nature portfolio

Peer Review File

Noncanonical calcium-independent TRPM4 signaling governs intestinal fluid homeostasis

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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)
Here, Liu, Hu, Xue and Huang et al. studied the mechanisms underlying bisacodyl treatment of constipation. Remarkably, they identified TRPM4 as the physiological target and further defined that the active metabolite, DAB, acts through a previously unknown binding site in the membrane domain of the receptor. Animal studies, electrophysiology, and cryo-EM are integrated with a very clear presentation to connect the mechanistic pieces. I found the study easy to follow and my impression is that it will be quite impactful in revealing both the physiological mechanism for a widely used drug and a new binding side in a TRP channel. I have no major concerns, just a few questions, mainly centered on the structure and biophysics.

1. Do the EC50's for BIC and DAB match reasonably with the concentrations achieved in vivo when humans take BIC?
2. Line 150 and Fig. 2: currents look larger at positive holding potentials than negative holding potentials, indicating outward, rather than inward rectification, correct? Maybe I am missing something. Regardless, can the authors speculate on the mechanistic basis of the rectification?
3. Does TRPM4 desensitize in the sustained presence of DAB? Might the DAB-bound structure represent a desensitized state, rather than an intermediate or pre-activated state? Or, do the top panels in Fig. 2 indeed report that DAB does not cause TRPM4 to desensitize (and ED Fig. 7A)?
4. Please elaborate on how asymmetric the Ca+DAB structure was, related to why symmetry expansion was needed and helpful- did this improve ligand density?

Reviewer #2

(Remarks to the Author)
This study identifies TRPM4 as the key intestinal epithelial target of the laxative metabolite deacetyl bisacodyl (DAB). DAB activates TRPM4 through a novel calcium-independent mechanism, triggering Na^+ influx that drives Cl^- secretion via the TRPM4→VGCC/NCX→ANO1 pathway. Structural analysis reveals a new ligand-binding site in TRPM4, highlighting a potential therapeutic target for chronic constipation and other gastrointestinal disorders.
This is a well-designed and compelling study, with mechanistic consistency and high-quality structural and functional data. The genetic validation and signaling pathway analyses are particularly robust. However, the broad impact on the field and therapeutic relevance beyond chronic constipation are not fully substantiated. The authors should better contextualize which gastrointestinal disorders are most likely to benefit from these findings and moderate claims regarding the immediacy of therapeutic application.

Detailed comments:
1. The identification of DAB as the first non-Ca^{2+} TRPM4 agonist represents a clear advance in TRPM4 biology. The discovery of a novel binding pocket is also noteworthy. However, the manuscript broadly claims a "novel therapeutic strategy" for gastrointestinal disorders, but the specific diseases beyond chronic constipation are not well delineated. The clinical relevance to diarrhea, inflammatory bowel disease, or other GI disorders remains speculative. The authors should clarify which conditions are most likely to benefit from TRPM4-targeted therapies, supported by preclinical and more extensive epidemiological rationale.
2. All in vivo studies have been focused on acute effects (24–36 h). How did the authors choose the time points? Chronic

effects, potential desensitization, or broader impacts on intestinal absorption/electrolyte homeostasis are not addressed. This point limits the translational significance of the findings.
3. In the paragraph “DAB activates TRPM4 via a TMD-focused mechanism” the authors conclude that the DAB-bound channel pore remains closed, possibly representing a pre-open or partially activated state. However, this appears inconsistent with the full channel activation observed in functional assays (in vitro and in vivo). Please clarify how this structural state correlates with full activation, and whether it reflects a true intermediate in the activation pathway.
4. While the mechanistic findings presented are compelling, the broader physiological significance of TRPM4 activation—either under normal homeostatic conditions or in disease contexts—is not sufficiently explored.
5. The use of HT-29 cells (colon cancer cell line) does not fully recapitulate the human intestinal physiology.
6. The rationale for the in vivo dosing should be justified, specifically in relation to clinically relevant concentrations.
7. Most figures report N = number of cells or mice, but it is sometimes unclear whether statistical tests account for technical or biological replicates.

Reviewer #3

(Remarks to the Author)
I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The authors have satisfyingly addressed my comments and questions.

