



Article

Ambroxol-Enhanced Frequency and Amplitude of Beating Cilia Controlled by a Voltage-Gated Ca^{2+} Channel, $\text{Cav}1.2$, via pH_i Increase and $[\text{Cl}^-]_i$ Decrease in the Lung Airway Epithelial Cells of Mice

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Abstract: Ambroxol (ABX), a frequently prescribed secretolytic agent which enhances the ciliary beat frequency (CBF) and ciliary bend angle (CBA, an index of amplitude) by 30%, activates a voltage-dependent Ca^{2+} channel ($\text{Ca}_v1.2$) and a small transient Ca^{2+} release in the ciliated lung airway epithelial cells (c-LAECs) of mice. The activation of $\text{Ca}_v1.2$ alone enhanced the CBF and CBA by 20%, mediated by a pH_i increase and a $[\text{Cl}^-]_i$ decrease in the c-LAECs. The increase in pH_i , which was induced by the activation of the $\text{Na}^+/\text{HCO}_3^-$ cotransporter (NBC), enhanced the CBF (by 30%) and CBA (by 15–20%), and a decrease in $[\text{Cl}^-]_i$, which was induced by the Cl^- release via anion channel 1 (ANO1), enhanced the CBA (by 10–15%). While a Ca^{2+} -free solution or nifedipine (an inhibitor of $\text{Ca}_v1.2$) inhibited 70% of the CBF and CBA enhancement using ABX, $\text{Ca}_v1.2$ enhanced most of the CBF and CBA increases using ABX. The activation of the $\text{Ca}_v1.2$ existing in the cilia stimulates the NBC to increase pH_i and ANO1 to decrease the $[\text{Cl}^-]_i$ in the c-LAECs. In conclusion, the pH_i increase and the $[\text{Cl}^-]_i$ decrease enhanced the CBF and CBA in the ABX-stimulated c-LAECs.

Keywords: voltage-gated Ca^{2+} channels; $[\text{Ca}^{2+}]_i$; intracellular pH ; intracellular Cl^- concentration; cell volume; $\text{Ca}_v1.2$; airway ciliated epithelial cells

1. Introduction

Mucociliary clearance (MC), which is a host defense mechanism of the lungs, consists of a surface fluid layer (surface mucous layer (SML) and periciliary layer (PCL)) and beating cilia lining the airway surface [1–3]. Inhaled small particles, such as bacteria, viruses and chemicals, are entrapped by the SML and swept away toward the oropharynx by the beating cilia in the PCL. The MC is compared to a belt conveyor system in sweeping away inhaled small particles from the airways, and the beating cilia are the engine that drives MC [1,3,4]. Impairment of the beating cilia, such as Kartagener syndrome or primary ciliary dyskinesia, causes serious respiratory diseases [1,3]. Therefore, the drugs activating beating cilia are of particular importance to prevent or improve respiratory diseases. Ambroxol (ABX), a frequently prescribed secretolytic agent for respiratory diseases, which increases $[\text{Ca}^{2+}]_i$ by activating a voltage-gated Ca^{2+} channel, $\text{Ca}_v1.2$, in the ciliated lung airway

epithelial cells (c-LAECs), has been shown to increase the CBF (ciliary beat frequency) and CBA (ciliary bend angle, an index of amplitude) [5,6]. However, the Ca^{2+} -regulated signals following $\text{Ca}_v1.2$ activation, which may enhance CBF and CBA, remain uncertain.

The beating cilia in the airways express many ion channels, including Ca^{2+} -permeable channels, such as $\text{Ca}_v1.2$, transient receptor potential (TRP) V4, TRPA1 and TRPM8 [6–8]. However, it is still controversial how these channels enhance the ciliary beating. The beating cilia in the airways are activated by many substances, such as cAMP, Ca^{2+} , ATP, β_2 -agonists, Cl^- and H^+ (intracellular pH (pH_i)) [4,6–12]. Among them, Ca^{2+} is an important ion that activates the beating cilia [3,4,6–8]. ABX stimulates $\text{Ca}_v1.2$ and a small transient Ca^{2+} release from the acidic stores in the c-LAECs [6,13]. These observations suggest that $\text{Ca}_v1.2$ plays an important role in the activation of ciliary beating during ABX stimulation. Moreover, ABX has been shown to stimulate anion secretion in lung epithelial cell lines [14]. The activation of $\text{Ca}_v1.2$ may stimulate Cl^- secretion in the c-LAECs, leading to a decrease in the intracellular Cl^- concentration ($[\text{Cl}^-]_i$), since anoctamine 1 (ANO1) exists in nasal ciliated epithelial cells [12]. Moreover, Saito et al. suggested that ABX stimulates a pH_i increase in c-LAECs [6]. Thus, $\text{Ca}_v1.2$ activation stimulated via ABX may increase the pH_i and decrease the $[\text{Cl}^-]_i$ in c-LAECs. Previous studies have demonstrated that a pH_i increase and an $[\text{Cl}^-]_i$ decrease enhance the CBF and CBA in airway ciliary cells [9,10,12]. The activation of $\text{Ca}_v1.2$ existing in the cilia may increase pH_i to enhance the CBF and CBA and decrease $[\text{Cl}^-]_i$ to enhance the CBA.

Cilia have two functionally distinct molecular motors, namely outer dynein arms (ODAs) and inner dynein arms (IDAs), which regulate the ciliary beat frequency (CBF) and ciliary bend angle (CBA), respectively [15,16]. Previous studies have demonstrated that a CBA increase, in addition to a CBF increase, enhances ciliary transport in the airway [12], although the CBF has been used to assess the activity of ciliary beating [3,4]. The signaling pathways regulating the CBF and CBA have been shown to be different [9,11,12]. ABX has been shown to enhance the CBF and CBA [6], suggesting that the activation of $\text{Ca}_v1.2$ may trigger two signaling pathways, increasing the CBF and CBA.

In this study, we examined the effects of $\text{Ca}_v1.2$ on the enhancement of the CBF and CBA in the ABX-stimulated c-LAECs of mice. In experiments using two solutions with and without $\text{CO}_2/\text{HCO}_3^-$, we found that the $\text{Ca}_v1.2$ activated using ABX stimulated two signaling pathways to increase the CBF and CBA, $\text{CO}_2/\text{HCO}_3^-$ -dependent and $\text{CO}_2/\text{HCO}_3^-$ -independent. This study is designed to clarify how $\text{Ca}_v1.2$ activates the two signalling pathways increasing the CBF and CBA in c-LAECs.

2. Results

In unstimulated c-LAECs perfused with the HCO_3^- -containing control solution, the CBF and CBA were 8–10 Hz and 70° – 90° , respectively [9,11]. Saito et al. demonstrated that ABX increases the CBF and CBA in a concentration-dependent manner and it maximally increases the CBF and CBA at 10 μM [6]. In this study, the concentration of ABX used was 10 μM . In this study, we used a nominally Ca^{2+} -free solution. Because an EGTA-containing Ca^{2+} -free solution increases the CBF by inhibiting the Ca^{2+} -dependent PDE1A existing in the metabolon, regulating the ODAs (CBF) in the cilia [11]. To inhibit $\text{Ca}_v1.2$, we used nifedipine [6]. To increase the pH_i , we applied a $\text{CO}_2/\text{HCO}_3^-$ -free solution, which enhances the CBF and CBA [9,10]. The application of a $\text{CO}_2/\text{HCO}_3^-$ -free solution is a well-known procedure to increase pH_i .

