About hysteresis in Shaker: Response to note by Villalba-Galea

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In this issue, Villalba-Galea (2023. *J. Gen. Physiol.* https://doi.org/10.1085/jgp.202313371) expresses interest in our recently published work (Cowgill and Chanda. 2023. *J. Gen. Physiol.* https://doi.org/10.1085/jgp.202112883). Our response points out the deficiencies in the alternative explanation proposed by Villalba-Galea to account for our findings on hysteresis (or lack thereof) in steady state charge–voltage curves of Shaker potassium channel.

Before we get into the specifics of the arguments made by Villalba-Galea, we want to be clear about a few ground truths. Thermodynamics dictates that at equilibrium, the distribution of charges in the membrane is determined solely by applied voltage. It does not matter what the voltage was initially; if the equilibrium conditions are met, the system has no memory of the past, and the final position of the charges is set by the applied force. Therefore, the equilibrium charge–voltage (Q–V) curves are going to be the same irrespective of the initial holding potential or the starting point.

The primary goal of our recent *JGP* article is to show how to obtain equilibrium Q–V curves so that they can be used to calculate free energy of channel gating. In our opinion, the lack of hysteresis is not controversial for the reasons outlined above.

Now, there can be two reasons for holding potential–dependent shifts in Q–V curve. Either the equilibrium conditions have not been met during these measurements or there is an unaccounted source of energy coming into play. Hysteresis of Q–V has been widely observed, and even when these measurements were carried out by increasing the length of the test pulse, these shifts persisted. We (and many others) suspected that these holding potential–dependent shifts reflect the inability of existing gating-current recording protocols to resolve slow-charge movements from a typical baseline drifts. Gating current is the first derivative of charge movement, and if the charge movement is very slow it may become impossible to resolve these currents from background. Although hysteresis of charge movement may be physiologically important, it is an artefact as far as equilibrium thermodynamics is concerned. Indeed, it is impossible to build a model which recapitulates hysteresis in steady state.

To overcome these issues with measuring steady-state Q–V curves, we devised a gating-current recording protocol that allowed us to measure the very slow charge movement in Shaker potassium channel. We showed that under steady state conditions the Q–V curves are essentially identical and do not exhibit any hysteresis. In his Letter, Villalba-Galea (2023) argues that the reason we did not observe any hysteresis is that our recording protocol is "artificially" making the Q–V curve shift disappear. He illustrates this point by creating a new model in his Fig. 2, and generates a pair of Q–V curves showing hysteresis. There are many misstatements and circular reasoning in this Letter, but we will strictly focus on two key issues. First, even with the model parameters provided in his Fig. 2, the shift in Q–V curves will disappear if integrated long enough. This is exactly what we showed in Fig. S2 B in our article (Cowgill and Chanda, 2023), and the same thing needs to be done here. Villalba-Galea does not provide any description as to how those Q–V curves were generated but we are absolutely sure that these purported shifts do not reflect equilibrium conditions. Note that the author states in the same letter that "It is yet to be clear what the minimum requirements of such a model would be." Second, the author has ignored the fact that the A359C fluorescence–voltage (F–V) curves, which mirrors gating-charge movement in the Shaker potassium channel, also do not exhibit hysteresis if we measure the change in fluorescence at steady state (Fig. 5 in Cowgill and Chanda, 2023). In fact, these F–V curves were obtained using standard recording protocols rather than the modified gating-current protocol! The advantage with F–V curves is that we are not measuring the rate of change but simply the extent of change.

In conclusion, the note by Dr. Villalba-Galea does not account for the lack of hysteresis in our measurements. In fact, it is incumbent on Dr. Villalba-Galea to prove why there should be hysteresis in steady state. It defies the principle of microscopic

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reversibility, and is inconsistent with our fundamental understanding of laws of nature.

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References

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