Reviewer #2

(Remarks to the Author)
The authors have made some revisions; however, our primary concerns remain largely unresolved, and the manuscript overall impact continues to be limited by the insufficient demonstration of disease relevance, translational context, and a fully supported structure–function relationship. Although some textual clarifications were added, these do not adequately address the substantive scientific gaps identified in the first revision.
The principal issues are as follows: the requested preclinical data and broader epidemiological justification for point 1 remain absent, thereby leaving the claims of disease relevance insufficiently supported; the response to point 3 persists as largely speculative in the absence of additional data; point 4 was not addressed in the revised manuscript; and the reply to point 5 still fails to provide a concrete experimental or methodological strategy to overcome the known limitations of the current model.
Overall, the revisions rely primarily on textual adjustments rather than the additional experimental or analytical work necessary to substantiate the central claims, and further data together with deeper mechanistic support would be needed for the manuscript to meet the level of rigor and impact expected for publication in Nature Communications.

Reviewer #3

(Remarks to the Author)
I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

We thank the reviewers for their thoughtful evaluation of our manuscript and for their constructive comments, which have helped us to improve both the clarity and rigor of the work. Below, we provide detailed responses to each point and describe the corresponding revisions made in the manuscript.

Reviewers' comments:

Reviewer #1 (Remarks to the Author)

Here, Liu, Hu, Xue and Huang et al. studied the mechanisms underlying bisacodyl treatment of constipation. Remarkably, they identified TRPM4 as the physiological target and further defined that the active metabolite, DAB, acts through a previously unknown binding site in the membrane domain of the receptor. Animal studies, electrophysiology, and cryo-EM are integrated with a very clear presentation to connect the mechanistic pieces. I found the study easy to follow and my impression is that it will be quite impactful in revealing both the physiological mechanism for a widely used drug and a new binding side in a TRP channel. I have no major concerns, just a few questions, mainly centered on the structure and biophysics.

Response: Thank you for your positive and insightful feedback on our manuscript. In response, we have carefully revised the manuscript to address your concerns.

1. Do the EC_{50}'s for BIC and DAB match reasonably with the concentrations achieved in vivo when humans take BIC?

Response: Thank you for the question. It is well established that bisacodyl (BIC) is converted by intestinal deacetylase enzymes into its active metabolite, bis-(p-hydroxyphenyl)-pyridyl-2-methane (DAB) [1]. Following the administration of BIC suppositories, the parent drug was undetectable in serum, while its metabolite DAB reached concentrations of 10-55 μg/L (approx. 36-198 nM). However, only 3.36±0.52% of the dosed drug was excreted in urine, indicating its low systemic absorption [2]. Thus, the absorption of the BIC or DAB is likely not required and the laxative effect is proposed to be occurred locally [3]. There is no data available regarding the BIC and DAB levels in the intestinal system. Nevertheless, *ex vivo* studies have established that a high concentration of BIC (10 μg/mL, ~27 μM) is required to enhance contractility in human large intestinal muscle strips [4]. In contrast, its active metabolite DAB exerts its effects—including enhanced colon mucosal secretion and increased muscle tone in both the small bowel and colon—at much lower concentrations (0.5-5 μM) [5]. In the manuscript, we demonstrate that DAB and BIC activate TRPM4 with EC_{50} values of 0.14 and 2.64 μM, consistent with the observation that DAB is much more potent than BIC to stimulate muscle contraction and mucosal secretion [4,5]. The discrepancy between the low EC_{50} values for TRPM4 activation (BIC: 2.64 μM; DAB: 0.14 μM) and the higher concentrations required in *ex vivo* muscle contraction/mucosal secretion is likely due to reduced bioavailability and system complexity. In the *ex vivo* setting, penetration through dense tissue barriers lowers the effective concentration of both compounds at their target site. Furthermore, to trigger a measurable functional

contraction, a threshold numbers of TRPM4 channels must be activated, a requirement that is absent in simplified *in vitro* systems. We have incorporated this information into the first paragraph of ***Discussion*** section (Lines 350-362).

2. Line 150 and Fig. 2: currents look larger at positive holding potentials than negative holding potentials, indicating outward, rather than inward rectification, correct? Maybe I am missing something. Regardless, can the authors speculate on the mechanistic basis of the rectification?

Response: Thanks reviewer points this out. We are sorry for the typo in the original manuscript (Inwardly rectified Na^+ current, Line 153). At the lower concentration ($\leqslant$ 0.3 μM), DAB elicits outwardly rectified Na^+ currents, whereas at higher concentrations, the I-V curves became linear. Such effect is consistent with voltage-dependent gating of TRPM4 channels [6]. We have changed the description in the manuscript accordingly (Lines 154-155).

3. Does TRPM4 desensitize in the sustained presence of DAB? Might the DAB-bound structure represent a desensitized state, rather than an intermediate or pre-activated state? Or, do the top panels in Fig. 2 indeed report that DAB does not cause TRPM4 to desensitize (and ED Fig. 7A)?

