in this experiment. When t-HDCA was absent, the amiloride response was 56 ± 28 μA/cm^2. When it was present, the amiloride response was 42 ± 22 μA/cm^2. Although the direction is consistent with t-HDCA inhibition, we did not detect differences between the groups ($p = 0.47$ by Student's t test). The traces shown reflect this similarity.

(3) The model is complicated, but the addition of the extra parameters does improve the fit. An F test is appropriate to determine if the extra parameters are justified, but a slightly better description of the implementation of the test would be appreciated.

Response: We have added a description of our implementation of the F-test to the end of the *Curve fitting for voltage-dependent data* section of the Methods (page 9).

(4) In equation (1), I believe you mean to have a greek delta (zFδnV).

Response: Corrected, thank you.

(5) The lack of binding to ENaC alpha seems inconsistent with the effect of t-CA on alpha expressed alone in oocytes. Have I misunderstood?

Response: You have not! We speculate that our inability to detect direct binding in crosslinking studies (Fig. 7B) stems from non-specific binding or multiple binding sites that masked the ability of any of our unlabeled bile acids to prevent crosslinking, but we cannot know. Consistent with this hunch, crosslinking measurements to alpha was relatively higher and noisier than to either beta or gamma in Figure 7.

(6) Amiloride is not competitive, but that does not seem to preclude t-CA from associating with the ion permeability pathway at a site distinct from the amiloride binding site.

Response: While we favor a voltage-dependent site outside of the ion permeation pathway, we agree that we cannot rule out a site in the pathway that avoids blocking channel currents. We have revised the Results to remove that interpretation, and the Discussion to replace "outside the ion permeation pathway", to read (page 18):

Our data also provide no evidence for competition between amiloride and bile acid binding. A binding site that avoids blocking channel currents near the Ilyaskin site could be consistent with the lack of competition and differences in both δ values and effects on ENaC function.

(7) In the section on curve fitting the voltage-dependent data, on the second line I believe you mean "... as a function of voltage ..."

Response: Corrected, thank you.

(8) Although not critical, I have been trying to imagine scenarios in which the two bile acids with opposing effects on ENaC in the colon might work in a physiological context. Any speculation on the question?

Response: The microbiome is responsible for converting primary bile acids, all of which activate ENaC, to secondary bile acids, some of which may inhibit ENaC. The composition of the bile acid pool depends on the specific microbes colonizing each individual. We speculate that the microbiome could influence fecal fluid content via the bile acid pool and its effects on ENaC activity. We have revised the second paragraph of the Discussion (page 17) to include this speculation.

Reflecting recycling, healthy subject bile acid pools include both primary and secondary bile acids (29654817), and both may be relevant in regulating ENaC in the biliary tree and gut. Here, ENaC has a role in regulating luminal fluids (18355814, 26475057). As bile acids variously regulate ENaC function, these compounds may account for part of the microbiome's influence on intestinal ion transport (27284010).

Dear Dr Kashlan,

Re: JP-RP-2022-283318R1 "Bile acids regulate the epithelial Na+ channel in native tissues through direct binding at multiple sites" by Xue-Ping Wang, Viktor Tomlin, Andrew J Nickerson, Runze Tian, Merve Ertem, Abagail McKernan, Xiaoguang Lei, Oleh Pochynyuk, and Ossama Kashlan

I am pleased to tell you that your paper has been accepted for publication in The Journal of Physiology.

NEW POLICY: In order to improve the transparency of its peer review process The Journal of Physiology publishes online as supporting information the peer review history of all articles accepted for publication. Readers will have access to decision letters, including all Editors' comments and referee reports, for each version of the manuscript and any author responses to peer review comments. Referees can decide whether or not they wish to be named on the peer review history document.

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Yours sincerely,

Dr Peying Fong
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EDITOR COMMENTS

Reviewing Editor:

We are grateful to the authors for responding to all comments raised by the referees and editors, all points have been sufficiently answered.

Senior Editor:

Thank you for your responsiveness to comments raised in the previous round of review.

The Referees and Reviewing Editor agree that all concerns were addressed adequately.

Please accept my congratulations on a job well-done.

REFEREE COMMENTS

Referee #1:

No further comments.

Referee #2:

I found the original paper to be interesting with a few minor problems. The authors have thoughtfully and completely addressed all of my concerns and suggestions.

1st Confidential Review **10-Aug-2022**