Jung and Lee have responded in this issue to our recent paper (Yu et al., 2014b) in which we concluded that calmodulin (CaM) does not alter anion permeability of the mouse ANO1/TMEM16A Ca\(^{2+}\)-activated Cl\(^{-}\) channel. In our paper, we suspected that the Ca\(^{2+}\)-CaM (abbreviated as CaM) effect observed by Jung et al. (2013) may result from technical complications such as series resistance and/or ion accumulation problems. One important observation in Jung et al. (2013) that supported the CaM modulation of TMEM16A's anion permeability was that the bi-ionic potentials in whole-cell patch-clamp recordings were different at low and high intracellular [Ca\(^{2+}\)]. However, comparing bi-ionic potentials between currents of vastly different amplitudes could be problematic. For example, series resistance (R\(_s\) in the circuit of Fig. 1 A) in patch-clamp recordings should not be a significant problem if the membrane resistance (R\(_m\)) is relatively high. However, as R\(_m\) is reduced substantially (such as activating large numbers of channels or increasing the leak current), the battery power (and thus the membrane voltage V\(_m\)) is shunted away significantly (see Fig. 1 A), and a higher R\(_s\) exacerbates the effect. This voltage-shunting problem exists whether the measurement is made by the voltage-clamp or current-clamp method. Jung and Lee (2015) use Fig. 1 D in their Letter to the Editor (abbreviated as “Letter”) to argue that they did not have such a problem. However, the V\(_m\) at low conductance in that figure is already near 0 mV, which would preclude the V\(_m\) reduction from being observed.

Ion accumulation may also be a problem in bi-ionic potential measurements. A significant change of ion concentrations adjacent to the membrane can occur in 10 s with merely 1 nA of current in whole-cell recordings (Vocke et al., 2013). Jung and Lee (2015) argue that accumulation of intracellular HCO\(_3^{-}\) in their whole-cell recordings cannot explain the increase of P\(_{\text{HCO}_{3}^{-}/\text{Cl}^{-}}\) because an increase of intracellular [HCO\(_3^{-}\)] should have decreased the P\(_{\text{HCO}_{3}^{-}/\text{Cl}^{-}}\) ratio. However, in their recording conditions (with low extracellular [Cl\(^{-}\]), a large TMEM16A conductance would not only accumulate intracellular HCO\(_3^{-}\) but also deplete intracellular Cl\(^{-}\). As both HCO\(_3^{-}\) and Cl\(^{-}\) gradients across the membrane were reduced, the measured reversal potential would approach 0 mV (or the calculated P\(_{\text{HCO}_{3}^{-}/\text{Cl}^{-}}\) ratio is ~0.3, a depletion of the ionic gradients would increase the calculated P\(_{\text{HCO}_{3}^{-}/\text{Cl}^{-}}\) ratio!

Jung and Lee also show in their Letter that different sources of CaM may differentially alter the HCO\(_3^{-}\) permeability of TMEM16A. We previously found no effect of recombinant bovine CaM (Sigma-Aldrich) in altering the anion permeability of TMEM16A (Yu et al., 2014b). The new results in Fig. 1 C of Jung and Lee’s Letter agree with our conclusion that the recombinant bovine CaM has little effect. However, they show that purified human brain CaM (EMD Millipore) significantly alters the bi-ionic potential (Fig. 1 A of their Letter). They suspect that the His-tag attached to the recombinant CaM may affect its properties, thus explaining the negative effect of the recombinant bovine CaM. It should be noted that inconsistent results were obtained from Jung and Lee’s experiments using recombinant CaM. In Fig. 4 D of Jung et al. (2013), it was shown that the recorded bi-ionic potential quickly approached 0 mV upon adding recombinant CaM. In Fig. 1 C of their Letter, such a robust effect of recombinant CaM is not observed.

