Peer Review File

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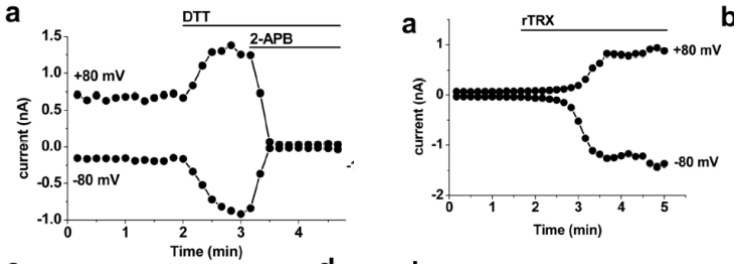
Reviewer A

Comment 1: The amplitude of Trek-1 currents with SBS present in figure 2 is roughly more than 10 times higher than that of the same type of currents in Figure 1. This apparent discrepancy needs to be explained.

Reply 1: First, we read the papers about TREK-1.

1) In P Miller's studies in hTREK-1 stably-expressed HEK 293 cells:
   a) Basal K^+ current densities at +60 mV were 92.5 ± 7.7 pA/pF, 165.2 ± 31.2 pA/pF, and 283.7 ± 83.3 (Acute hypoxia occludes hTREK-1 modulation: re-evaluation of the potential role of tandem P domain K^+ channels in central neuroprotection. J Physiol. 2003 Apr 1;548(Pt 1):31-7.).
   b) Basal current densities at +60 mV were 173 ± 53 pA/pF and 410 ± 80 pA/pF at pipette pH7.2 (Polymodal regulation of hTREK1 by pH, arachidonic acid, and hypoxia: physiological impact in acidosis and alkalosis. Am J Physiol Cell Physiol. 2004 Feb;286(2):C272-82.). There was a five-fold difference betweenthe basal TREK-1 currents.
2) As for the other channels, for example, TRPC5, the baseline currents were no more than 0.1nA (one experiment), 0.75nA (another experiment) in TRPC5 stably-expressed HEK293 cells (TRPC channel activation by extracellular thioredoxin. Nature. 2008 Jan 3;451(7174):69-72.). There was a seven-fold difference between the basal currents.



A possible reason for the different basal currents of these over-expressed channels was that there might be spontaneous activities in different cells. However, this phenomenon has less effect on analysis during different treatments, where the changes were recorded before and after one treatment in the same cell. Also, t-test analysis showed that there were no statistical differences between the basal currents of Figures 1 and 2 (10.33±3.53 pA/pF vs.51.28±45.66 pA/pF), only the five-fold difference between the basal TREK-1 current density, which was similar to previous studies.

Changes in the text: To avoid this discrepancy, we directly added the mean current density of each data point in the results (see Page 7, lines 10–12, lines 17–19).

Comment 2: Dose-response curves in Figure 2 and 3 suggest that the effects of bepridil on baseline or BL1249-induced Trek-1 are significantly different, even with a big difference of Hill coefficient (1.1 versus 3.2). It is quite puzzling! One possibility is that different channels were targeted in these two sets of experiments. Another possibility which seems more likely here is that the concentrations chosen for fitting these curves in Figure 2 and 3 are so incomplete that the error of curves is inevitably big: e.g. in Figure 3c, the curve is technically determined by only three data points at 1, 3 and 10, so a dose-response curve could be plotted in different ways, depending on where the actual I value at 7 is. The authors need to put more efforts on these experiments and provide justification.

Reply 2: This is very important advice for us to discuss the effect of bepridil on TREK-1 in depth.

In Figure 3C, there were five concentrations of bepridil 0.1, 0.3, 1, 3, and 10 μM, which was the same as in Figure 2. To clearly show the fitting curves' concentrations, we changed the scale of the x-axis to the logarithm of the original data (Figures 2 and 3).

In fact, antiarrhythmic drugs are not simply combined with ion channels but are closely related to the channel's dynamic state, resulting in "use dependence" and "reverse use dependence."

Bepridil, a nonspecific inhibitor of Ca^{2+}, Na^{+}, and K^{+} channels in cardiomyocytes, can significantly accelerate the inactivation of homotetrameric Kv1.5 channels. In one study, bepridil blockage of recombinant human cardiac IKs current exhibited a time-dependent unblocking; its binding affinity to the channel was greater in the closed state channel, as evidenced by unblocking during prolonged depolarization.

Wang et al. found that the inhibition of I_{Ks} by bepridil was assuming one-to-one interactions between the channel and the drug molecule, with an IC_{50} 6.2μM. High concentrations (>10 μM) of bepridil depressed I_{Kr}, with an IC_{50} of 13.2 μM, and the blockade occurred in a cooperative manner (Hill coefficient=3.03). Therefore, the different effects of bepridil on baseline or BL1249-induced TREK-1 might be one characteristic of bepridil's blockade of TREK-1 due to reverse use dependence.

