Review of: "An allosteric agonist activates BK channels by perturbing coupling between Ca2+ binding and pore opening."

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Potential competing interests: The author(s) declared that no potential competing interests exist.

Using in silico screening, patch-clamp electrophysiology and molecular modeling and dynamics, Zhang et al. have identified the compound termed BC5 (net charge=+2) as a tool to further understand allosteric coupling between calcium-driven activation and pore opening in slo1 channels. Data underscore the importance of T245 and E219. The methods are solid and most of the conclusions are supported by the data (yet see major points #2 and 4). A few concerns (major and minor) are listed below:

Major:

1-Results: Fig. 2b. How specific is the effect of 10 mM Mg^{2+} vs. the G/G_{max}-V being shifted to the left maximally by each modulator, so neither modulator can further activate on top of the other)?

2-Contrary to the authors' hypothesis, Fig. 2f shows that the differences in V0.5 between control and BC5 are similar in wt and mutants of relevance. Please elaborate.

3-Page 5 of Results, second parag., line 3: "...rather the competition between calcium and BC5 is allosteric." The several poses of BC5 show the molecule directly interacting with E374, E399 and, probably too, D367. Please elaborate.

4-Results: data from Fig. 4d do not support the conclusion that the responses of V0.5 delta to BC50 or calcium differ between wt and T245W channels. Explain what statistical test(s), number of samples and power level were used to claim the contrary.

5-Discussion: modulation of slo1 channel activity by several ligands has been reported to depend on calcium levels: alcohol (Kuntamallappanavar & Dopico, 2016) and cholesterol (North et al., 2022) modify slo1 channel activity only in the presence of calcium. More important, PIP2 has dual actions on slo1 channel activity, with voltage-dependent inhibition of activity in absence of calcium, a mechanism that has been related to shielding of surface charge by PIP2 negative charges in its headgroup, and voltage-independent potentiation of channel activity in the presence of calcium. Moreover, PIP2 sensing-sites in slo1 channel proteins have been mapped to the interface between the VSD and the gating ring (Tian et al., 2015), as authors advance for BC5 in the present study. Surprisingly, none of these modulators and their possible mechanisms of action common to/different from those of BC5 are discussed. Please elaborate on

how PIP2 and BC5 having opposite net charges seem to induce a similar effect (voltage-dependent current inhibition) by probably acting at a similar VSD-gating ring interface.

Minor:

1-Introd, first page, line 6: “The function of BK channels…elevated intracellular calcium.” This is not accurate as a global increase in cytosolic calcium is not needed (nor achieved under physiological conditions) to activate BK channels. Rather, a local increase near the channel calcium sensors suffices (Jaggar et al., 2000). Please reword.

2-Results, page 2, second parag., line 3 from bottom of parag: “…is an off-target effect.” Authors refer to a site(s) different from the VSD-gating ring interface within the same target subunit. Please rephrase.

3-English: the manuscript is plagued with rather colloquial language, BK electrophysiology jargon and just plain convoluted sentences. A few examples follow:

Throughout text: BK type Ca2+-activated K+ channels; rephrase to “Ca2+- and voltage-activated K+ channels of large conductance” or to “large conductance, Ca2+- and voltage-activated K+ channels”

Throughout text: when introducing a conditional replace “if” with “whether”.

Abstract: “BC5…enhances channel activity by specifically affecting the Ca^{2+}-dependent mechanism..activates the channel in the absence of Ca^{2+} binding.” For the reader who does not have access to the whole set of data, the statements may appear contradictory. Please rephrase.

Introd., page 2, line 1: use “resulting from the transmembrane segments…”

Introd., second parag., lines 1-2: “What…remain unclear.” Please rephrase.

Introd., second parag., line 11: split to “…and pore opening. This hypothesis seemed…”

Disc., first parag., Edit to: “Our results…activation of the channel required neither voltage sensor activation nor indirect modification of voltage dependent gating.”

Also, replace “such that its effect” with “such that BC5 effect” (line 7).