2.1. ABX-Stimulated Cellular Events

2.1.1. ABX-Stimulated Increases in the CBF and CBA

Supplementary Videos S1 and S2 show video images of a c-LAEC before and 15 min after ABX stimulation. ABX gradually increased the ratio of the CBF and CBA (normalized CBF and CBA) by 30% within 10 min, and the ratios of the CBF and CBA at 10 min after stimulation were 1.25 ($n = 13$) and 1.27 ($n = 8$), respectively (Figure 1A). We examined the effects of Ca^{2+} on the CBF and CBA stimulated by ABX. The switch to a

nominally Ca^{2+} -free solution decreased the ratios of CBF and CBA by 5% within 5 min, and then stimulation using ABX increased the ratios of the CBF and CBA by 10–13% within 5 min. The ratios of the CBF and CBA at 5 min after the switch were 0.96 ($n = 8$) and 0.96 ($n = 6$) and those 5 min after the addition of ABX were 1.04 and 1.09, respectively (Figure 1B). The same experiments were carried out using nifedipine (20 μM) instead of the Ca^{2+} -free solution. The addition of nifedipine decreased the ratios of the CBF and CBA by 5% and then ABX stimulation increased them by 10%. A previous study has shown similar results in c-LAECs [6]. Experiments were also carried out in the absence of $\text{CO}_2/\text{HCO}_3^-$ (Figure 1C,D). The switch to the $\text{CO}_2/\text{HCO}_3^-$ -free control solution immediately increased the CBF and CBA, and the ratios of the CBF and CBA 5 min after the switch were 1.32 ($n = 8$) and 1.21 ($n = 6$), respectively. Further ABX stimulation increased only the CBA, but not the CBF. The ratios of the CBF and CBA at 5 min after ABX stimulation were 1.32 ($n = 6$) and 1.30 ($n = 8$), respectively. The effects of nifedipine (10 μM) on the CBA increase were examined (Figure 1D). The addition of nifedipine decreased the CBF and CBA by 5%. Then, the switch to a $\text{CO}_2/\text{HCO}_3^-$ -free solution immediately increased the CBF and CBA. The ratios of the CBF and CBA 5 min after the switch were 1.28 ($n = 4$) and 1.20 ($n = 4$), respectively. Further ABX stimulation did not increase the CBA (Figure 1D). Similar results were obtained using a Ca^{2+} -free solution. The CBA increase stimulated by ABX in the application of the $\text{CO}_2/\text{HCO}_3^-$ -free solution appears to be controlled by a $[\text{Ca}^{2+}]_i$ increase via the $\text{Ca}_v1.2$ activation.

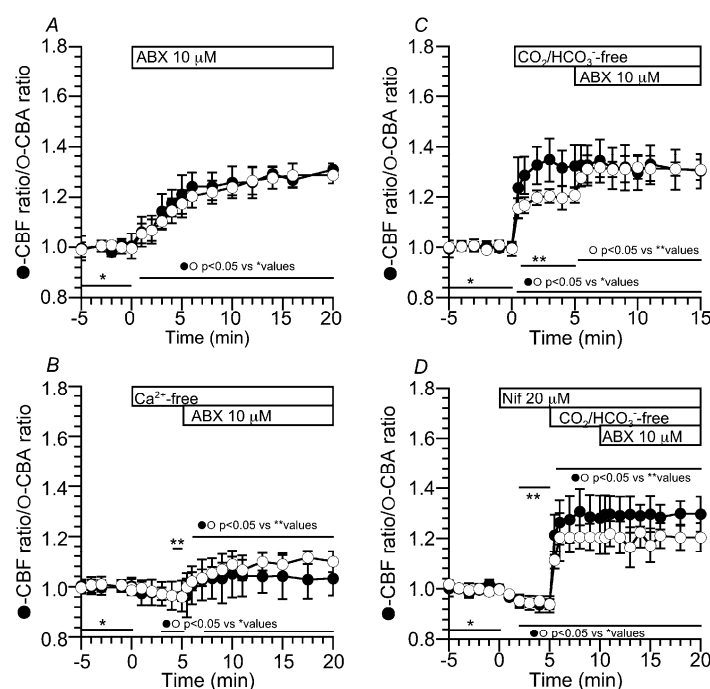


Figure 1. The effect of ABX on the ratios of the CBF and CBA in c-LAECs. (A): ABX (10 μM) stimulation in the presence of $\text{CO}_2/\text{HCO}_3^-$. In the $\text{CO}_2/\text{HCO}_3^-$ -containing control solution, ABX stimulation (10 μM) gradually increased the ratios of the CBF and CBA by 30% within 10 min. (B): Effects of Ca^{2+} -free solution on the ABX-stimulated CBF and CBA in the presence of $\text{CO}_2/\text{HCO}_3^-$. The switch to a nominally Ca^{2+} -free solution decreased the CBF and CBA ratios by 5% within 5 min. Then, ABX stimulation gradually increased the CBF and CBA ratios by 10%. (C): ABX stimulation in the absence of $\text{CO}_2/\text{HCO}_3^-$. The switch to the $\text{CO}_2/\text{HCO}_3^-$ -free solution immediately increased the CBF ratio by 30% and the CBA ratio by 20%. Then, ABX stimulation increased the CBA ratio by 10%, but not the CBF ratio. (D): Effects of nifedipine on the ABX-stimulated CBF and CBA ratios in the absence of $\text{CO}_2/\text{HCO}_3^-$. The addition of nifedipine (20 μM) decreased the CBF and CBA ratios by 5%. The switch to the $\text{CO}_2/\text{HCO}_3^-$ -free solution immediately increased the CBF ratio by 30% and the CBA ratio by 20%. Further ABX stimulation did not induce any increase in the CBF ratio or the CBA ratio.

Prior addition of BAPTA-AM (10 μM , a membrane permeable analog of BAPTA, a Ca^{2+} chelator, that binds the intracellular calcium after the acetoxymethyl group is removed by the cytoplasmic esterase) completely inhibited the increases in the CBF and CBA stimulated by ABX in a Ca^{2+} -free solution [6]. Thus, Ca^{2+} entry via the ABX-activated $\text{Ca}_v1.2$ triggers two signal pathways, $\text{CO}_2/\text{HCO}_3^-$ -dependent and $\text{CO}_2/\text{HCO}_3^-$ -independent pathways.

2.1.2. ABX-Stimulated Increases in $[\text{Ca}^{2+}]_i$

Changes in $[\text{Ca}^{2+}]_i$ were monitored via the fura-2 fluorescence ratio (F340/F380) in the c-LAECs. In the control solution, ABX stimulation gradually increased the F340/F380, which reached a plateau within 15 min, and then, the addition of nifedipine decreased the F340/F380 to the pre-stimulation level within 10 min (Figure 2A). Changes in the F340/F380 stimulated by ABX were measured in the c-LAECs perfused with a Ca^{2+} -free solution. ABX induced a small transient increase in the F340/F380 (Figure 2B), as shown in a previous report [6]. ABX has been shown to stimulate the Ca^{2+} release from acidic stores in alveolar type II cells (ATII cells), which was inhibited by an increase in pH_i [6,13]. Experiments were then carried out in a $\text{CO}_2/\text{HCO}_3^-$ -free solution. The switch from the control solution to the $\text{CO}_2/\text{HCO}_3^-$ -free solution did not change the F340/F380, and ABX stimulation did not increase the F340/F380. The addition of nifedipine did not change the F340/F380 (Figure 2C). An increase in pH_i induced by the application of the $\text{CO}_2/\text{HCO}_3^-$ -free solution inhibited ABX-stimulated Ca^{2+} release from the acidic stores, as shown in a previous report [6]. Moreover, the $\text{CO}_2/\text{HCO}_3^-$ -free solution may hyperpolarize the membrane potential by inhibiting the NBC [6], which decreases the $[\text{Ca}^{2+}]_i$ to a low level.