Changes in the text: We added this finding in the results section (see Page 7, lines 24–28). In the previous version, we had explained this difference in the discussion (see Page 9, lines 24–28). In the revised version, we added one important reference to support this finding further (see Page 10, lines 2–8)

Comment 3: If bepridil exerts different effects on baseline TREK-1 and BL1249-induced TREK-1 activities, I wonder how the authors will justify the accuracy and reliability of the conclusions made from Figures 4 and 5.

Reply 3: Please refer to Reply 2.

Comment 4: The authors claim that effect of bepridil on Trek-1 channels are quick. But the experimental results presented in the manuscript fail to provide such supportive evidence. Time-course for bepridil effect should be added

Reply 4: Thank you for your suggestion. We added the time-course for bepridil effect (see Figure2B).

Comment 5: The experimental data for Trek-1 expression in U251MG and effects of 4-AP/TEA should be shown, especially for Trek-1 expression in U251MG which is quite important for the current study. In current version, the authors explained the

relevant results with ‘data not shown’. It is more persuasive to present these data directly.

Reply 5: Thank you for your suggestion. We detected the mRNA expression of TREK-1 and added the mRNA expression data as supplemental data.

Comment 6: In Figure 1, 10 micromolar BL 1249 certainly is not a saturating concentration for activation of Trek-1 though the authors didn’t further use higher concentrations. The dose-response curve could be significantly different if the actual saturating concentration is unexpectedly higher than estimated.

Reply 6: Thank you for your expert review. When we attempted to add more than 10 μM BL1249, the cells were consistently intolerant to the stimulation. Also, 10 mM BL1249 was usually the highest concentration used in previous studies. For example, in cultured human bladder myocytes, BL1249 produced concentration-dependent TREK-1 currents, with an EC_{50} of 1.49 μM. Two-electrode voltage clamp (TEVC) currents measured from Xenopus oocytes expressing TREK-1 revealed an EC_{50} of 5.5 μM. Due to the BL1249 solubility limits in DMSO, it was difficult to achieve a higher concentration. Therefore, 10 μM BL1249 is near saturation to activate TREK-1.

Comment 7: It remains ambiguous how the concentration-dependent curves were fitted. The current traces for comparison were from ramp recording. So, the individual voltage is particularly important for fitting such curve as the current value at different voltage varies significantly.

Reply 7: Thank you for your suggestion. The pulse protocol included both a ramp and a step pulse to +20mV: a 200ms step pulse to 20mV followed by a 500ms ramp from –90 mV to +20 mV (HEK-293 cell). We then analyzed the currents at individual +20mV to fit the curves.

Changes in the text: To clearly describe the curve fit method, we added the following explanation in the Methods section:"The currents at +20 mV were used for the analysis of the differences between the different treatments, and the currents of ramp pulse were used for the I-V relationship analysis."(see Page 5, lines 11–13). We

also changed "a was the amplitude of current"to "a was the amplitude of current at +20 mV"in the equation explanation of the Statistical analysis section (see Page 6, lines 25 and 27).

Comment 8: In the legend for Figure 4B, the concentration of BL1249 should be 30 micromolar rather than 310 micromolar. A statistics figure should conclude the finding how Bepridil inhibits the activity evoked by BL1249 in figure 4.

Reply 8: We corrected this. A statistics figure was added to show the effect of bepridil on the BL1249-evoked current.

Changes in the text: We added a statistics figure in Figure 4 (see Figure 4D) and a description of the mean current density of each data point in the results:"The basal current was 335.89±141.27 pA/pF and BL1249 (30 μM) induced current was 1543.14±124.88 pA/pF, which was decreased to 921.95±841.79 pA/pF by bepridil (Figure 4D, $P<0.05$)." (see Page 8, lines 9–11).

Comment 9: A statistics figure at least should be provided to support the observations for Figure 5C.

Reply 9: We followed the reviewer's advice to analyze the resting membrane potential, action potential amplitude, and action potential duration between the different treatments.

Changes in the text: We directly added a description of the mean resting membrane potential, action potential amplitude, and action potential duration in the results (see Page 8, lines 16–23).

Comment 10: In figure 4B and 5B, the authors used current amplitude directly instead of current density so that the error bar looks too big to assume the accuracy of the conclusion (e.g., error bar for BL1249 in figure 5B).

Reply 10: Thank you for your suggestion. When we used current density, the error bar was still large. A possible reason for this is that there are different channel activities between different primary cells. To overcome this limitation, we normalized

current by calculating the ratio between the current with different drugs and the same cell's basal current.

Changes in the text: see Figures 4D and 5B.

Comment 11: The authors should clearly articulate the dose information of bepridil in introduction, especially on the relationship between the dose administrated in clinical practice for patients and the dose used in current experiments.

