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Supplementary Discussion

Stoichiometry

The pentameric architecture is consistent with previous characterization[5] but was somewhat unexpected given a recent study using the single-molecule photobleaching technique that suggested that bestrophins are tetramers[25]. This apparent discrepancy is most likely due to uncertainties in determining precise stoichiometries of high oligomeric forms using fluorescence photobleaching[63,64].

Anion permeability of BEST1$_{cryst}$

In light of previous reports of low but measurable permeability of mammalian BEST1 or BEST2 to gluconate, glutamate and aspartate[29,32,65], our results for these anions, for which permeation was not detected (Fig 1b), might be due to the detection limit of the assay, specifics of chicken BEST1 or other reasons. The permeation of GABA in its anionic form was not apparent using our assay (not shown). In addition to the possibility of its permeation being below the detection limit of the assay, the assay would not detect permeation of GABA in its zwitterionic form.

Channel modulation

Amino acid changes within the C-terminal tail of mouse BEST3 yield robust whole cell currents through channels that were otherwise silent[26,66], suggesting a modulatory role for this element. In human BEST1, phosphorylation of Ser 358, which is located in the C-terminal tail, has also been shown to modulate channel activity[67]. This residue is conserved in chicken BEST1 and not phosphorylated in the structure (Extended Data Fig. 3c). In addition to phosphorylation at this and potentially other residues (for example, see refs 67-69), *Drosophila* BEST1, but apparently not mammalian homologs, is activated by cell swelling[29,70].

Acidic cluster residues

Glu 300, Asp 302, and Asp 303 are part of the highly conserved acidic cluster but do not coordinate Ca^{2+} in the structure. Asp 303 is buried and forms hydrogen bonds with main chain amide nitrogen atoms of Val 3 and Leu 234. Glu 300 and Asp 302 participate in ion pair interactions (with Lys 149 and Lys 30, respectively) but they also project into the cytosol and may serve to increase the local concentration of Ca^{2+}.

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