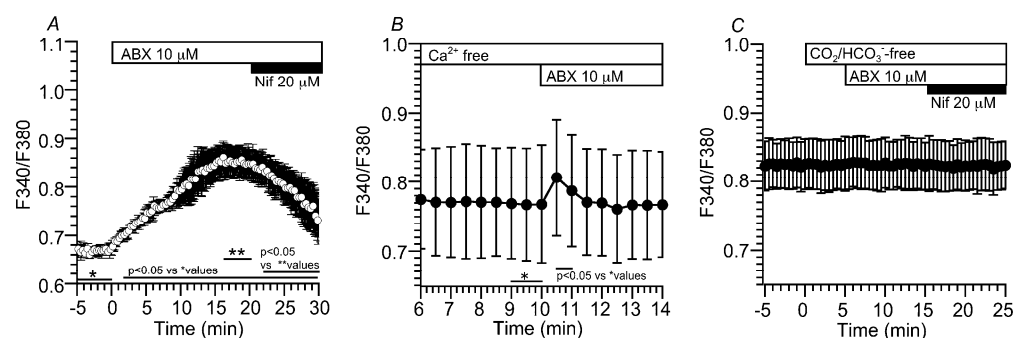


Figure 2. Effects of ABX on $[\text{Ca}^{2+}]_i$ in c-LAECs. Changes in $[\text{Ca}^{2+}]_i$ were monitored via the fura-2 fluorescence ratio (F340/F380). (A): ABX stimulation gradually increased F340/F380, which reached a plateau within 15 min. Then, the addition of nifedipine gradually decreased F340/F380 to the control level before ABX stimulation within 10 min. (B): Effects of the Ca^{2+} -free solution on the ABX-stimulated $[\text{Ca}^{2+}]_i$. In the Ca^{2+} -free solution, ABX stimulation induced a small transient increase in F340/F380. (C): Effects of ABX on $[\text{Ca}^{2+}]_i$ in the absence of $\text{CO}_2/\text{HCO}_3^-$. The switch to the $\text{CO}_2/\text{HCO}_3^-$ -free solution did not change F340/F380 and then ABX stimulation did not change F340/F380. Further addition of nifedipine did not change F340/F380. The $\text{CO}_2/\text{HCO}_3^-$ -free solution increased pH_i , which inhibits Ca^{2+} release from acidic stores, and it inhibits Na^+ entry via the NBC. The $\text{CO}_2/\text{HCO}_3^-$ -free solution decreases $\text{Ca}_v1.2$ channel activity.

2.1.3. ABX-Stimulated Changes in pH_i

Changes in pH_i were measured via the ratio of SNARF1 fluorescence (F645/F592). In the control solution, ABX stimulation gradually increased the pH_i from 7.49 to 7.65 ($n = 8$) within 15 min (Figure 3A). The cells were also stimulated by ABX in a Ca^{2+} -free solution (Figure 3B) or in the presence of nifedipine (Figure 3C). ABX increased the pH_i from 7.51 to 7.58 in the Ca^{2+} -free solution ($n = 4$, Figure 3B) and from 7.52 to 7.60 in the presence of nifedipine ($n = 5$, Figure 3C). Thus, the activation of $\text{Ca}_v1.2$ significantly increased the pH_i in the ABX-stimulated c-LAECs. However, the Ca^{2+} release from the acidic stores also increased the pH_i , but its extent was small (Figure 3B,C). Changes in pH_i were measured upon applying the $\text{CO}_2/\text{HCO}_3^-$ -free solution. An application of the $\text{CO}_2/\text{HCO}_3^-$ -free

solution transiently increased and sustained the pH_i . The further ABX stimulation did not change the pH_i (Figure 3D).

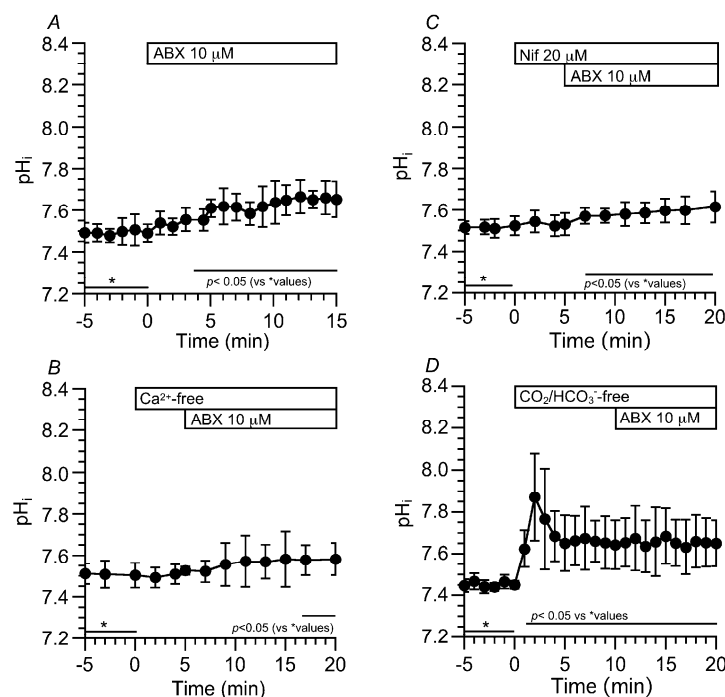


Figure 3. Effects of ABX on pH_i in c-LAECs. Changes in pH_i were measured using the SNARF1 fluorescence ratio (F645/F592). (A): ABX stimulation gradually increased pH_i from 7.49 to 7.65 within 15 min ($n = 8$). (B): Effects of a Ca^{2+} -free solution on the ABX-stimulated pH_i . The switch to a Ca^{2+} -free solution did not change pH_i . Then, ABX stimulation gradually increased pH_i from 7.51 to 7.58 ($n = 4$). (C): Effects of nifedipine on the ABX-stimulated pH_i . The addition of nifedipine did not change pH_i . Then, ABX stimulation gradually increased pH_i from 7.52 to 7.60 ($n = 5$). (D): Effects of $\text{CO}_2/\text{HCO}_3^-$ -free solution on the ABX-stimulated pH_i . The switch to a $\text{CO}_2/\text{HCO}_3^-$ -free solution induced a transient increase, followed by a sustained increase in pH_i . The values of pH_i before, 2 min after and 10 min after the switch were 7.45, 7.87 and 7.65 ($n = 6$), respectively. Further ABX stimulation did not change pH_i .

2.1.4. ABX-Stimulated Cell Shrinkage and a Decrease in $[\text{Cl}^-]_i$

Video Images of ABX-Stimulated Cell Shrinkage and Enhancement of MQAE Fluorescence in c-LAECs

Figure 4A shows a phase contrast video image of a typical c-LAEC perfused with the control solution before ABX stimulation (Figure 4A) and that at 15 min after ABX stimulation (Figure 4B). The outline of a c-LAEC before ABX stimulation is shown in Figure 4A and was superimposed in Figure 4B. The c-LAECs stimulated by ABX were smaller than those before ABX stimulation, indicating that ABX decreased the cell volume. Cell shrinkage is known to decrease $[\text{Cl}^-]_i$ [9,12]. We monitored the $[\text{Cl}^-]_i$ using a Cl^- -sensitive fluorescence dye in the c-LAECs (MQAE) [9,12]. ABX stimulation increased the intensity of MQAE fluorescence in one of the c-LAECs (Figure 4C,D), indicating that ABX stimulation decreases $[\text{Cl}^-]_i$.

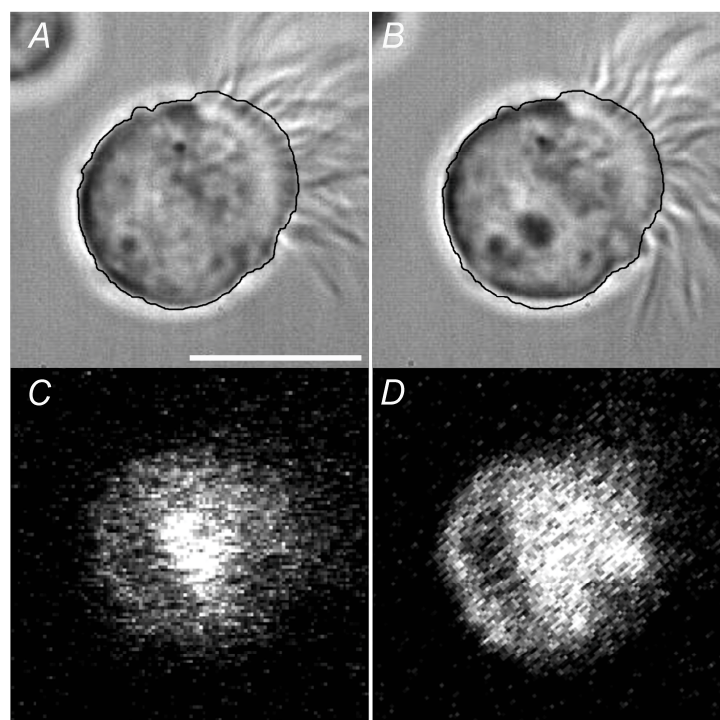


Figure 4. Video frame images and MQAE fluorescence of a c-LAEC. (A,B): Video frame images of c-LAECs before (A) and at 15 min after ABX stimulation (B). The outline of a c-LAEC before ABX stimulation is traced by the black line (A). ABX stimulation induced cell shrinkage. The traced outline of the c-LAECs in the panel (A) was superimposed onto the c-LAEC in the panel (B). The panel (B) shows that the outline of the c-LAEC stimulated by ABX was smaller than that before ABX stimulation. (C,D): Changes in MQAE fluorescence of a c-LAEC before (C) and at 15 min after ABX stimulation (D). ABX stimulation increased the intensity of MQAE fluorescence, indicating that ABX decreased $[Cl^-]_i$ in the c-LAEC.