Response: Thank you very much for raising your concern, and we fully agree that it could represent a desensitized state. However, we prefer to interpret this structure as representing a pre-open pore conformation, for the following reasons:

Functionally, DAB at low concentrations gradually increased the TRPM4 currents [both inward (-100 mV) and outward (+100 mV)] with the higher concentrations reach the plateau much faster. During the recording (10 min), we didn't observe channel desensitization. Moreover, the laxative effect of DAB persists for at least 48 h indicating its sustained activation *in vivo*. Calcium, the canonical activator, rapidly stimulates the TRPM4 currents with rapid desensitization to a plateau level that can be reversed by addition of PIP_2 [7]. However, DAB activation of TRPM4 is not dependent on the Ca^{2+} ions.

Structurally, although it could represent a desensitized state, we think it is more likely to be a pre-open state. First, how TRPM4 becomes desensitized structurally is not clear, so we do not have evidence to support that this is a desensitized state. Second, we speculate that opening of the channel requires other factors besides DAB in our cryo-EM experiment. The absence of those factors, together with this DAB-bound conformation being in between the closed and open conformations, makes us think this is more likely to be a pre-open or intermediate state rather than a desensitized state.

Together, we interpret the $TRPM4_{DAB}$ structure as a pre-open state, while acknowledging that a desensitized conformation cannot be entirely ruled out.

4. Please elaborate on how asymmetric the Ca^{2+} DAB structure was, related to why

symmetry expansion was needed and helpful- did this improve ligand density?

Response: We thank the reviewer for this insightful question. The Ca^{2+}-DAB structure is overall C4 symmetric, with all four subunits clear and consistent density for both the Ca_{TMD} and DAB in the consensus C4 reconstruction. Symmetry expansion was not applied to enhance the ligand or transmembrane domain density, which were already well resolved in the initial consensus map. Instead, it was specifically used to improve the local resolution of the MHR1/4 region, which exhibited conformational variability and reduced local resolution in the consensus reconstruction. By performing symmetry expansion followed by focused classification, we were able to sort out this heterogeneity and obtain improved density for the MHR1/4 region. We have clarified this point in the ***Methods*** (Lines 595-596).

Reviewer #2 (Remarks to the Author):

This study identifies TRPM4 as the key intestinal epithelial target of the laxative metabolite deacetyl bisacodyl (DAB). DAB activates TRPM4 through a novel calcium-independent mechanism, triggering Na^+ influx that drives Cl^- secretion via the TRPM4→VGCC/NCX→ANO1 pathway. Structural analysis reveals a new ligand-binding site in TRPM4, highlighting a potential therapeutic target for chronic constipation and other gastrointestinal disorders.
This is a well-designed and compelling study, with mechanistic consistency and high-quality structural and functional data. The genetic validation and signaling pathway analyses are particularly robust. However, the broad impact on the field and therapeutic relevance beyond chronic constipation are not fully substantiated. The authors should better contextualize which gastrointestinal disorders are most likely to benefit from these findings and moderate claims regarding the immediacy of therapeutic application.

Response: Thank you very much for reviewing our manuscript and providing constructive comments to improve our manuscript. We fully agree with your point that the broad impact on the field and the therapeutic relevance beyond chronic constipation could be better substantiated.

1. The identification of DAB as the first non-Ca^{2+} TRPM4 agonist represents a clear advance in TRPM4 biology. The discovery of a novel binding pocket is also noteworthy. However, the manuscript broadly claims a "novel therapeutic strategy" for gastrointestinal disorders, but the specific diseases beyond chronic constipation are not well delineated. The clinical relevance to diarrhea, inflammatory bowel disease, or other GI disorders remains speculative. The authors should clarify which conditions are most likely to benefit from TRPM4-targeted therapies, supported by preclinical and more extensive epidemiological rationale.

Response: Thank you for these constructive comments. We agree that the statement "novel therapeutic strategy for gastrointestinal disorders" remains speculative. Thus, in

the revised manuscript, we have revised the statement "*novel therapeutic strategy for gastrointestinal disorders*" to be *"a novel therapeutic strategy for constipation arising from a variety of etiologies, including neurogenic bowel dysfunction, opioid-induced constipation, colorectal malignancy, and constipation-predominant irritable bowel syndrome (IBS-C)"* in the ***Introduction*** (Lines 38-43; 84) and ***Discussion*** section (Lines 405-407). This is because bisacodyl is widely used to treat acute and chronic constipation arising from a variety of etiologies, including neurogenic bowel dysfunction, opioid-induced constipation, colorectal malignancy, and constipation-predominant irritable bowel syndrome (IBS-C) [3,8]. It is also used to prepare the colon for colonoscopy.