Reply 11: Thank you for your constructive advice. We added the IC_{50} of bepridil's inhibition on every channel of references and the information about concentration relevant to the therapeutic plasma concentrations in clinical use (2-3 μM) in the Introduction section (see Page 3, lines 22–27). We added this information to support the present study's concentrations in the Methods section (see Page 5, lines 15–18).

Reviewer B

Comment 1: Clear conclusion on the basis of the authors' experiments are difficult. Most of the experiments use BL1249 to activate TREK-currents. This procedure introduces different unknown aspects into the system. Effects may be mediated by interaction between both drugs. The authors should provide data about effects on basal current density.

Reply 1: BL1249 has previously been validated to be specifically active Trek-1 channel not only in heterologous expression systems but in primary cells. BL-1249 stimulates K2P2.1(TREK-1)-like currents in bladder smooth muscle cells (J Pharmacol Exp Ther. 2005 Apr;313(1):250-9.), primary mouse alveolar type-2 cells (Sci Rep. 2020 Dec 15;10(1):22011.) and Pancreatic ductal adenocarcinoma cells (Biochim Biophys Acta. 2016 Oct;1862(10):1994-2003.).

BL-1249 action is occluded by mutations that stabilize the C-type gate (Mol Pharmacol. 2014 May;85(5):671-81.). A study published on Science reveals that BL-1249, negatively charged activator (NCA), act on a gating mechanism that involves a site that overlaps with the conserved QAL+-binding site located below the

selectivity filter (Science. 2019 Feb 22;363(6429):875-880.). Therefore, BL-1249 can act as a specific activator for Trek-1 channel. In addition, Trek-1 stably expressed HEK-293 cell line could overcome the possible disturbance.

The effects of bepridil on basal Trek-1 current density have been included in our work (Figure 2), which is carried out before the experiment about the effect of bepridil on BL-1249 induced currents. Bepridil could decrease the baseline Trek-1 currents with an IC_{50} 0.59 μM.

Changes in the text: We have modified our text to explain the background effect of BL1249 on Trek-1 in the part of Introduction (see Page 3, line 28; Page 4, line 1-4): "Furthermore, BL1249 has previously been validated to be specifically active Trek-1 channel not only in heterologous expression systems but in primary cells (20, 21), which act on a gating mechanism involving a site located below the selectivity filter and the C-type gate (2)".

Comment 2: Why are effects on U251MG cells not shown? Please provide an additional figure.

Reply 2: Thank you for your constructive advice. In the 3.3 part of the Results, there are already data about the effects of BL1249 and bepridil on the Trek-1 current in U251MG cells (see Page 8, line 5-11). The figure 4 also shows these effects.

Comment 3: Effects on mouse ventricular cells are difficult to interpret since effects of bepredil may be reduced due to simultaneous inhibition of ICa,L currents. One solution may be to repeat those experiments in presence of another ICa,L inhibitor, that may not effects TREK-1 currents. Again, experiments with bepredil only.

Reply 3: We agree with the reviewer. Bepridil is a muti-channel blocker for Na^+, Ca^{2+} and K^+ channels. To evaluate the effect of bepridil on action potential (AP), it is impossible to inhibit voltage dependent calcium channel (VDCC), which play a key role in AP.

On the basis of our part 3.2 and 3.3 results, in Trek-1 stable expressed HEK-293cells, bepridil decreased baseline and BL-1249 induced Trek-1 currents.

Because HEK-293 cells have no VDCC, this effect of bepridil on heterologous trek-1 was almost impossible to be caused by the inhibition of calcium channel. In addition, the mean amplitude of BL-1249 induced Trek-1 currents was 5.6731 nA, a very large outward current which was not possible interfered by the smaller inward calcium current.

However, Liu H et al found that selected DHP L-type Ca2+ channel antagonists potently inhibit native bTREK-1 channels in bovine adrenal zona fasciculata (AZF) cells (J Pharmacol Exp Ther. 2007 Oct;323(1):39-48.). They had not confirmed whether the outward current was carried by TREK-1 using specific inhibitor, activator, or siRNA knock down of TREK-1. The result from bovine TREK-1 channels should be verified in human and mouse cells.

Therefore, it might be more complicated when using Ca2+ channel antagonist. However, the further study should be carried out to reveal the exact effect of all kinds of Ca2+ channel antagonists on human TREK-1.

Comment 4: Please provide n-numbers within the figures or legends.

Reply 4: We have done so.

Changes in the text: We added n-numbers within the figure legends.

Comment 5: Please discuss potential clinical applications. Which cardiac diseases are characterized by TREK-1 upregulation? Importance of K2P channels for cardiac arrhythmias has been demonstrated before: PMID: 25951834 and PMID: 28057773.

Reply 5: Thank you for your constructive advice. We added the discussion of potential clinical applications of Trek-1 and bepridil (see Page 11, line 13-23).