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Recent advances in the structure, function, and regulation of the volume-regulated anion channels and their role in immunity

Sergei Yanushkevich, Aleksandra Zieminska, Joshua Gonzalez, Francisca Añazco, Richard Song, Alejandra Arias-Cavieres, Sara T. Granados, Junyi Zou, Yan Rao, and Axel R. Concepcion
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Corresponding author(s): Axel Concepcion (aconcepcion@uchicago.edu)

The following individual(s) involved in review of this submission have agreed to reveal their identity: Rajan Sah (Referee #2)

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1st Editorial Decision **01-Nov-2024**

Dear Professor Concepcion,

Re: JP-TR-2024-285200 "Recent advances in the structure, function, and regulation of the volume-regulated anion channels and their role in immunity" by Sergei Yanushkevich, Aleksandra Zieminska, Joshua Gonzalez, Francisca Añazco, Richard Song, Alejandra Arias-Cavieres, Sara T. Granados, Junyi Zou, Yan Rao, and Axel R. Concepcion

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We look forward to receiving your revised submission.

If you have any queries, please reply to this email and we will be pleased to advise.

Yours sincerely,

Laura Bennet

Senior Editor
The Journal of Physiology

EDITOR COMMENTS

Reviewing Editor:

I agree with the reviewers' comments-this is a timely and well-constructed review that thoroughly summarizes our current understanding of LRRC8 proteins in immunity. Please implement the minor reviewers' suggestions.

Please also see 'Required Items' below.

REFEREE COMMENTS

Referee #1:

Review

Since the identification of LRRC8 proteins as the pore-forming subunits- with LRRC8A being essential-of the volume-regulated anion channels (VRACs), substantial progress has been made in understanding the structure-function relationship and physiological roles of VRACs. These roles include various functions of VRACs in immune cells.

In this timely review, the authors comprehensively cover the current knowledge about the structure of LRRC8 complexes, highlight open questions, provide insights into potential activation mechanisms of these channels, and offer a focused summary of the various functions of VRACs in both the innate and adaptive immune system.

The authors succeed not only in presenting the current knowledge clearly and thoroughly, but also in pointing out ambiguities and contradictions in the literature and proposing intriguing hypotheses. Given the exponential growth of literature on the different aspects of LRRC8-formed VRACs, it is understandable that the authors do not attempt to cover all areas. Instead, they focus on selected topics, such as activation mechanisms and the physiological role of VRACs in the immune system.

This review is up-to-date, presents also the newest literature in a balanced manner, and is written with great clarity. Below are some suggestions that could further improve the manuscript (without prioritization, in the order they appear in the manuscript):

1) In the well-written historical section, the work by Grinstein and colleagues on volume-induced anion permeability (in B lymphocytes) prior to the electrophysiological characterization (Grinstein et al., J Gen Physiol 1982; Grinstein et al., Am J Physiol 1983) could be mentioned for completeness. This could also be considered as part of the discovery of VRACs (Figure 1).

2) Lines 171f: It can be mentioned that the topology was also determined by immunolabeling and protease protection assays (Qiu et al., Cell 2014; Voss et al., Science 2014; Lee et al., J Biol Chem 2014).

3) Lines 260ff: It is worth mentioning that interestingly, this region is not only involved in selectivity, but also determines the subunit-specific voltage-dependent inactivation (Ullrich et al., J Biol Chem 2016).

4) Line 292: Not only Lutter, but also, for example, Lee et al. (2014), Planells-Cases et al. (2015), Schober et al., J Physiol 2017, should be cited here.

5) Lines 508f: if the ratio in a hexamer is 2:1 to 3:1, would it not be expected to have 2-3 LRRC8A subunits rather than 4-6? Or is this referring specifically to the 3xKO cells?

6) Lines 593ff: This is an interesting hypothesis/speculation. The authors could present it more clearly: Do they mean that in ebo, LRRC8 complexes (including LRRC8Aebo) still reach the plasma membrane, do not mediate a swelling-activated current, but might be activated by other mechanisms? Are the authors suggesting that LRRC8 complexes with LRRC8Aebo mediate osmolyte transport but not chloride conductance (similar to the separation of channel activity and transporter function recently shown for connexins (Gaete et al., PNAS 2024), which the authors discuss in a different context (lines 900ff))? Another possible explanation could be a channel-independent role of LRRC8A, such as a scaffolding function, which is conserved in the ebo mutant.