ABX-Stimulated Decreases in Cell Volume and $[Cl^-]_i$

In the control solution, the changes in cell volume (V/V_0 , index of cell volume) and $[Cl^-]_i$ (F_0/F , MQAE fluorescence ratio) stimulated by ABX were measured in the c-LAECs (Figure 5). ABX stimulation decreased the V/V_0 to 0.81 ($n = 5$, 6 min after ABX stimulation) (Figure 5A) and the F_0/F to 0.70 ($n = 4$, 10 min after the ABX stimulation) (Figure 5B). Then, the addition of nifedipine immediately recovered the V/V_0 to the level before ABX stimulation (V/V_0 at 5 min after nifedipine addition = 1.03) (Figure 5A). Thus, ABX stimulation decreased the cell volume (V/V_0) and MQAE fluorescence ratio (F_0/F) by activating $Ca_v1.2$.

To examine the relationship between the CBA and V/V_0 (or $[Cl^-]_i$) without a pH_i change, experiments were also carried out using the CO_2/HCO_3^- -free solution, in which ABX stimulation increased only the CBA. The switch to the CO_2/HCO_3^- -free solution decreased the V/V_0 and F_0/F to 0.90 ($n = 5$, 5 min after the switch) and 0.84 ($n = 4$, 5 min after the switch), respectively. Further ABX stimulation decreased the V/V_0 and F_0/F to 0.78 ($n = 5$, 10 min after ABX stimulation, Figure 5C) and 0.69 (10 min after ABX stimulation, Figure 5D), respectively. Thus, in the CO_2/HCO_3^- -free solution, ABX still induced cell shrinkage, leading to $[Cl^-]_i$ decrease, although ABX did not increase the $[Ca^{2+}]_i$ (Figure 2C). The question is whether or not the cell shrinkage or $[Cl^-]_i$ decrease was $Ca_v1.2$ -dependent. The effects of nifedipine on ABX-stimulated cell shrinkage and $[Cl^-]_i$ decrease were examined in the CO_2/HCO_3^- -free solution (Figure 5E,F). In the control solution, the addition of nifedipine increased the V/V_0 and F_0/F to 1.12 ($n = 4$, 5 min after nifedipine addition) and 1.15 ($n = 5$, 5 min after nifedipine addition), respectively. The switch to the CO_2/HCO_3^- -free solution decreased the V/V_0 and F_0/F to 0.93 and 0.82,

and then ABX stimulation did not change the V/V_0 (0.91 at 10 min after ABX stimulation) (Figure 5E) and F_0/F (0.82 at 10 min after ABX stimulation) (Figure 5F). The ABX-stimulated cell shrinkage and $[Cl^-]_i$ decrease in the CO_2/HCO_3^- -free solution are likely to be induced by the activation of $Ca_v1.2$.

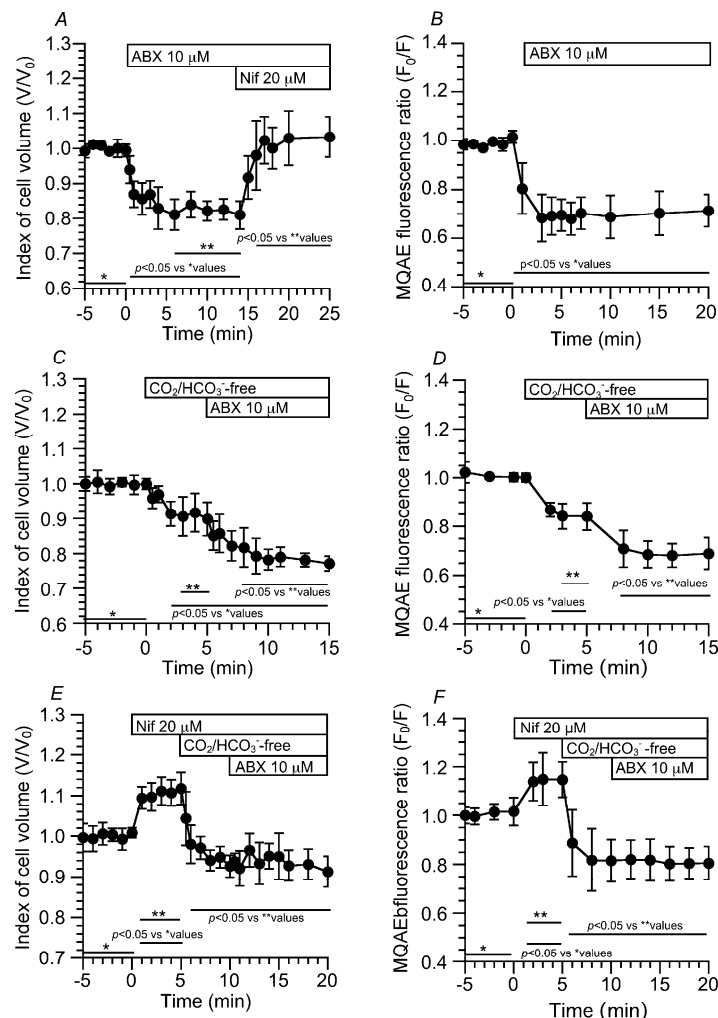


Figure 5. Changes in the cell volume and $[Cl^-]_i$ induced by ABX stimulation in c-LAECs. (A): In the CO_2/HCO_3^- -containing control solution, ABX stimulation decreased V/V_0 by 20%. Then, the addition of nifedipine immediately increased V/V_0 to the prestimulation level. (B): In the CO_2/HCO_3^- -containing control solution, ABX stimulation decreased the ratio of MQAE fluorescence (F_0/F) by 30%. (C): The switch to the CO_2/HCO_3^- -free control solution decreased V/V_0 by 10%. Then, ABX stimulation gradually decreased V/V_0 . In the CO_2/HCO_3^- -free control solution, ABX still activates $Ca_v1.2$ channels. (D): The switch to the CO_2/HCO_3^- -free control solution decreased F_0/F by 15%, and then, ABX stimulation decreased F_0/F by 20%. (E): Effects of nifedipine on V/V_0 stimulated using ABX. The addition of nifedipine increased V/V_0 by 10%. Then, the switch to the CO_2/HCO_3^- -free solution decreased V/V_0 by 20%. Further ABX stimulation did not decrease V/V_0 . (F): The addition of nifedipine increased F_0/F , and the switch to the CO_2/HCO_3^- -free control solution decreased F_0/F . Further ABX stimulation did not decrease F_0/F .

2.2. CO_2/HCO_3^- -Dependent Pathway (pH_i Pathway)

The pH_i pathway (pH_i increase) is activated by the HCO_3^- entry. The epithelial cells in airways express two HCO_3^- transporters, $Na^+-HCO_3^-$ cotransport (NBC) and Cl^-/HCO_3^- exchange (anion exchange, AE) [17].

2.2.1. Effects of DIDS on the CBF, CBA and pH_i

The effects of 4,4'-Diisothiocyano-2,2'-stilbenedisulfonic acid (DIDS, 100 μM , an inhibitor of the NBC and AE) on the CBF, CBA and pH_i were examined (Figure 6). The addition of DIDS (100 μM) gradually increased the CBF, but not the CBA (Figure 6A). The CBF reached a plateau within 15 min. The CBF and CBA ratios 15 min after DIDS addition were 1.12 ($n = 6$) and 1.01 ($n = 4$), respectively. The addition of DIDS also gradually increased the pH_i from 7.45 to 7.52 ($n = 5$, 10 min after DIDS addition), and ABX stimulation did not affect the gradual pH_i increase induced by DIDS (7.55 ($n = 4$), 10 min after ABX stimulation, Figure 6B). The effects of ABX on the CBA and CBF were examined in the presence of DIDS (Figure 6C). The addition of DIDS gradually increased only the CBF. Then, stimulation using ABX immediately increased only the CBA by 17% without any increase in the CBF (Figure 6C). In the DIDS-treated c-LAECs, ABX did not increase the CBF and pH_i , but it increased the CBA (Figure 6C). Changes in the MQAE fluorescence ratio induced by ABX were measured in the presence of DIDS. DIDS alone did not change the F_0/F , but stimulation with ABX decreased the F_0/F (Figure 6D). DIDS did not affect the $[\text{Cl}^-]_i$ decrease stimulated by ABX, suggesting that a $[\text{Cl}^-]_i$ decrease increased the CBA.

