# 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



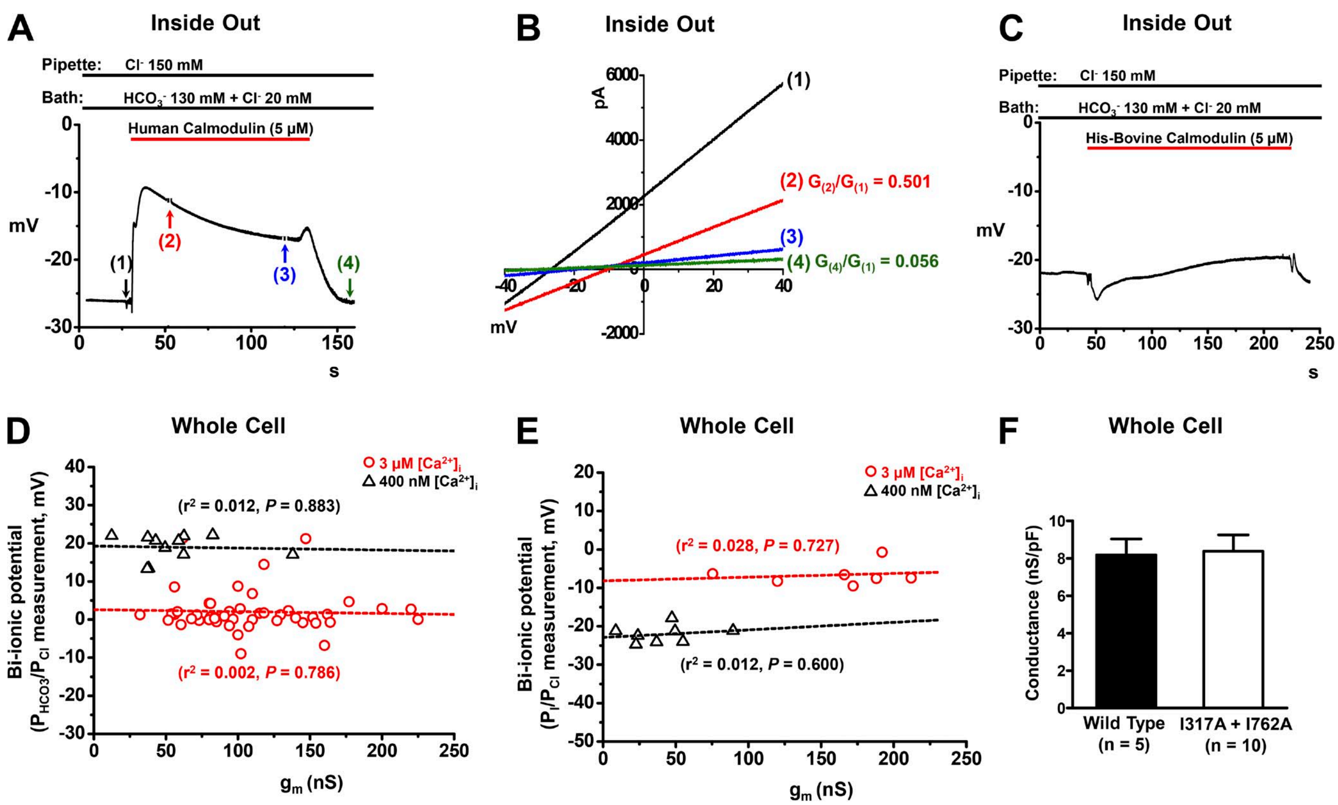


**Figure 1.** Regulation of ANO1 $P_{HCO3}/P_{Cl}$ by calmodulin. (A–C) Supplementation of calmodulin in inside-out patches increases $P_{HCO3}/P_{Cl}$ of hANO1. Cytosolic solution includes 3 µM of free $Ca^{2+}$. (A) The addition of human calmodulin altered bi-ionic potential of $Cl^-/HCO_3^-$ and increased $P_{HCO3}/P_{Cl}$ 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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