Does calmodulin regulate the bicarbonate permeability of ANO1/TMEM16A or not?

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The role of calmodulin in the activation of Ca^{2+}-activated Cl^- channels (CaCCs) has been an important topic over the past three decades of CaCC research. Recently, the discovery of ANO1/TMEM16 as a component of CaCC raised the question of whether calmodulin modulates ANO1 activity (Tian et al., 2011; Terashima et al., 2013; Vocke et al., 2013; Yu et al., 2014a). Apart from its activation by calmodulin, our group reported that HCO_3^- permeability of human ANO1 (hANO1) can be dynamically modulated by Ca^{2+}/calmodulin (Jung et al., 2013). We read with interest the article titled "Calcium-calmodulin does not alter the anion permeability of the mouse TMEM16A calcium-activated chloride channel" (Yu et al., 2014b) that challenges the conclusions of our study (Jung et al., 2013). The two major concerns raised by Yu et al. are: (1) they were unable to reproduce the calmodulin-dependent ion permeability changes in inside-out patch recordings of mouse ANO1/TMEM16A (mANO1) channel, and (2) whole-cell recordings used in our study to measure the bi-ionic potentials are not suitable to obtain the reversal potential (E_{rev}) because of the series resistance and/or ion accumulation problems. However, we believe that the conclusions of Yu et al. were based on several inappropriate assumptions and technical issues.

First, our conclusion of Ca^{2+}/calmodulin-induced regulation of ANO1 HCO_3^- permeability is based on an integrated approach of biochemical and electrophysiological methods. For example, in whole-cell recordings we used (a) the calmodulin-binding inhibitor J-8, (b) calmodulin knockdown by siRNAs, and (c) mutation of calmodulin-binding domains (CBDs) in hANO1 to examine the involvement of calmodulin in the high Ca^{2+}-induced regulation of hANO1 HCO_3^- permeability. In addition, inclusion of calmodulin to the cytoplasmic side of outside-out and inside-out patches reproduced the results of the whole-cell recordings. On the other hand, Yu et al. (2014b) used exclusively inside-out patches expressing mANO1 and recombinant tagged-bovine calmodulin, and concluded that calmodulin does not alter the anion permeability of ANO1. Yu et al. did not examine the effects of calmodulin using other approaches. Suspecting that the differences in calmodulin and approach used by Yu et al. may have led to the disparate findings, we attempted to reproduce their findings using similar inside-out patch recordings and, importantly, the same His_6-tagged recombinant bovine calmodulin used by Yu et al. (C4874; Sigma-Aldrich) and the calmodulin purified from human brain (208698; EMD Millipore). Although the human calmodulin reproduced our results of calmodulin-induced regulation of ANO1, the effect of the tagged-bovine recombinant calmodulin was much smaller than that observed with human calmodulin (Fig. 1, A–C). In Fig. 1 A, a strong blockade or reduction in the current magnitude during calmodulin treatment might shift E_{rev} to 0. When we analyzed the I-V relationship during zero-current clamping, the channel conductance decreased time dependently as a result of the rundown of ANO1 current in excised patches (Fig. 1 B). However, only 5.6% of the initial conductance at point (4) was enough to maintain E_{rev}, indicating that the ANO1 channel conductance (g_C) is far greater than the background conductance (g_B) during the entire E_{rev} measurement, and that the reduction in g_C cannot account for the elevation in E_{rev}.

The reason for the discrepancy between the two calmodulins is unclear at the present time. Although the amino acid sequence of human and bovine calmodulins is identical, the His_6 tag attached to the recombinant bovine calmodulin seems to affect its properties and the effect of calmodulin on ANO1 HCO_3^- permeability. In our previous study (Jung et al., 2013), we used recombinant human calmodulin after removing the GST-tag by thrombin digestion for patch-clamp experiments. Calmodulin is a strongly negatively charged molecule and commonly binds to an amphipathic α-helical segment that is positively charged. The CBDs of hANO1 belong to an α-helical "1–8–14 motif" with a weak net positive charge (+1) between 1 and 14 residues (Jung et al., 2013). Therefore, because of the relatively weak electrostatic interaction, it is conceivable to speculate that high fidelity calmodulin structure is required to

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modulate ANO1 activity, and that especially positive charges from the His_6 tag might hamper the proper protein–protein interaction between calmodulin and ANO1.