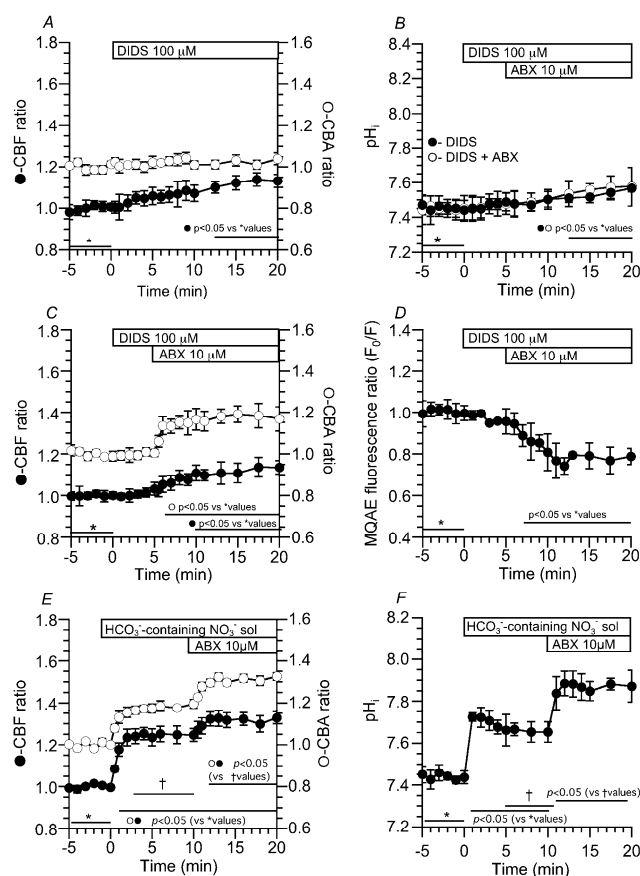


Figure 6. Effects of DIDS on the CBF, CBA, pH_i and $[\text{Cl}^-]_i$ stimulated by ABX in c-LAECs. (A): Effects of DIDS on CBF and CBD. The addition of DIDS gradually decreased the CBF, but not the CBA in c-LAECs. (B): Effects of DIDS on pH_i s with or without ABX. The addition of DIDS gradually increased pH_i (J) The ABX stimulation did not affect the gradual pH_i increase induced by DIDS (E). (C): Changes in the CBF and CBA ratios stimulated by ABX in the presence of DIDS. The addition of DIDS gradually increased the CBF ratio by 15%, but not the CBA ratio. Then, ABX stimulation increased the CBA ratio but not the CBF ratio. (D): Effects of ABX on $[\text{Cl}^-]_i$ of c-LAECs in the presence of DIDS. The addition of DIDS slightly decreased F_0/F and then ABX stimulation decreased

F_0/F . (E): Effects of ABX on the CBF and CBA ratios in the HCO_3^- -containing NO_3^- solution. The switch to the HCO_3^- -containing NO_3^- solution increased the CBF and CBA ratios. Then, ABX stimulation further increased the CBF and CBA ratios. (F): Effects of ABX on pH_i in the HCO_3^- -containing NO_3^- solution. The switch to the HCO_3^- -containing NO_3^- solution increased pH_i . Then, the ABX stimulation further increased pH_i . The HCO_3^- -containing NO_3^- solution enhanced the pH_i increase stimulated by ABX.

2.2.2. Effects of HCO_3^- -Containing NO_3^- Solution on CBF, CBA and pH_i

To confirm HCO_3^- entry via the NBC, we used a HCO_3^- -containing Cl^- -free NO_3^- solution, in which the NBC is functional, but not the AE (Figure 6E,F). The switch to the HCO_3^- -containing Cl^- -free NO_3^- solution immediately increased the CBF and CBA, and then stimulation using ABX increased both. The CBF and CBA ratios at 10 min after the switch were 1.24 ($n = 7$) and 1.20 ($n = 8$), and those at 10 min after the ABX stimulation were 1.33 and 1.33, respectively (Figure 6E). The HCO_3^- -containing Cl^- -free NO_3^- solution alone increased the pH_i from 7.43 to 7.65 ($n = 5$) and then, the ABX stimulation increased the pH_i to 7.91. The HCO_3^- -containing Cl^- -free NO_3^- solution potentiated the CBF, CBA and pH_i being increased by ABX (Figure 6F). The HCO_3^- -containing Cl^- -free NO_3^- solution, which inhibits HCO_3^- extrusion via the AE while maintaining HCO_3^- influx via the NBC, may increase the concentration of HCO_3^- in the c-LAECs, leading to an increase in pH_i . ABX appears to stimulate the NBC to increase the pH_i .

2.3. $\text{CO}_2/\text{HCO}_3^-$ -Independent Pathway (Cl^- Pathway)

An increase in the CBA appears to be activated by an $[\text{Cl}^-]_i$ decrease (Cl^- pathway), coupled with cell shrinkage [12]. To inhibit the $[\text{Cl}^-]_i$ decrease, we used inhibitors of the Cl^- channel, 5-nitro-2-(3-phenylpropylamino)benzoic acid (NPPB, 20 μM) and T16Ainh (10 μM , an inhibitor of ANO1). A previous study demonstrated that anoctamin-1 (ANO1), a Ca^{2+} -activated Cl^- channel, functions in ciliated nasal epithelial cells [12,18].

2.3.1. Effects of NPPB on CBF, CBA and $[\text{Cl}^-]_i$

The addition of NPPB decreased the CBF and CBA ratios to 0.92 and 0.96 within 5 min, respectively. Then, the ABX stimulation immediately increased the CBF and CBA ratios to 1.06 and 1.10, respectively (Figure 7A). The addition of NPPB increased the MQAE fluorescence ratio (F_0/F) from 0.99 ($n = 6$) to 1.14 (5 min after the addition). Then, the ABX stimulation gradually increased the F_0/F (F_0/F at 15 min after ABX stimulation = 1.25) (Figure 7B). In the experiments using the $\text{CO}_2/\text{HCO}_3^-$ -free solution, NPPB did not affect the increases in the CBF and CBA induced by the switch to the $\text{CO}_2/\text{HCO}_3^-$ -free solution. However, ABX stimulation did not increase the CBF or CBA (Figure 7C). The CBF and CBA ratios before and 15 min after ABX stimulation were 1.03 ($n = 4$) and 1.11 ($n = 6$) and 1.03 and 1.06 in the $\text{CO}_2/\text{HCO}_3^-$ -free solution with NPPB, respectively. Changes in $[\text{Cl}^-]_i$ were monitored via the MQAE fluorescence ratio (F_0/F) (Figure 7D). The addition of NPPB increased the F_0/F by 15%. The switch to a $\text{CO}_2/\text{HCO}_3^-$ -free solution, in which there was decreased Na^+ entry due to inhibition of the NBC, decreased the F_0/F to 0.88 ($n = 5$) at 5 min after the switch, and stimulation with ABX did not change the F_0/F (0.86 at 15 min after ABX stimulation). An increase in $[\text{Cl}^-]_i$ appears to inhibit the CBF and CBA increase stimulated by ABX [12]. No decrease in $[\text{Cl}^-]_i$ induced no further increase in the CBA during ABX stimulation in the $\text{CO}_2/\text{HCO}_3^-$ -free solution.