We have previously explored the role of TRPM4 on the onset and development of ulcerative colitis. However, deletion of *Trpm4* had no significant effect on the onset and development in mouse ulcerative colitis model (data not shown) suggesting that TRPM4 is not critical in the ulcerative colitis progression. We haven't tested the role of TRPM4 in other GI disorders that we will continue to explore. In this manuscript, we indeed aimed to discover a TRPM4 probe that is not readily available in literature, and we identified BIC and its active metabolite DAB are potent TRPM4 activators. Given BIC is a widely used laxative in clinical practice, we explored its effect on the intestinal fluid homeostasis and demonstrated that DAB triggers its laxative effect through TRPM4, extending the role of TRPM4 in the GI system. DAB thus may represent a molecular probe to investigate the role of TRPM4 pathophysiology.

2. All in vivo studies have been focused on acute effects (24–36 h). How did the authors choose the time points? Chronic effects, potential desensitization, or broader impacts on intestinal absorption/electrolyte homeostasis are not addressed. This point limits the translational significance of the findings.

Response: Thank you for raising this constructive question. In the manuscript, we only showed the DAB laxative effect at 24 h. In the preliminary study, we explored the DAB laxative effects every 12 h as shown in **Fig. R1**. After 12 h of DAB oral gavage, DAB caused laxative effects that reached plateau at 24 h and persisted to 48 h. We are not able to do longer time points since the IUCAU is not permitted.

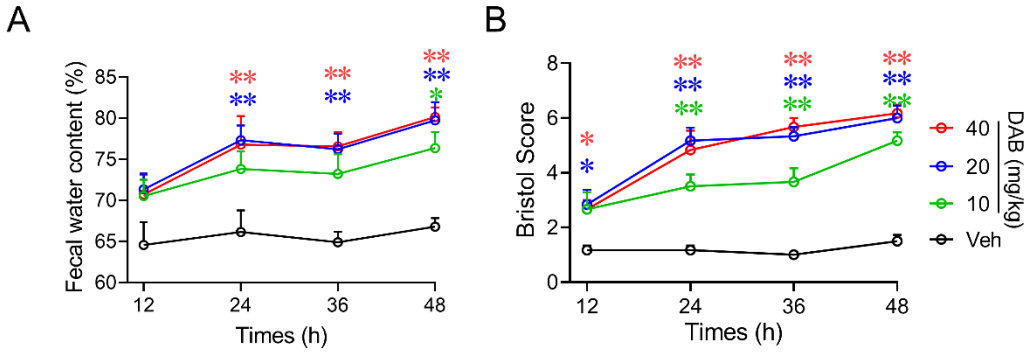

Figure R1: Laxative effects of different dose of DAB in mice. (A) Fecal water content and (B) Bristol score of the stool collected at different time points. N = 6 mice. *, P <

0.05; **, P < 0.01, vs. Veh at respective time point. Two-Way ANOVA

3. In the paragraph "DAB activates TRPM4 via a TMD-focused mechanism" the authors conclude that the DAB-bound channel pore remains closed, possibly representing a pre-open or partially activated state. However, this appears inconsistent with the full channel activation observed in functional assays (in vitro and in vivo). Please clarify how this structural state correlates with full activation, and whether it reflects a true intermediate in the activation pathway.

Response: We thank the reviewer for raising this insightful point. We conclude that the structures determined in the cryo-EM experiments represent a pre-open or intermediate state, as the pore remains closed. Under our cryo-EM conditions, DAB binding alone is likely insufficient to trigger full channel opening, since many key cellular factors are absent compared to *in vitro* and *in vivo* environment, including membrane potential and endogenous lipids. These differences likely account for the discrepancy between the structural and functional results, an observation commonly encountered in ion channel studies. Since all *in vitro* and *in vivo* data were obtained under physiological conditions, we believe that the functional assays best reflect the true effect of DAB.

4. While the mechanistic findings presented are compelling, the broader physiological significance of TRPM4 activation—either under normal homeostatic conditions or in disease contexts—is not sufficiently explored.

Response: Thank you for this thoughtful comment. The primary goal of the current study was to identify a TRPM4 probe, such tools have been lacking. We identified BIC and its active metabolite, DAB, are potent TRPM4 activators. Given BIC is a widely used laxative in clinical practice, we further explored its effect on the intestinal fluid homeostasis and demonstrated that DAB triggers its laxative effect through TRPM4 activation, thereby extending the role of TRPM4 in the GI system. We fully agree that the broader implications of TRPM4 activation in health and disease are of great interest. Ongoing studies in our lab are addressing these questions, but they are beyond the scope of the present work.