7) Line 473: Schober et al., J Physiol 2017, was another early publication showing heteromeric VRAC, and it deserves to be cited here.

8) Line 475: Voss et al. had also already shown that different subunits determine distinct biophysical properties.

9) Line 554: Delete the word "and".

10) Lines 657ff / Figure 8: Deneka et al., 2021 should be cited again for the structures discussed / shown here.

11) Line 803: lease spell out "NLR".

12) Line 833: A reference is needed here.

13) Line 870: As only Zhou et al., 2020 is meant, rather "this study".

14) Lines 904-906: This section could be more clearly expressed. It seems that the authors suggest that the potentially different permeation for osmolytes and chloride, or different activation mechanisms, account for the directionality of the transport substrates. However, this difference is of course due to the respective electrochemical gradients.

15) Lines 1043ff: Is there any evidence supporting channel function, let alone a gain of function, of LRRC8AΔ91/+35?

16) In general: The sections on structure-function relationships and physiological roles in immune cells are both quite comprehensive, almost each being a review on its own. The authors might consider strengthening the connection between these parts to make the review more cohesive, rather than it feeling like two separate reviews.

Referee #2:

This review article by Sergei Yanushkevich and Axel Concepcion entitled "Recent advances in the structure, function, and regulation of the volume-regulated anion channels and their role in immunity" is exceptionally well written, thoughtful and thought-provoking. It is a veritable tour de force description and digestion of the field of LRRC8 channel biology from structure-function and immunity perspectives. However, the article is also very long and the structure-function and immunity pieces are almost independent sections. One wonders if this article could and perhaps should be broken up into 2 separate review articles, and in fact, would have a greater impact as 2 separate articles than bundled into one mega review article.

Otherwise, there were only a few points to address:

1. In line 387-393, the authors use the ebo/ebo mouse as evidence that the LRRD is important for channel activation, however, it is not known if the reduction in currents in LRRC8Aebo/ebo is due to impaired LRRC8 trafficking to the plasma membrane. This possibility should be suggested.

2. Lines 621-626, the authors are listing papers that show VRAC activation in response to mechanical swelling, independent of changes in ionic strength, due to injection of isotonic solution into cells. The authors should also reference Zhang, Y, et al Nature Cell Biology, 2017 among those papers. In this paper, adipocytes were inflated with isotonic solution to activate VRAC in a quantitative manner. Moreover, this was the first (and may remain the only) study to show that the mechanical swelling induced VRAC current is entirely LRRC8A-dependent, by using LRRC8A null adipocytes. All the other articles cited only show an injection activated VRAC but all predated the identification of LRRC8 channels as the molecular identity - so none could test the LRRC8A dependence.

3. Paragraph lines 1077-1086, describes DCPIB as a very non-specific VRAC inhibitor as compared to dicoumarol. This is inaccurate. DCPIB does have off-target effects at higher concentrations, but what should be compared are the IC50 for VRAC of ~4 uM for DCPIB versus the IC50 for other channels. From that perspective, it does remain one of the more potent and selective VRAC inhibitors. Dicoumarol IC50 is worse than DCPIB and also had been clinically used previously as an

anti-coagulant by inhibiting vitamin K synthesis. So, it is not possible for dicoumarol to be described as "selective" and for dicoumarol to have a "promising role" as a "clinically available VRAC inhibitor".

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- Please provide high quality and high resolution images and figures.

END OF COMMENTS

EDITOR COMMENTS

Reviewing Editor:

I agree with the reviewers' comments-this is a timely and well-constructed review that thoroughly summarizes our current understanding of LRRC8 proteins in immunity. Please implement the minor reviewers' suggestions.

Please also see 'Required Items' below.

Response: We appreciate the thorough evaluation of our manuscript by the reviewing editor and both referees. We have addressed all the comments raised by the referees (*Cf.* point-by-point responses below) and, as requested, have included additional references to enhance the manuscript. Additionally, we have incorporated new findings published within the last few months that relate to the topic of this review, which are highlighted in red in the revised manuscript.

REFEREE COMMENTS

Referee #1:

Review

Since the identification of LRRC8 proteins as the pore-forming subunits- with LRRC8A being essential-of the volume-regulated anion channels (VRACs), substantial progress has been made in understanding the structure-function relationship and physiological roles of VRACs. These roles include various functions of VRACs in immune cells.

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This review is up-to-date, presents also the newest literature in a balanced manner, and is written with great clarity. Below are some suggestions that could further improve the manuscript (without prioritization, in the order they appear in the manuscript):

Response: We thank the reviewer for their thorough evaluation of our review and the insightful summary and comments provided.