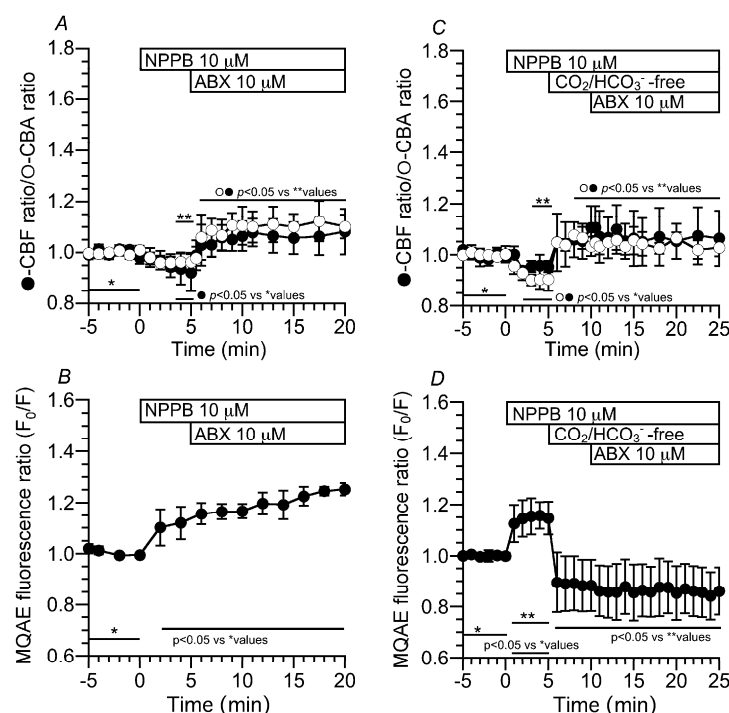


Figure 7. Effects of NPPB (a Cl^- channel blocker) on the CBF, CBA and $[\text{Cl}^-]_i$ stimulated by ABX. (A): In the $\text{CO}_2/\text{HCO}_3^-$ -containing control solution, the addition of NPPB decreased the CBF and CBA ratios by 5%. Further ABX stimulation increased the CBF and CBA ratios by 10%. (B): The addition of NPPB increased F_0/F . Then, ABX stimulation gradually increased F_0/F . (C): The addition of NPPB decreased the CBF and CBA ratios by 5% and then the switch to the $\text{CO}_2/\text{HCO}_3^-$ -free solution increased the CBF and CBA ratios. Further ABX stimulation did not change the CBF and CBA ratios. (D): The addition of NPPB increased F_0/F , and then, the switch to the $\text{CO}_2/\text{HCO}_3^-$ -free control solution decreased F_0/F . Further ABX stimulation did not change F_0/F .

2.3.2. Effects of an ANO1 Inhibitor (T16Ainh) on CBF, CBA and $[\text{Cl}^-]_i$

The addition of T16Ainh (10 μM , an inhibitor of ANO1) decreased the CBA (0.95 ($n = 5$) at 5 min after the addition), but not the CBF (1.02 ($n = 7$) at 5 min after the addition). ABX stimulation increased the CBF and CBA to 1.12 and 1.12 (at 10 min after the ABX stimulation), respectively (Figure 8A). Experiments were then carried out in a $\text{CO}_2/\text{HCO}_3^-$ -free solution. T16Ainh did not affect the CBF and CBA increased by the switch to the $\text{CO}_2/\text{HCO}_3^-$ -free solution. Then, further ABX stimulation did not change the CBF or CBA. The CBF and CBA ratios before and after ABX stimulation were 1.23 ($n = 7$) and 1.18 ($n = 5$) at 5 min after the T16Ainh addition and 1.23 and 1.20 at 10 min after the ABX stimulation, respectively (Figure 8B). These results indicate that the activation of ANO1 decreases the $[\text{Cl}^-]_i$ in ABX-stimulated c-LAECs.

2.4. Effects of a $\text{CO}_2/\text{HCO}_3^-$ -Free Cl^- -Free NO_3^- Solution on CBF, CBA and $[\text{Cl}^-]_i$

These results suggest that an increase in pH_i and a decrease in $[\text{Cl}^-]_i$ stimulated by ABX increased the CBF and CBA. We used a $\text{CO}_2/\text{HCO}_3^-$ -free Cl^- -free NO_3^- solution, which increases the pH_i and decreases the $[\text{Cl}^-]_i$ [9]. The switch to the $\text{CO}_2/\text{HCO}_3^-$ -free solution increased the CBF and CBA. The CBF and CBA ratios at 5 min after the switch were 1.25 ($n = 5$) and 1.16 ($n = 5$), respectively. Then, the second switch to the $\text{CO}_2/\text{HCO}_3^-$ -free Cl^- -free NO_3^- solution increased the CBA without any CBF increase. The CBF and CBA ratios at 5 min after the second switch were 1.27 ($n = 5$) and 1.28 ($n = 5$), respectively. Further ABX stimulation did not increase the CBA or CBF. The CBF and CBA ratios at 5 min after ABX stimulation were 1.31 ($n = 5$) and 1.29 ($n = 5$), respectively (Figure 9A). Changes in $[\text{Cl}^-]_i$ were also monitored via the MQAE fluorescence ratio using the same protocol. The $\text{CO}_2/\text{HCO}_3^-$ -free solution decreased the F_0/F to 0.90 (5 min after the switch, $n = 4$)

and the second switch further decreased the F_0/F to 0.73 (5 min after the second switch, $n = 4$). ABX stimulation did not decrease the F_0/F (0.69 at 10 min after the ABX stimulation, $n = 4$) (Figure 9B). Thus, the effects of ABX on the CBA and CBF were mimicked by the $\text{CO}_2/\text{HCO}_3^-$ -free Cl^- -free NO_3^- solution.

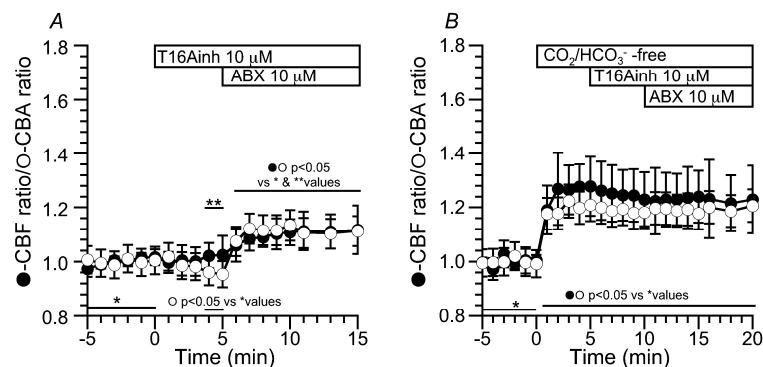


Figure 8. Effects of T16Ainh (an inhibitor of ANO1) on the CBF and CBA stimulated by ABX. (A): In the $\text{CO}_2/\text{HCO}_3^-$ -containing control solution, the addition of T16Ainh (10 μM) decreased the CBA ratio by 5%, but not the CBF ratio. Further ABX stimulation increased the CBF and CBA ratios by 10%. (B): The switch to the $\text{CO}_2/\text{HCO}_3^-$ -free control solution increased the CBF ratio by 30% and the CBA ratio by 20%, and then, the addition of T16Ainh did not change the CBF and CBA ratios. The addition of ABX did not change the CBF and CBA ratios.

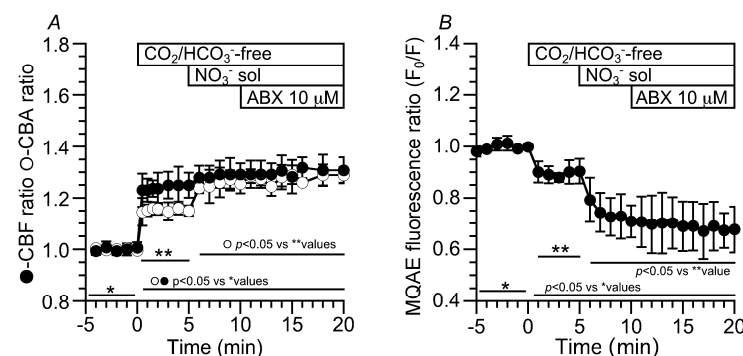


Figure 9. Effects of $\text{CO}_2/\text{HCO}_3^-$ -free Cl^- -free NO_3^- solution on the CBF and CBA. (A): The switch to the $\text{CO}_2/\text{HCO}_3^-$ -free solution increased the CBF and CBA ratios and then the switch to the $\text{CO}_2/\text{HCO}_3^-$ -free Cl^- -free NO_3^- solution increased the CBA ratio, but not the CBF ratio. Further ABX stimulation did not increase the CBF and CBA ratios. Thus, the increases in CBA and CBF stimulated by ABX were mimicked by the $\text{CO}_2/\text{HCO}_3^-$ -free Cl^- -free NO_3^- solution. (B): Changes in $[\text{Cl}^-]_i$ monitored via the MQAE fluorescence ratio (F_0/F). The switch to the $\text{CO}_2/\text{HCO}_3^-$ -free solution decreased F_0/F and then the second switch to the $\text{CO}_2/\text{HCO}_3^-$ -free Cl^- -free NO_3^- solution decreased F_0/F . Further ABX stimulation did not change F_0/F .

