LETTER

What is the correct stoichiometry of Kv2.1:Kv6.4 heteromers?

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Möller et al. (1) propose that the predominant native stoichiometry of Kv2.1:Kv6.4 heteromeric K^+ channels is 2:2, a finding that is unexpected based on past analyses which support a 3:1 stoichiometry for Kv2.1:KvS regulatory subunit heteromers, including Kv6.4 and Kv9.3 (2-4). Like Möller et al. (1), we used a combination of total internal reflection fluorescence (TIRF) microscopy and electrophysiology to examine the native stoichiometry of Kv2.1:Kv6.4 heterotetramers (5). However, we reached the conclusion that 3:1 heteromers are the predominant functional species for these heteromers. We suspect the differences in our findings result primarily from the TIRF data collection strategies. We note that both studies find support for their conclusions from electrophysiological analyses, but we defer on discussing those here since they may be considered insufficient to definitively determine stoichiometry in isolation from TIRF data.

Our approach, in Pisupati et al. (5), to determining the Kv2.1:Kv6.4 stoichiometry using TIRF relied on maximizing detection of green fluorescent protein (GFP) photobleaching events (0.69) and eliminating the confound of channel colocalization, since this can lead to overestimation of stoichiometry if not properly accounted for (6). We noted a slow GFP bleaching rate ($t = 68$ s) and utilized very low channel expression levels. We observed a large majority of Kv2.1:Kv6.4−GFP channels bleaching in a single step and calculated that 2:2 heteromers never exceeded 30% of the total heteromers, even at highly Kv6.4−GFP-biased expression ratios. We also showed that mutations to the Kv6.4 activation gate can almost double the fraction of Kv2.1:Kv6.4−GFP heteromers bleaching in two steps without appearance of channels bleaching in more than two steps (despite our comparatively high GFP detection efficiency). This result is compatible with the established principle of KvS regulatory subunit self-incompatibility during assembly (7–10), which theoretically caps Kv6.4 at two diagonally opposed subunits per tetramer. It strongly supports the interpretation that the wild-type stoichiometry for Kv2.1:Kv6.4−GFP heteromers is 3:1.

In contrast, Möller et al. (1) note a rapid GFP bleach rate (<10 s estimated) which we suspect contributes, in part, to their unusually low GFP detection efficiency (0.49) via increases in prebleaching and simultaneous bleaching. They also conducted TIRF experiments at high channel expression levels (>100× our RNA concentration), which required simultaneous deconvolution of detection efficiency and channel colocalization. Using this method, ∼40 to 50% of their Kv2.1:Kv6.4−GFP channel spots bleached in two or more steps, despite a two-step detection efficiency of only 0.24 (0.49^2). This reflects an approximate fourfold increase in spots with two or more Kv6.4−GFPs compared to Pisupati et al. (5), where only ∼15 to 25% of spots bleached in two steps (despite a detection efficiency 0.48 [0.69^2]) and channels bleaching in more than two steps were not observed. We believe these fundamental differences in data collection and raw step counts led to the contrasting conclusions of the two studies. We suspect that the combination of low GFP detection efficiency and high channel expression in Möller et al. (1) led to an underestimation of the extent of channel colocalization and a subsequent overestimation of Kv6.4 stoichiometry. We therefore stand by our conclusion presented in Pisupati et al. (5) that Kv2.1:Kv6.4 heteromers preferentially assemble in a 3:1 stoichiometry.

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