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April 10, 2016

Dear Editors,

Thank you for reviewing our manuscript entitled "TRPV4-dependent calcium influx and ATP release in mouse and rat gastric epithelia" (Manuscript No. 24955) that we submitted for publication in *World Journal of Gastroenterology*.

The manuscript has been thoroughly revised to address the points raised by the reviewers. Please find attached the revised version of the manuscript in which the changes are highlighted, and our point-by-point responses to the reviewers' comments. We hope that the revised manuscript is now acceptable for publication in *World Journal of Gastroenterology*.

We would like to thank the referee for his/her helpful comments and suggestions.

Yours sincerely,

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Replies to the comments from the reviewer

Report on the manuscript entitled: "TRPV4-dependent calcium influx and ATP release in mouse gastric epithelium" by Mihara and colleagues

The manuscript describes an original research performed in mouse stomach and in rat epithelium cell line focusing on the expression and function of TRPV4 ion channels. The study concept is based on previous findings of the authors regarding TRPV4-mediated ATP release on the esophagus. The series of experiments in this manuscript demonstrated delayed gastric emptying in TRPV4 knockout mice compared to their WT littermates, as well as TRPV4 expression in mRNA and protein levels in both mouse and rat gastric epithelial cell line. Increased intracellular Ca2+ concentrations and/or evoked TRPV4-like current activities were measured in WT and RGE1-01 cells, but not TRPV4KO cells by fluorescent Ca2+-imaging after the application of GSK1016790A, a TRPV4-specific agonist. The authors also measured ATP release using luciferin-luciferase assay after GSK1016790A or mechanical stimuli from RGE1-01 cells. The results of the present study suggest a novel diagnostic and therapeutic target for functional gastrointestinal disorders.

In general, the idea is interesting, the various morphological and functional methods are sophisticated, the figures are demonstrative. The following points should be taken into consideration:

Comments:

1) *The title of the manuscript suggests that the experimental work of the authors was focused only on mouse gastric epithelium. I would recommend to alter the title so that it refers to the examined rat gastric epithelial cell line as well.*

We thank the reviewer for this recommendation. Accordingly, we revised our title to "TRPV4-dependent calcium influx and ATP release in mouse and rat gastric epithelia".

2) *The last sentence of the abstract, where the authors state that TRPV4 is expressed in gastric epithelium is too general, it should include mouse and rat gastric epithelium to be more precise.*

We appreciate the reviewer's point. We have revised the final sentence of the abstract to read: "TRPV4 is expressed in mouse and rat gastric epithelia and contributes to ATP release and gastric emptying."

3) *Part of the experimental work is done with GSK1016790A, a TRPV4-specific agonist. At*

least a reference or a whole sentence should be dedicated to this compound in the introduction.

We thank the reviewer for raising this point. We now state that GSK1016790A is a specific agonist: "TRPV4 is also activated by the specific agonist GSK1016790A that elicits whole-cell currents in mouse and rat TRPV4-expressing cells with EC_{50} values of 18.5 and 10 nM, respectively[3]."

Reference

3 Willette RN, Bao W, Nerurkar S, Yue TL, Doe CP, Stankus G, Turner GH, Ju H, Thomas H, Fishman CE, Sulpizio A, Behm DJ, Hoffman S, Lin Z, Lozinskaya I, Casillas LN, Lin M, Trout RE, Votta BJ, Thorneloe K, Lashinger ES, Figueroa DJ, Marquis R, Xu X. Systemic activation of the transient receptor potential vanilloid subtype 4 channel causes endothelial failure and circulatory collapse: Part 2. *J Pharmacol Exp Ther* 2008; **326**(2): 443-452 [PMID: 18499744 DOI: 10.1124/jpet.107.134551]

4) *"TRPV4 expressed in intestinal epithelial cells causes increases in intracellular calcium concentrations, chemokine release and colitis[4] as well as increased paracellular permeability[5]." – The authors should differentiate between physiological funtions of TRPV4 and its role in different pathological processes.*

We understand the reviewer's concern and revised our manuscript to differentiate the two functions by stating: "Although the physiological function of TRPV4 expression in intestinal epithelial cells is unknown, TRPV4 activation in these cells causes increases in intracellular calcium concentrations, chemokine release, and incidence of colitis, as well as increased paracellular permeability".

5) *Stating the purpose of the present study at the end of the introduction the authors focus only on mouse stomach solely, while experiments on rat cell line compose considerable part of the experimental work.*

We agree with the reviewer's comment. Accordingly we revised relevant passages in our manuscript to "mouse and rat".

6) *How much did the mice weigh at the time of the experiment?*

The mice weighed between 23 and 25 g at the time of the experiments. We added this information to the Methods section.

7) *In the Materials & Methods part the authors should mention that RGE-01 is a rat cell line.*

We agree with the reviewer's suggestion. Accordingly, we revised our manuscript to indicate that RGE1-01 is a rat gastric epithelial cell line.