5. The use of HT-29 cells (colon cancer cell line) does not fully recapitulate the human intestinal physiology.

Response: Thank you very much for your comments. We agree it is better to use human enterocytes to perform the experiment. Given the ethical restriction associated with using primary human enterocytes, we turned to the HT-29 cell line, a standard model for intestinal epithelium, to investigate the laxative mechanism of DAB. In this model, we delineated a pathway where DAB activates TRPM4, leading to Ca^{2+} influx predominantly through L-type Ca^{2+} channels, which in turn stimulates Ca^{2+}-activated Cl^- channels (CaCCs). This role of CaCCs is confirmed by our *in vivo* experiments using the inhibitor $CaCC_{inh}$-A01, as presented in the manuscript (**Fig. 4H&I**). Moreover, our conclusion that L-type Ca^{2+} channels primary mediate Ca^{2+} influx is consistent with the established finding that DAB-induced secretion in the human intestine is

independent of voltage-gated sodium channels but dependent on L-type Ca^{2+} channels [5]. A sentence "*This VGCC-mediated Ca^{2+} influx is consistent with the established finding that DAB-induced secretion in the human intestine is dependent on L-type Ca^{2+} channels* [5]." has been added into the revised manuscript ***Discussion*** section (Lines 388-390).

6. The rationale for the in vivo dosing should be justified, specifically in relation to clinically relevant concentrations.

Response: We thank the reviewer for this comment. In clinical practice, the dose of BIC is 5-15 mg/day, that equivalent to doses in mice 1.3 mg/kg- 5 mg/kg. We initially performed the dose-response relationships of DAB laxative effect (**Fig. R1**). We chose 20 mg/kg for further study because it produced robust and sustained response. This dose is relatively higher than BIC used in clinical practice. However, it should be noted that, the BIC used in clinical practice is formulated to be enteric-coated tablet that enables the drugs to be released in the intestine while in mice, because of the technique difficulty, no enteric-coated tablet for mice is available. Thus, in our mouse study, DAB may be distributed in entire gastrointestinal system while in human, BIC is only distributed in the intestinal system and converted to be DAB. Such different formulations may explain why higher DAB dose should be used in mice.

7. Most figures report N = number of cells or mice, but it is sometimes unclear whether statistical tests account for technical or biological replicates.

Response: We thank the reviewer for the helpful suggestions. In this revised manuscript, we have incorporated the information of the N numbers.

Reference

1 Kamm, M. A. *et al.* Oral Bisacodyl Is Effective and Well-Tolerated in Patients With Chronic Constipation. *Clin Gastroenterol Hepatol* **9**, 577-583, doi:10.1016/j.cgh.2011.03.026 (2011).

2 Flig, E., Hermann, T. W. & Zabel, M. Is bisacodyl absorbed at all from suppositories in man? *Int J Pharm* **196**, 11-20, doi:10.1016/S0378-5173(99)00385-3 (2000).

3 Corsetti, M., Landes, S. & Lange, R. Bisacodyl: A review of pharmacology and clinical evidence to guide use in clinical practice in patients with constipation. *Neurogastroenterol Motil* **33**, 14123, doi:10.1111/nmo.14123 (2021).

4 Mitznegg P *et al.* Mode of action of bisacodyl (dulcolax) on isolated muscles of human colon (author's transl). *Klin Wochenschr* **53**, 493-495, doi:10.1007/BF01468637 (1975).

5 Krueger, D., Demir, I. E., Ceyhan, G. O., Zeller, F. & Schemann, M. bis-(p-hydroxyphenyl)-pyridyl-2-methane (BHPM)—the active metabolite of the

laxatives bisacodyl and sodium picosulfate—enhances contractility and secretion in human intestine in vitro. *Neurogastroenterol Motil* **30**, e13311, doi:10.1111/nmo.13311 (2018).

6 Nilius, B. *et al.* Voltage dependence of the Ca2+-activated cation channel TRPM4. *J Biol Chem* **278**, 30813-30820, doi:10.1074/jbc.M305127200 (2003).

7 Nilius, B. *et al.* The Ca2+-activated cation channel TRPM4 is regulated by phosphatidylinositol 4,5-biphosphate. *EMBO J* **25**, 467-478, doi:10.1038/sj.emboj.7600963 (2006).

8 Müller-Lissner, S. *et al.* Bisacodyl and Sodium Picosulfate Improve Bowel Function and Quality of Life in Patients with Chronic Constipation—Analysis of Pooled Data from Two Randomized Controlled Trials. *Open J. Gastroenterol* **07**, 32-43, doi:10.4236/ojgas.2017.71005 (2017).