2.5. Expression of Anoctamin-1 in c-LAECs

The expression of ANO1 (TMEM16A) was examined using Western blotting and immunofluorescence. In the Western blotting, a single band of ANO1 was detected at 110 kDa (Figure 10). The immunofluorescence analysis of ANO1 revealed that the cilia and cell bodies were positively stained for ANO1 in the c-LAECs (Figure 11A), and the cilia were positively stained for acetylated tubulin (α -tubulin, a marker of cilia) (Figure 11B). The merged image shows that ANO1 exists in the cilia (Figure 11C). A phase contrast image of the c-LAECs is shown in Figure 11D.

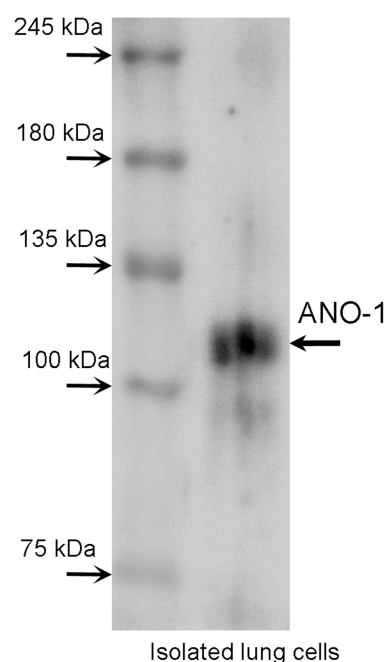


Figure 10. Western blotting for ANO1 in isolated lung cells. A single band for ANO1 was detected at 110 kDa in isolated lung cells.

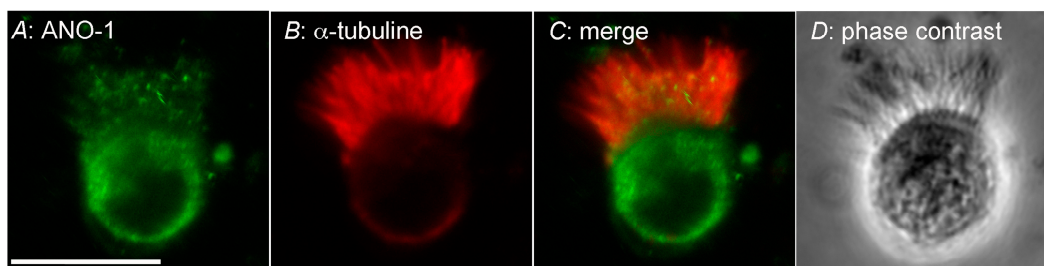


Figure 11. Immunofluorescence examination of ANO1. (A): ANO1. (B): α -tubuline. (C): Merged image. (D): Phase contrast image. Cilia were positively stained for ANO1.

3. Discussion

The activation of $\text{Ca}_v1.2$, a voltage-dependent Ca^{2+} channel, maintained the sustained increase in $[\text{Ca}^{2+}]_i$ in ABX-stimulated-cLAECs, in which the contribution of Ca^{2+} release from the stores was small [6]. This study demonstrated that the $[\text{Ca}^{2+}]_i$ increase using ABX triggers CBF and CBA increases in the c-LAECs by activating two signaling pathways: the pH pathway (an increase in pH_i) and the Cl^- pathway (a decrease in $[\text{Cl}^-]_i$). The pH pathway increased the CBF by 30% and the CBA by 15–20%, and the Cl^- pathway increased the CBA by 10–15%.

The $[\text{Ca}^{2+}]_i$ increase is essential to increase the CBF and CBA in the ABX-stimulated c-LAECs, since prior treatment with BAPTA-AM abolished their increases. This suggests that the non-specific actions of ABX, which are not mediated by an $[\text{Ca}^{2+}]_i$ increase, are negligibly small in the regulation of the CBF and CBA. DIDS, which inhibited the pH pathway, did not affect the ABX-stimulated increase in $[\text{Ca}^{2+}]_i$, since ABX stimulation increased only the CBA, but not the CBF, although the $[\text{Ca}^{2+}]_i$ increase appears to be sufficient to increase the CBF. Moreover, in the presence of NPPB or T116Ainh, ABX stimulation induced small increases in the CBF and CBA, suggesting the activation of the pH pathway. ABX is likely to increase $[\text{Ca}^{2+}]_i$ to a sufficient level to activate the pH pathway. A $[\text{Cl}^-]_i$ increase on the part of the inhibitors of the Cl^- channels appears to decrease the CBF and CBA enhanced by the pH pathway. Yasuda et al. showed that an increase in $[\text{Cl}^-]_i$ decreases the CBF and CBA [12]. Moreover, in the $\text{CO}_2/\text{HCO}_3^-$ -free

solution, ABX increased only the CBA and decreased the cell volume and $[Cl^-]_i$, effects that were eliminated using a Ca^{2+} -free solution or nifedipine. In a CO_2/HCO_3^- -free solution, ABX may increase the $[Ca^{2+}]_i$ to a level activating ANO1 in the cilia. Saito et al. suggest that ABX increases the $[Ca^{2+}]_i$ only in the cilia, although no increase in the $[Ca^{2+}]_i$ of the cell body was detected in the CO_2/HCO_3^- -free solution [6]. In the olfactory cilia, the $[Ca^{2+}]_i$ increase is limited in the same vicinity for a long time [19]. Moreover, we measured the $[Ca^{2+}]_i$ in c-LAECs stimulated by ABX and ionomycin (IM, 1 μ M), using fluo4 fluorescence. The increases in the fluorescence ratio (F/F_0) stimulated by ABX were 10–15% of those stimulated by IM (1 μ M) (Supplementary Figure S1). The direct activation of the CBF by a $[Ca^{2+}]_i$ increase has been established in the beating cilia of the airways [3,4,20]. However, in the ABX-stimulated c-LAECs, increases in $[Ca^{2+}]_i$ may have been too small to directly increase the CBF and CBA.

An increase in pH_i enhances the CBF and CBA in airway ciliary cells [9,10] and enhances the CBF in sperm flagella [21,22]. In the c-LAECs, the pH pathway is activated by HCO_3^- entry via the NBC, which is inhibited by DIDS. There are two bicarbonate transporters in the c-LAECs: the NBC and AE [17]. The DIDS-sensitive AE exists in the apical membrane and mediates HCO_3^- secretion in the bronchiole epithelial cells [17]. Moreover, in the HCO_3^- -containing Cl^- -free solution, ABX enhanced the increase in pH_i , indicating that the AE does not transport HCO_3^- into the cell in c-LAECs. In this solution, no HCO_3^- secretion via the AE occurs because of no extracellular Cl^- . The inhibition of AE (no HCO_3^- extrusion via the AE retaining HCO_3^- uptake via the NBC) increases the intracellular HCO_3^- concentration to elevate the pH_i . Based on these results, we concluded that ABX stimulated the NBC, mediated by a $[Ca^{2+}]_i$ increase, leading to an increase in pH_i . Fois et al. demonstrated that ABX increases pH_i in type II pneumocytes [13]. The messenger RNAs of all NBC isoforms are expressed in the airway epithelial cells [17], but the membrane localization of the NBC isoforms has not been identified, although NBCe1 and NBCe2 have been identified in the basolateral membrane of Calu-3 cells [23]. Moreover, DIDS did not affect the decrease in $[Cl^-]_i$, indicating that the $[Cl^-]_i$ decrease is not induced by DIDS-sensitive Cl^- channels in the c-LAECs.

