LETTER TO THE EDITOR

Reply to Chadha et al.

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To the editor:

The letter from Drs Chadha, Greenwood, Zhong and Cole correctly points out that the drug XE991, which is commonly used as a specific inhibitor of K_v7 channels, may also inhibit other subtypes of voltage-activated K^+ (K_v) channels. In an article published in 2011 in this same journal (Ng *et al.*, 2011), Dr Greenwood and colleagues state: 'XE991 and linopirdine block all K_v7 channels with IC_{50} values $\sim 3 \mu\text{mol}\cdot\text{L}^{-1}$ (Wang *et al.*, 2000) and at concentrations $<100 \mu\text{mol}\cdot\text{L}^{-1}$ are not known to have effects on any other ion channel.' In 2010, Zhong *et al.* demonstrated inhibitory effects of $10 \mu\text{M}$ XE991 on cloned $K_v1.2$ / $K_v1.5$ and $K_v2.1$ / $K_v9.3$ channels in an expression system (HEK 293 cells) (Zhong *et al.*, 2010). We had observed that $10 \mu\text{M}$ XE991 induced membrane depolarization in rat basilar artery myocytes and constriction of pressurized basilar arteries and concluded that this effect was likely due to its inhibition of K_v7 channels in the myocytes (Mani *et al.*, 2011). What we neglected to point out in our article was that, at the resting membrane voltage of the basilar artery myocytes (~ -60 mV), the other XE991-sensitive channels ($K_v1.2$ / $K_v1.5$ and $K_v2.1$ / $K_v9.3$), would not be appreciably active because their threshold for voltage-dependent activation is more positive (~ -45 to -40 mV) (Zhong *et al.*, 2010). Furthermore, we had previously shown that 4-aminopyridine (4-AP), a blocker of other classes of K_v channels, including $K_v1.2$ / $K_v1.5$ and $K_v2.1$ / $K_v9.3$ (Nelson and Quayle, 1995; Cox, 2005), did not significantly depolarize rat mesenteric artery myocytes (which had resting membrane voltages ~ -61 mV, similar to basilar artery myocytes) or constrict pressurized rat mesenteric arteries (Mackie *et al.*, 2008). The specificity of XE991 as a blocker of K_v7 channels is supported by our finding that knocking down expression of $K_v7.5$ channels in A7r5 vascular smooth muscle cells completely eliminated the XE991-sensitive currents (Mani *et al.*, 2009). We would assert that at resting membrane voltages of -60 to -45 mV, K_v7 channels are the only K_v channels that have an appreciable open probability under physiological conditions and therefore the effects of XE991 (figure 3 of Mani *et al.*, 2011) that we observed can reasonably be attributed to inhibition of K_v7 channels.

Vascular myocytes express a wide variety of ion channels, making it a challenge to isolate the contribution of a particular subset of channels. In some cases, the biophysical properties of the channels can be used to effectively isolate them from other channels using patch clamp electrophysiology. We have utilized the perforated patch configuration, 5 s voltage steps from -4 mV holding potential, and an external solution supplemented with gadolinium chloride to effectively isolate K_v7 currents in vascular myocytes over the

physiological voltage range between -65 and -20 mV. Gadolinium chloride blocks Ca^{2+} influx that might activate Ca^{2+} -activated K^+ channels (K_{Ca}) and also shifts the voltage dependence of activation of 4-AP-sensitive K_v channels ~ 15 mV in the positive direction (Mani *et al.*, 2011). The perforated patch configuration is essential because we find that the K_v7 currents run down significantly within a few minutes in a ruptured patch configuration (L.I. Brueggemann and K.L. Byron, unpublished experiments.).

The native vascular K_v7 currents measured with our recording conditions have electrophysiological characteristics of cloned K_v7 channels, including kinetics of deactivation, voltage-dependence of activation, etc. (Brueggemann *et al.*, 2011). We have also shown that these currents are fully inhibited by pharmacological K_v7 channel blockers (XE991 or linopirdine), but insensitive to pharmacological blockers of other classes of vascular K^+ channels, including drugs that inhibit K_{Ca} , K_{ATP} and other subtypes of K_v channels (Mackie *et al.*, 2008). The inhibitory effect of XE991 on currents recorded at voltages ≤ -20 mV was irreversible in every vascular smooth muscle preparation we have tested (L.I. Brueggemann and K.L. Byron, unpubl. obs.), whereas the enhancement of the currents by drugs such as flupirtine and celecoxib was fully reversed on washout of drugs (Brueggemann *et al.*, 2007; 2009). Wladyka and Kunze similarly found that inhibition of the K_v7 -mediated M-currents in nodose neurons was sustained on washout of XE991, while the inhibition of other subtypes of K_v currents was rapidly reversed (Wladyka and Kunze, 2006). Thus, the irreversibility of block by XE991 further supports our contention that the currents we record are mediated by K_v7 channels.

By contrast, the strategies employed by Zhong *et al.* to record K_v7 currents in vascular myocytes have yielded a mix of several currents, only a small fraction of which is blocked (reversibly) by XE991 or linopirdine (Zhong *et al.*, 2010). This may be attributed to use of a ruptured patch configuration, short (≤ 500 ms) voltage steps from a hyperpolarized holding potential, and test voltages at which other types of ion channels are predominant. The contribution of K_v7 channels is inferred by subtracting the majority of the signal to reveal the XE991- or linopirdine-sensitive current, typically at $+20$ to $+40$ mV. At these voltages, other types of K^+ channels, including 4-AP-sensitive K_v channels, are likely to overwhelm the smaller K_v7 currents (Mackie *et al.*, 2008). The results of Zhong *et al.* indicate that both XE991 and linopirdine can inhibit these 4-AP-sensitive channels at positive voltages.

In summary, we respectfully suggest that the electrophysiological and pharmacological approaches we have employed in our studies enable us to conclude with reasonable

confidence that K_v7 channels have an important functional role in determining basilar artery myocytes' resting membrane voltage. We remain convinced that the effects of XE991 on membrane voltage and basilar artery constriction that we reported in our article can be attributed primarily to its actions as an inhibitor of K_v7 channels.

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