1) In the well-written historical section, the work by Grinstein and colleagues on volume-induced

anion permeability (in B lymphocytes) prior to the electrophysiological characterization (Grinstein et al., J Gen Physiol 1982; Grinstein et al., Am J Physiol 1983) could be mentioned for completeness. This could also be considered as part of the discovery of VRACs (Figure 1).
Response: Good point, thank you! We cannot overlook the early studies that examined anion permeability changes induced by alterations in cell volume. We have added the following sentence to enhanced the historical overview of VRACs in immunity section: “The initial reports on cell swelling and its connection to increased anion permeability were first documented in Ehrlich ascites cells (Hoffmann, 1978) and human lymphocytes (Grinstein *et al.*, 1982a; Grinstein *et al.*, 1982b; Grinstein *et al.*, 1983)”.

2) Lines 171f: It can be mentioned that the topology was also determined by immunolabeling and protease protection assays (Qiu et al., Cell 2014; Voss et al., Science 2014; Lee et al., J Biol Chem 2014).
Response: As pointed by the reviewer, we have included the following in the text: “This LRRC8 protein topology has been further validated experimentally through immunolabeling and protease protection assays (Lee *et al.*, 2014; Qiu *et al.*, 2014; Voss *et al.*, 2014)”.

3) Lines 260ff: It is worth mentioning that interestingly, this region is not only involved in selectivity, but also determines the subunit-specific voltage-dependent inactivation (Ullrich et al., J Biol Chem 2016).
Response: Good point. We have included the following in the text: “Amino acid residues K98 and D100 in LRRC8A and equivalent residues in LRRC8C and LRRC8E, which are located immediately upstream of the EL1H, have been shown to be responsible for the subunit-specific voltage-dependent inactivation kinetics of VRACs (Ullrich *et al.*, 2016).”

4) Line 292: Not only Lutter, but also, for example, Lee et al. (2014), Planells-Cases et al. (2015), Schober et al., J Physiol 2017, should be cited here.
Response: We have included the references indicated by the reviewer.

5) Lines 508f: if the ratio in a hexamer is 2:1 to 3:1, would it not be expected to have 2-3 LRRC8A subunits rather than 4-6? Or is this referring specifically to the 3xKO cells?
Response: In the paper by Rutz et al. 2021, the authors reported that they found LRRC8-A and -C subunits at a ratio of 1.8:1 in wild-type HEK cells and a ratio of 2.9:1 in LRRC8$^{B,D,E-/-}$ HEK cells. Assuming a hexameric arrangement of channels isolated from LRRC8$^{B,D,E-/-}$ HEK cells, and that their approach captured all proteins containing LRRC8A, their result indicated “a predominance of LRRC8A component in populations presumably containing four to six copies of the subunit”.

Upon reviewing the calculations, we confirm the authors correctly stated that LRRC8A would be the predominant component in a hexameric complex. However, it is important to clarify that the maximum number of LRRC8A subunits per hexameric channel in a ~3:1 ratio of 8A/8C would be five (in average, based on the Figure 1b, Rutz et al. 2021), rather than six as stated by the authors. To construct hexameric channels with a ~3:1 ratio of 8A/8C, only multiples of three can yield the exact number of subunits required to form such channels.

For example:

For a 3:1 ratio of 8A:8C, if we multiply by 3, we get 9 copies of 8A and 3 copies of 8C, totaling 12 subunits. This configuration would yield one channel with a 4:2 ratio and one channel with a 5:1 ratio of LRRC8A to LRRC8C.

If we multiply by 6, we have: 18 copies of 8A and 6 copies of 8C, totaling 24 subunits. This would yield 2 channels with a 4:2 ratio and 2 channels with a 5:1 ratio of LRRC8A to LRRC8C.

It important to highlight that the authors find pool of channels with ratios over 3:1 in some experiments (dots distributed over the 3:1 ratio in Figure 1b, Rutz et al. 2021). This suggests that in some circumstances, homohexameric LRRC8A channels might be found. To be accurate with the language, we have revised the text to indicate that four to five copies of LRRC8A subunits would be found in “heterohexameric” channels.