Second, regarding the concerns related to series resistance and ion accumulation in whole-cell recording, we think that Yu et al. (2014b) assumed extreme conditions, which maximize the effect of those problems that are not relevant to our recordings. In our study, zero-current clamping mode was used to measure the membrane potentials, and these values were used for all mechanistic analyses, including statistical comparisons. During zero-current clamping, we occasionally applied ramp pulse to obtain I-V curves, which were used only to confirm ANO1 currents. Therefore, our ion permeability (P_x/P_{Cl}) analysis was entirely based on the membrane potential obtained from zero-current clamping. Theoretically, in zero-current clamping, there will be no potential change problems caused by series resistance ($V_{err} = I_{cmd} \times R_{series}$. If $I_{cmd} = 0$ pA, then $V_{err} = 0$. Where V_{err} = voltage error, I_{cmd} = command current, and R_{series} = series resistance; Armstrong and Gilly, 1992; Sakmann and Neher, 1995). Consistent with this notion, the voltage drop was negligible when we analyzed the plots of membrane potential versus conductance that had been used for our P_{HCO3}/P_{Cl} and P_I/P_{Cl} measurements (Fig. 1, D and E). This result contradicts the findings in Fig. 4 of the paper by Yu et al. (2014b). The voltage drop caused by large current amplitude is problematic only in the voltage-clamp recording of reversal potential (E_{rev}) measurements using I-V curves. In this case, V_{err} caused by the large currents (R_{series} = ~2 MΩ and R_m = ~10 MΩ in our whole-cell recording) is usually within 20% of the E_{rev} (a maximum of ~4 mV in HCO_3^-/Cl^- bionic potential). The value corresponds to ~0.1 of