8) *The reverse transcription PCR analysis method description is lacking some pieces of information regarding how the isolated RNA content was measured and whether the samples underwent DNase digestion or not.*

We appreciate the reviewer's concern. We revised our manuscript to include additional detail: "Total RNA (1 μg) was isolated using the RNeasy Mini Kit (Qiagen, Courtaboeuf, France) and measured with a NanoDrop device (Thermo Fisher Scientific Inc., Wilmington, USA). Genomic DNA was eliminated in the process of reverse transcription (QuantiTect Reverse Transcription Kit, QIAGEN)."

9) *The mTRPV4 primer sets did not yield TRPV4 specific sequence in the primer blast, was there a mispelling in the sequence?*

We confirmed these sequences again. The mTRPV4 primer sets have been used for mouse TRPV4 by several researchers, including us, as referenced in the following publications:

http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3417458/

http://www.jbc.org/content/284/32/21257.full.pdf

http://www.jbc.org/content/early/2009/06/12/jbc.M109.020206.full.pdf

We attach a PDF file that lists the primer sets that specifically recognize mouse TRPV4 mRNA. Even if the primer sets do not recognize the sequence for AMPA3 (alpha 3) (Gria3), *Mus musculus* fer-1-like 5 (*C. elegans*) (Fer1l5) or others, we would still be able to differentiate the products based on the predicted 401 bp size.

10) *Figure 1: the gel photo of the RT-PCR reaction should include a DNA ladder, otherwise the result can be questionable. Either figure captions or Table 1 with the primer sequences should include information on product length.*

We agree with the reviewer's suggestion, which is related to Comment 9. We revised the Figure 1 legend to read: "The expected sizes of the amplified fragments for *TRPV4* and *GADPH* were 404 and 545 bp for mouse and 524 and 268 bp for rat, respectively.".

11) *Regarding Ca2+-imaging measurement the authors should clarify the % of reacting cells. Or if the n=20 is the number of reacting cells, then how many cells did the authors measure? In what % of the TRPV4KO cells did GSK induce Ca2+ influx?*

Please explain why it is necessary to incubate cells with GSK for 100 sec, should not 30 sec be enough?

We understand the reviewer's concern. First, please see the attached supplemental figure that shows representative traces of intracellular Ca^{2+} concentrations of gastric epithelial cells isolated from (A) WT and (B) TRPV4 knockout mice. Responses to GSK varied from cell to cell (strong, weak, or no response) in WT mice, while no responses were observed in TRPV4 knockout mice. As response rates were dependent on its threshold, which makes the rates arbitrary, we decided to calculate a response delta ratio in all ionomycin-responsive cells between WT and TRPV4 knockout mice. We isolated 53 and 41 ionomycin-responsive cells from six WT mice and five TRPV4 knockout mice, respectively. According to our immunohistochemical (Fig. 2) and electrophysiological study that shows five responsive cells in five trials using WT gastric epithelial cells (Fig. 4), the majority of gastric epithelial cells express TRPV4 to some degree.

Regarding the incubation time, as shown in the attached supplemental figure, all cells had distinctive response times. Since some cells responded 30 sec after GSK application, we decided to incubate cells with GSK for 100 sec.

According to the reviewer's concerns, we have revised our manuscript to state: "Since the degree of responses (strong, weak, or no response) to GSK varied from cell to cell in WT gastric epithelial cells, we expressed the observed changes in all ionomycin-responsive cells as a ratio between WT and TRPV4 knockout mice (Δ). We evaluated 53 and 41 ionomycin-responsive cells from six WT mice and five TRPV4 knockout mice, respectively. Given the variations in response times, and that some WT cells responded 30 sec after GSK application, we decided to incubate cells with GSK for 100 sec."

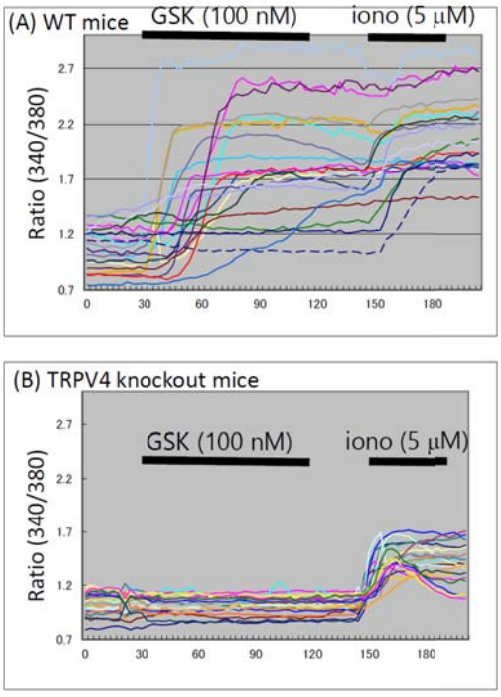

Fig Representative traces of intracellular Ca^{2+} concentrations of gastric epithelial cells isolated from (A) WT and (B) TRPV4 knockout mice.