6) Lines 593ff: This is an interesting hypothesis/speculation. The authors could present it more clearly: Do they mean that in ebo, LRRC8 complexes (including LRRC8Aebo) still reach the plasma membrane, do not mediate a swelling-activated current, but might be activated by other mechanisms? Are the authors suggesting that LRRC8 complexes with LRRC8Aebo mediate osmolyte transport but not chloride conductance (similar to the separation of channel activity and transporter function recently shown for connexins (Gaete et al., PNAS 2024), which the authors discuss in a different context (lines 900ff))? Another possible explanation could be a channel-independent role of LRRC8A, such as a scaffolding function, which is conserved in the ebo mutant.
Response: The reviewer is correct; this is exactly the message we wanted to convey. We have rewritten the entire section to clarify our point and hypothesis and added the reviewer's suggestion regarding LRRC8A playing other potential, uncharacterized channel-independent roles.

7) Line 473: Schober et al., J Physiol 2017, was another early publication showing heteromeric VRAC, and it deserves to be cited here.
Response: We have included the reference indicated by the reviewer.

8) Line 475: Voss et al. had also already shown that different subunits determine distinct biophysical properties.
Response: We have included the reference indicated by the reviewer.

9) Line 554: Delete the word "and".
Response: Done, thanks!

10) Lines 657ff / Figure 8: Deneka et al., 2021 should be cited again for the structures discussed / shown here.
Response: We have included the reference indicated by the reviewer.

11) Line 803: lease spell out "NLR".
Response: As suggested by the reviewer, we have spelled out NLR in the text, specifically in lines 122-123 of the current text, where it was first mentioned.

12) Line 833: A reference is needed here.
Response: As suggested by the reviewer, we have included 3 references to support this part of the text.

13) Line 870: As only Zhou et al., 2020 is meant, rather "this study".
Response: We have made this correction.

14) Lines 904-906: This section could be more clearly expressed. It seems that the authors suggest that the potentially different permeation for osmolytes and chloride, or different activation mechanisms, account for the directionality of the transport substrates. However, this difference is of course due to the respective electrochemical gradients.

Response: We appreciate this comment by the reviewer and have revised the text for clarity. It now reads: “This suggests that cGAMP can be transported along its electrochemical gradient independently of Cl^- fluxes that are triggered by hypotonic cell swelling, potentially through a completely different mechanism and under distinct conditions. However, to mechanistically describe these different modes of actions, more structural studies of VRACs are needed. One potential alternative mechanism could involve the presence of a pervasive molecular cue that modulates VRAC activation”.

15) Lines 1043ff: Is there any evidence supporting channel function, let alone a gain of function, of LRRC8AΔ91/+35?
Response: Based on the existing literature, there is no evidence to support either loss-of-function or gain-of-function channel activity for the LRRC8A$^{\Delta 91/+35}$ mutant. However, the current literature suggests that a loss-of-function in LRRC8 does not impact lymphocyte development. We speculate that the LRRC8A$^{\Delta 91/+35}$ mutation may exhibit a gain-of-function because our studies suggest that LRRC8 channels may play a negative regulatory role in lymphocyte function, with their activity being linked to the suppression of lymphocyte function. Therefore, we propose that the LRRC8A$^{\Delta 91/+35}$ mutant could lead to a gain-of-function and may also introduce novel, uncharacterized features necessary for lymphocyte development.

16) In general: The sections on structure-function relationships and physiological roles in immune cells are both quite comprehensive, almost each being a review on its own. The authors might consider strengthening the connection between these parts to make the review more cohesive, rather than it feeling like two separate reviews.
Response: We appreciate the reviewer's comment. In response to the overall feedback on our manuscript and the exponential growth of the VRAC field, we made an effort to address two areas suggested by the editor, leveraging our expertise in ion channels and transporters in immunity. We aimed to highlight the discrepancies in lymphocyte biology related to LRRC8-VRACs, particularly concerning the LRRC8 topology and its function under normal tonicity. Additionally, we sought to broaden our discussion beyond lymphocyte biology by including insights on VRACs in innate immunity. Given that the study of ion channels and transporters in immunity is an emerging area within immunology, we believe that combining these two sections in the same manuscript adds significant value by integrating these seemingly unrelated fields. Therefore, we respectfully request to keep these two sections together, as doing so will enrich the current literature in this evolving area of immunology.