To address the effect of the purified human brain CaM from EMD Millipore shown in Fig. 1 A of Jung and Lee’s Letter, we first confirmed the effectiveness of CaM by showing that this CaM readily inhibits olfactory cyclic nucleotide–gated (CNG) channels (Fig. 1 B). One feature of the CaM effect on the olfactory CNG channel (regardless of the source of CaM) is that after the channel is inhibited by CaM, if the inside-out patch is perfused with a solution containing saturating [Ca\(^{2+}\)] without CaM, the current cannot be recovered (Fig. 1 B; also see Fig. 3 of Yu et al., 2014b). The current recovery is only observed after exposing the patch to a 0-Ca\(^{2+}\) solution containing Ca\(^{2+}\) chelators. In contrast, for all of Jung and Lee’s recordings that show a positive CaM effect on TMEM16A, the CaM effect disappears in the Ca\(^{2+}\)-containing washout solution.
To examine the effect of the purified human brain CaM on TMEM16A, we first prepared a 5-µM CaM solution using our standard saturated [Ca\(^{2+}\)] solution containing 0.12 mM of total [Ca\(^{2+}\)] and 0.1 mM EGTA. The estimated free [Ca\(^{2+}\)] would be \(\sim 20 \mu M\) if no other Ca\(^{2+}\) chelator (including CaM) is in the solution. However, this purified human brain CaM was lyophilized from a solution containing 2 mM EDTA, resulting in \(\sim 90 \mu M\) [EDTA] in the 5-µM CaM solution (information from the vendor). Part of the EDTA molecules probably have been bound with Ca\(^{2+}\) in the CaM-preparation process, in which EDTA was likely used for eluting substrate-bound CaM. When we delivered this 5-µM CaM solution to the cytoplasmic side of the patch using the SF-77 fast-solution exchanger (Warner Instruments), a small change of the recorded voltage in \(I = 0\) current-clamp recording mode (\(E_{I=0}\)) was immediately observed (Fig. 1 C). Most strikingly, this “CaM effect” disappeared immediately after

![Figure 1.](image-url)
CaM was removed by switching the intracellular solution back to the control HCO₃⁻ solution containing ~20 µM of free [Ca²⁺]!

We suspected that the contaminating EDTA in the CaM solution may be the culprit of altering E₁=₀ because the membrane potential (Vₘ), namely E₁=₀, is a weighted sum of the reversal potential of TMEM16A current (E_C) and that of the background current (E_B) according to the equation:

\[ V_m = \left( \frac{g_C}{g_C + g_B} \right) \times E_C + \left( \frac{g_B}{g_C + g_B} \right) \times E_B, \]

where gc and gb are the TMEM16A conductance and the background conductance, respectively. Therefore, a contaminating EDTA could chelate free Ca²⁺, reduce the gc/gb ratio, and therefore render Vₘ approaching E_B. This problem can be demonstrated by changing the total [Ca²⁺] in the HCO₃⁻ solution to 0.105 mM (Fig. 1 D) and 0.25 mM (Fig. 1 E). With a lower [Ca²⁺] (Fig. 1 D), the CaM effect is stronger. When the total [Ca²⁺] is 0.25 mM (Fig. 1 E), the CaM effect is negligible. We also conducted similar experiments by adding 90 µM Na-EDTA without CaM (Fig. 1 F). In this solution (free [Ca²⁺] estimated to be ~75 nM), no detectable TMEM16A current was observed (comparing the two voltage-clamp experiments in the bottom panels of Fig. 1 F), and the E₁=₀ values, which can be considered as the reversal potential of the background conductance (or E_B), were in the positive range. Finally, if the CaM effect is caused by a low gc as a result of insufficient free [Ca²⁺], we expect that this source of CaM should not generate an effect in the solution used in Jung et al. (2013) because 10 mM EGTA provides a large Ca²⁺-buffering power. This is indeed observed as shown in Fig. 1 G (n = 3).

We thus conclude that the effect of the purified human brain CaM from EMD Millipore in our experiments is not a genuine CaM effect. (If it is a CaM effect, why is the effect weaker when more Ca²⁺ ions are present in the solution?) The effect we observed can be explained by a reduction of gc caused by the extra Ca²⁺-chelating power from the contaminating EDTA molecules. Although the CaM effect in our experiments is similar to that of Jung and Lee in that the effect disappears upon removing “CaM” in the presence Ca²⁺, we do not know if the result in Fig. 1 A of Jung and Lee’s Letter can be explained by the extra Ca²⁺-chelating power because the information of the total [Ca²⁺] and Ca²⁺-buffering power in that experiment is not available to us.

Various laboratories have provided evidence arguing that CaM may or may not modulate the functions of TMEM16A (Tian et al., 2011; Jung et al., 2013; Terashima et al., 2013; Vocke et al., 2013; Tien et al., 2014a; Yu et al., 2014a,b). In our experiments, whether CaM is a recombinant bovine CaM or the purified human brain CaM, we have not yet observed any genuine CaM effect in altering the anion permeability of the TMEM16A Ca²⁺-activated Cl⁻ channel, although both types of CaM are effective in inhibiting the olfactory CNG channel.

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Purified human brain calmodulin does not alter the bicarbonate permeability of the ANO1/TMEM16A channel
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In a revised version of Jung and Lee’s Letter to the Editor, two additional panels were added to their Fig. 1. Consequently, Yu and Chen’s references to Jung and Lee’s Fig. 1, B and C, should refer instead to Fig. 1, C and D.

The HTML and PDF versions of the article have been corrected.