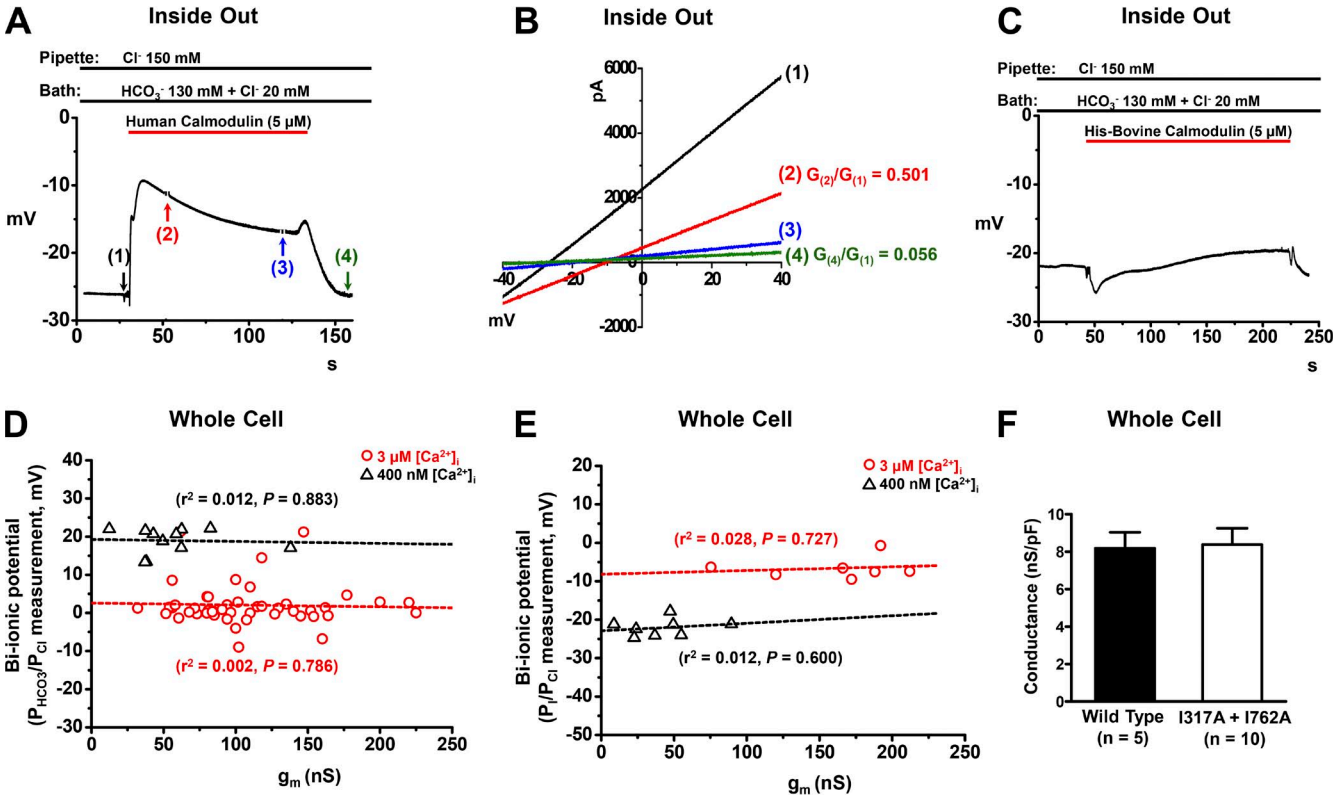

Figure 1. Regulation of ANO1 P_{HCO3}/P_{Cl} by calmodulin. (A–C) Supplementation of calmodulin in inside-out patches increases P_{HCO3}/P_{Cl} of hANO1. Cytosolic solution includes 3 μM of free Ca^{2+}. (A) The addition of human calmodulin altered bi-ionic potential of Cl^-/HCO_3^- and increased P_{HCO3}/P_{Cl} of hANO1 from 0.27 to 0.64. (B) I-V relationships were analyzed during the zero-current clamp recording in A. The anion outward chord conductance (G) between E_{rev} and E_{rev} plus 25 mV was calculated by linear plotting. (C) Effect of the recombinant calmodulin (His_6-tagged bovine calmodulin) was much less than that of human calmodulin. (D and E) Bi-ionic potentials as a function of membrane conductance are plotted. The hANO1 membrane potential data from zero-current clamping in whole-cell configuration were obtained from our previous study (Jung et al., 2013). The bath solution was replaced from 150 mM Cl^- to 130 mM HCO_3^- plus 20 mM Cl^- (D) or to 130 mM I^- plus 20 mM Cl^- (E). Currents were activated by 400 nM (black) or 3 μM of free Ca^{2+} (red) in the pipette. The linear lines represent the result of regression analyses. Large current amplitude did not produce a significant voltage drop. (F) Effect of the CBD mutation (I317A + I762A) on the Cl^- conductance of hANO1. Whole-cell currents were activated by 3 μM of free Ca^{2+} in the pipette. Cl^- membrane conductance was obtained by voltage clamping at 40 mV, and the values were normalized by cell capacity. The normalized conductance of wild-type and CBD-mutated ANO1s were 8.19 ± 0.84 (n = 5) and 8.38 ± 0.87 (n = 10) nS/pF, respectively. Data are means ± SEM. There were no significant differences between two groups ($P > 0.05$). The electrophysiological recordings were performed as described previously (Jung et al., 2013).

P_{HCO3}/P_{Cl} changes in our measurements, where P_{HCO3}/P_{Cl} was increased from 0.38 to 1.07 by high Ca^{2+} (Jung et al., 2013). Therefore, even in the E_{rev} measurements using I-V curves, V_{err} caused by series resistance would influence only 15% of the total P_{HCO3}/P_{Cl} change induced by high Ca^{2+}. In addition, Yu et al. (2014b) suspected that the absence of P_{HCO3}/P_{Cl} change by high cytosolic Ca^{2+} in the CBD-mutated ANO1 might be caused by the smaller current size of the mutant ANO1, because the voltage drop would be smaller if the CBD mutation itself negatively affected ANO1 current. However, there was no difference between the current size of wild-type and CBD-mutated ANO1 (Fig. 1 F). Collectively, the above results indicate that the series resistance problems caused by large current amplitude do not account for the increase in P_{HCO3}/P_{Cl} by high cytosolic Ca^{2+}.

We are fully aware of the ion accumulation problem in bi-ionic potential measurements during whole-cell recording. However, this problem would be negligible in zero-current clamping experiments, as discussed by Yu et al. (2014b). Moreover, even in the E_{rev} measurements using I-V relationships, we injected a ramp pulse of 250 ms, which is significantly shorter than that used by Yu et al. (3 s; 12 times longer than Jung et al., 2013), and which minimizes any potential ion accumulation. Therefore, the condition that Yu et al. used for demonstrating a potential ion accumulation problem was many folds more favorable than our conditions to induce ion accumulation. Another point that we would like to stress is that the accumulation of anion X^- in the cytosolic side always reduces the P_x/P_{Cl} value according to the Goldman–Hodgkin–Katz (GHK) flux equation. The main focus of our study is the narrowing of the P_x/P_{Cl} intervals by Ca^{2+}/calmodulin. Therefore, high cytosolic Ca^{2+} decreased ANO1 permeability to highly permeable anions such as I^- (P_I/P_{Cl}), whereas it increased ANO1 permeability to the poorly permeable ions such as HCO_3^- and F^- (P_{HCO3}/P_{Cl} and P_F/P_{Cl}). Yu et al. attempted to address the ion accumulation problem and P_x/P_{Cl} changes by only measuring anions highly permeable to ANO1, such as SCN^- and I^-, and showing a drop in the SCN^-/Cl^- bi-ionic potential and P_{SCN}/P_{Cl} by the large current amplitude. However, ion accumulation in the cytosolic side cannot explain the increase in P_{HCO3}/P_{Cl} by high cytosolic Ca^{2+}, which is the main finding of our study. If the arguments by Yu et al. were valid and HCO_3^- was indeed accumulated during our whole-cell recordings, the P_{HCO3}/P_{Cl} should have decreased by the high cytosolic Ca^{2+}-induced large currents according to the GHK equation. Our findings show the opposite! One might argue that depletion of cytosolic Cl^- during ANO1 activation in the whole-cell patch would increase P_{HCO3}/P_{Cl}. However, because the pipette resistance (2 MΩ) is much lower than the membrane resistance (10 MΩ, even in the case of large whole-cell current), intracellular Cl^- replenishment from the pipette solution will exceed the Cl^- depletion through the channel, and thus we do not think that simple cytosolic Cl^- depletion elevates P_{HCO3}/P_{Cl} from 0.38 to 1.07 in zero-current clamp recordings. Therefore, we believe that all our inside-out, outside-out, and whole-cell patch-clamp data together with the results of biochemical analyses are valid to demonstrate the dynamic modulation of hANO1 anion permeability by Ca^{2+}/calmodulin.

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