Referee #2:

This review article by Sergei Yanushkevich and Axel Concepcion entitled "Recent advances in the structure, function, and regulation of the volume-regulated anion channels and their role in immunity" is exceptionally well written, thoughtful and thought-provoking. It is a veritable tour de force description and digestion of the field of LRRC8 channel biology from structure-function and immunity perspectives. However, the article is also very long and the structure-function and immunity pieces are almost independent sections. One wonders if this article could and perhaps should be broken up into 2 separate review articles, and in fact, would have a greater impact as 2 separate articles than bundled into one mega review article.
Response: We appreciate the reviewer’s thoughtful comments regarding our manuscript. Regarding the suggestion to split the review into two separate manuscripts, I had a similar thought. However, as mentioned in the last response to Reviewer 1, we believe that keeping these two sections in one manuscript will significantly enrich the current literature in the evolving field of ion channels and transporters in immunity. We have made considerable efforts to

connect the concepts derived from studies related to protein biochemistry, as well as the structure and topology of VRACs, to address the discrepancies observed in VRAC studies concerning lymphocyte physiology and function. Additionally, we have speculated on the physiological role VRACs may play in immune cell function and how their structural components, properties, and gating mechanisms might influence immune cell biology in physiological contexts.

Otherwise, there were only a few points to address:

1. In line 387-393, the authors use the ebo/ebo mouse as evidence that the LRRD is important for channel activation, however, it is not known if the reduction in currents in LRRC8Aebo/ebo is due to impaired LRRC8 trafficking to the plasma membrane. This possibility should be suggested.
Response: We thank the reviewer for this comment. We have rewritten the text addressing this topic in Section 4 (VRAC activation and gating), specifically in lines 597-619. Along with Table 3, we provide compelling arguments to suggest that LRRC8^{ebo} mutant has the potential to be expressed at the cell surface (lines 612-619): “Our hypothesis is supported by several observations: 1) the normal expression of LRRC8A^{ebo} mutant protein in T cells from *Lrrc8a*$^{ebo/ebo}$ mice (Platt *et al.*, 2017), 2) the LRRC8A^{ebo} mutant protein retains the IL1 sequence motif that is crucial for membrane trafficking, and 3) the LRRC8A^{ebo} mutant maintains intact NT and TM1-4 regions, which are necessary to generate a functional pore domain. Consequently, it is possible that the transport activity of VRACs is preserved in the *Lrrc8a*$^{ebo/ebo}$ mutants compared to *Lrrc8a*$^{-/-}$ mice (Table 3). Nevertheless, this hypothesis requires further testing as it is important to consider that *Lrrc8a*$^{ebo/ebo}$ and *Lrrc8a*$^{-/-}$ mice originate from different genetic backgrounds”. While this is currently a hypothesis, bring this up in the discussion will encourage researchers in this field to test this hypothesis. We also comment, as reviewer 1 suggested, the possibility of LRRC8A (and potentially the LRRC8A^{ebo} mutant) playing a channel-independent role (e.g. as a scaffolding protein).

2. Lines 621-626, the authors are listing papers that show VRAC activation in response to mechanical swelling, independent of changes in ionic strength, due to injection of isotonic solution into cells. The authors should also reference Zhang, Y, et al Nature Cell Biology, 2017 among those papers. In this paper, adipocytes were inflated with isotonic solution to activate VRAC in a quantitative manner. Moreover, this was the first (and may remain the only) study to show that the mechanical swelling induced VRAC current is entirely LRRC8A-dependent, by using LRRC8A null adipocytes. All the other articles cited only show an injection activated VRAC but all predated the identification of LRRC8 channels as the molecular identity - so none could test the LRRC8A dependence.
Response: The reviewer is correct; we overlooked including Zhang et al. (2017) in our citations regarding VRAC activation by mechanical swelling, independent of ionic strength. We have now added this citation to our list of references. Thank you for bringing this to our attention.

3. Paragraph lines 1077-1086, describes DCPIB as a very non-specific VRAC inhibitor as compared to dicoumarol. This is inaccurate. DCPIB does have off-target effects at higher concentrations, but what should be compared are the IC50 for VRAC of ~4 uM for DCPIB versus the IC50 for other channels. From that perspective, it does remain one of the more potent and selective VRAC inhibitors. Dicoumarol IC50 is worse than DCPIB and also had been clinically used previously as an anti-coagulant by inhibiting vitamin K synthesis. So, it is not possible for dicoumarol to be described as "selective" and for dicoumarol to have a "promising role" as a "clinically available VRAC inhibitor".
Response: We appreciate the reviewer’s comment but respectfully disagree. In section 6, which discusses the immunotherapeutic potential of LRRC8 modulation, we have reviewed the current literature on DCPIB, one of the most potent inhibitors of VRACs identified to date (IC_{50} ~4.1

μM). DCPIB has served as the gold standard for validating $I_{Cl,swell}$ in electrophysiology experiments for over 20 years (Decher et al., 2001), giving researchers ample time to examine the off-target effects of this drug in more detail. DCPIB has been shown to inhibit other channels and transporters with better IC_{50} than those for VRACs. For example, it inhibits connexin Cx43 with an IC_{50} of ~1 μM (Bowens et al., 2013), as well as K_2P channels, including TRESK (IC_{50} ~0.14 μM) and TASK1 (IC_{50} ~0.95 μM) (Lv et al., 2019). Additionally, it inhibits the heteromeric Kir3.1/Kir3.4 channels with an EC_{50} of ~3.3 μM in inside-out macropatches, in a heterologous expression system using Xenopus oocytes (Deng et al. 2016). More importantly, DCPIB was shown to have an off-target effect in LRRC8A-KO cells, as demonstrated by recent findings that involve uncoupling the mitochondria (Afzal *et al.*, 2019). The Qiu lab identified dicumarol as an additional inhibitor of VRACs, with the rationale of drug-repurposing as DCPIB is not approved by the FDA. Although dicumarol has long been known as a competitive inhibitor of vitamin K epoxide reductase, leading to its use as an anticoagulant, its IC_{50} for VRAC inhibition is reported to be similar to that of DCPIB, ranging between 3.8 μM and 4.1 μM in HEK 293 cells and microglia, respectively (Chu et al. 2023). The authors also tested the effect of dicumarol in blocking other chloride channels, such as PAC channel and TMEM16A/CaCC, and observed no significant impact. Moreover, an important observation from Chu and colleagues is that the LRRC8A-dependent hypotonic-induced ATP release observed in HeLa was significantly reduced in WT HeLa cells treated with 5 μM dicumarol (>50%). In contrast, the same concentration of DCPIB had no effect, suggesting that in certain contexts, dicumarol may act as a more potent VRAC inhibitor than DCPIB. We appreciate the reviewer's comment and have made revisions to the text, which include: 1) the report of IC_{50} values for these drugs, 2) an emphasis on the anticoagulant effect of dicumarol, and 3) the introduction of a new dicumarol-derivative drug identified by the same research group, bromadiolone (a widely used rodenticide), along with its notable anticoagulant effect. We hope the revised text addresses the reviewer's concerns.

Dear Professor Concepcion,

Re: JP-TR-2024-285200R1 "Recent advances in the structure, function, and regulation of the volume-regulated anion channels and their role in immunity" by Sergei Yanushkevich, Aleksandra Zieminska, Joshua Gonzalez, Francisca Añazco, Richard Song, Alejandra Arias-Cavieres, Sara T. Granados, Junyi Zou, Yan Rao, and Axel R. Concepcion

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

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Authors should note that it is too late at this point to offer corrections prior to proofing. Major corrections at proof stage, such as changes to figures, will be referred to the Editors for approval before they can be incorporated. Only minor changes, such as to style and consistency, should be made at proof stage. Changes that need to be made after proof stage will usually require a formal correction notice.

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Are you on Twitter? Once your paper is online, why not share your achievement with your followers? Please tag The Journal (@jphysiol) in any tweets and we will share your accepted paper with our 30,000 followers!

Yours sincerely,

Laura Bennet
Senior Editor
The Journal of Physiology

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EDITOR COMMENTS

Reviewing Editor:

I agree with the reviewers that this revision on volume-regulated anion channels is both timely and exciting. The issue raised by Reviewer 2 needs to be appropriately acknowledged.

REFEREE COMMENTS

Referee #1:

The authors have thoroughly addressed both reviewers' comments and have done an excellent job in the revision. They not only responded to the concerns and suggestions raised but also updated the review by incorporating recent studies.

Referee #2:

The authors have satisfactorily addressed my comments and concerns and the modifications to the manuscript are appropriate. In the rebuttal, I would say that dicoumarol specificity has not been tested as rigorously as DCPIB, and the cited absence of effect on PAC and TMEM16A are just a few token examples, but to be fair dicoumarol would need to be tested against ALL the same off-targets as DCPIB in order to state it is any better. It is just a new finding of an established molecule with anti-coagulant activity on target activity, so it is a stretch to say it is any better than DCPIB - especially since it has known anti-coagulant activity and can cause lethal bleeding - enough to kill rodents - as it's primary indication in the case of bromadiolone.

Otherwise, this is really an incredible document and I congratulate the authors on a nice piece of work.