The pH pathway was still activated by ABX in the presence of nifedipine or in a Ca^{2+} -free solution. This finding indicates that the ABX-stimulated small transient Ca^{2+} release from the acidic stores activates the NBC in the c-LAECs, leading to a pH_i increase. Lieb et al. reported that a transient $[Ca^{2+}]_i$ increase induced by short-term ATP stimulation has been shown to induce a prolonged CBF increase in human airway ciliary cells, and they suggested that a transient $[Ca^{2+}]_i$ increase phosphorylates target proteins to induce a prolonged CBF increase [20]. The ABX-stimulated small transient $[Ca^{2+}]_i$ increase appears to phosphorylate target proteins, including the NBC, to activate the pH pathway.

This study also demonstrated that a pH_i increase enhances not only the CBF but also the CBA in the c-LAECs. Cilia have two functionally distinct molecular motors: the ODAs and the IDAs. The ODAs control the CBF and the IDAs control the waveform, including the CBA [15]. An increase in the pH_i is suggested to directly act on the ODAs in sperm flagella [21,22]. Although there is no report showing that pH_i affects the IDAs, a pH_i increase may activate the IDAs, in a similar mechanism to activating the ODAs, to increase the CBA. The activation mechanisms of ODAs or IDAs due to a pH_i increase remain uncertain. Previous reports have suggested that pH_i -induced changes in the histidine charge may affect the activity of dynein ATPase [24]. A study on sperm flagella suggests that an increase in the pH_i activates dynein via the pH -dependent and cAMP-independent phosphorylation of dynein components and/or other axonemal proteins [22].

Previous studies have demonstrated that a decrease in $[Cl^-]_i$ enhances the CBA at 37 °C, but not the CBF [9,12]. $[Cl^-]_i$ is decreased by the cell shrinkage under iso-osmotic conditions [12]. ABX stimulated a cell shrinkage and a $[Cl^-]_i$ decrease in c-LAECs. Previous studies have demonstrated that $Ca_v1.2$ is expressed in the cilia and is activated by ABX [6]. The Ca^{2+} influx via $Ca_v1.2$ activated the Cl^- pathway in the c-LAECs, but the Ca^{2+} release

from the acidic stores had little effect on the Cl^- pathway, because ABX still decreases $[\text{Cl}^-]_i$ without a Ca^{2+} release in a $\text{CO}_2/\text{HCO}_3^-$ -free solution.

The Cl^- pathway, which was independent of HCO_3^- (pH_i pathway), was activated by a Cl^- -free NO_3^- solution and inhibited by Cl^- channel blockers (NPPB and T16Ainh) in the c-LAECs. This study demonstrated that ANO1 (a Ca^{2+} -activated Cl^- channel [18]) is activated by $\text{Ca}_v1.2$. Moreover, both channels are expressed in the cilia of the c-LAECs [6,12]. The coupling of ANO1 and $\text{Ca}_v1.2$ in the cilia plays a key role in activating the Cl^- pathway in ABX-stimulated c-LAECs. However, ANO1 was also expressed in the cell body. The ANO1 expressed in the cell body ($\text{ANO1}_{\text{cell body}}$) may be activated by the $[\text{Ca}^{2+}]_i$ increase caused by $\text{Ca}_v1.2$ during ABX stimulation. The activation of ANO1 in the cell body stimulates the Cl^- release from the basolateral membrane and enhances cell shrinkage in c-LAECs. However, increases in $[\text{Ca}^{2+}]_i$ are small in the cell body during ABX stimulation (Supplementary Figure S1). In a previous report, no $[\text{Ca}^{2+}]_i$ increase was detected in an airway cell line during ABX stimulation [14]. Moreover, in this study, no $[\text{Ca}^{2+}]_i$ increase was detected in the $\text{CO}_2/\text{HCO}_3^-$ -free solution [6]. However, experiments using nifedipine or a Ca^{2+} -free solution revealed that ABX still activated both $\text{Ca}_v1.2$ and ANO1 in this solution. In the cilia, the activation of $\text{Ca}_v1.2$ may increase $[\text{Ca}^{2+}]_i$ to a significant level, activating ANO1 in the $\text{CO}_2/\text{HCO}_3^-$ -free solution. Based on these observations, the activities of ANO1 in the cell body may be low in ABX-stimulated c-LAECs, although we cannot neglect the contribution of the ANO1 in the cell body to decreased $[\text{Cl}^-]_i$.

The Cl^- pathway increased the CBA, but not the CBF. However, an increase in $[\text{Cl}^-]_i$ induced by NPPB decreased both the CBF and CBA. Thus, the effects of $[\text{Cl}^-]_i$ on the CBA and CBF are different. Yasuda et al. suggested that the concentration–response curve of the CBA to $[\text{Cl}^-]_i$ shifts to a lower concentration than that of the CBF [12]. A level of $[\text{Cl}^-]_i$ under the control conditions may maintain the IDA activity, controlling the CBA at the lowest level, but it may maintain the ODA activity, controlling the CBF at the highest level.

There are many reports showing that decreased $[\text{Cl}^-]_i$ enhances cellular functions in many cell types, including the airway ciliary cells [25–29]. These observations suggest that a Cl^- sensor exists in these cells. With-no-lysine kinase1 (WNK1) and WNK4 have been shown to be intracellular Cl^- sensors [27–29]. Piali et al. demonstrated that the Cl^- binds to the kinase domain of WNK1 and suppresses its activity by inhibiting autophosphorylation [28]. The actions of WNK1 and WNK4 were studied in the NaCl cotransporter of distal nephrons [27–29]. There are four subtypes of WNK, WNK1–4, and the chloride binding site of the kinase domain is conserved among them, but the activities of the WNK subtypes are inhibited by different Cl^- concentrations, that is, WNK1 by 60–150 mM, WNK3 by 100–150 mM and WNK4 by 0–40 mM [27,28]. WNK1 and WNK4 have been shown to regulate epithelial Na^+ channels [30,31], CFTR [32], NKCC1 [33] and NCC [26,34]. Based on these observations, our hypothesis is that WNK1 controls the ODAs and WNK4 controls the IDAs in the c-LAECs. Further experiments are needed to verify this hypothesis.

ABX at a high concentration, such as 100 μM , inhibits voltage-gated Na^+ channels (Na_vs) [35]. ABX at 1 μM is unlikely to inhibit Na_vs . Ciliated LAECs express epithelial Na^+ channels, the activation of which induces a dry airway surface by accelerating the fluid absorption observed in cystic fibrosis patients. An increase in Na^+ influx induces cell swelling to increase $[\text{Cl}^-]_i$. An increase in $[\text{Cl}^-]_i$ decreases the CBF and CBA [12]. In healthy airways, the c-LAECs secrete Cl^- [14]. The impairment of Cl^- secretion, such as in cystic fibrosis, may increase $[\text{Na}^+]_i$, leading to an $[\text{Cl}^-]_i$ increase, which may decrease the CBF and CBA [12]. The inhibition of the Na_vs using 10 μM ABX appears to have little effect on the CBF and CBA in c-LAECs.

Figure 12 shows a schematic diagram of the ABX-stimulated c-LAECs. ABX increases $[\text{Ca}^{2+}]_i$ by stimulating the nifedipine-sensitive $\text{Ca}_v1.2$ in the c-LAECs. The increases in $[\text{Ca}^{2+}]_i$ activate two signaling pathways: the pH_i pathway and the Cl^- pathway. The activation of $\text{Ca}_v1.2$ maintains most of the increase in $[\text{Ca}^{2+}]_i$. The $[\text{Ca}^{2+}]_i$ increase activates NBC-entering HCO_3^- to increase the pH_i . The pH_i pathway (an elevation in pH_i) increases the CBF and CBA by 30% and 15–20%, respectively, in ABX-stimulated c-LAECs. The

$[Ca^{2+}]_i$ increase also activates the ANO1 existing in the cilia to accelerate Cl^- release, leading to the decrease in $[Cl^-]_i$, and may also activate the ANO1 existing the cell body. The Cl^- pathway (an $[Cl^-]_i$ decrease) enhances the CBA by 10–15%, and was completely inhibited by nifedipine or T16Ainh. The Ca^{2+} release from the acidic stores, which is small and transient, also increases $[Ca^{2+}]_i$ and activates the NBC in the c-LAECs.

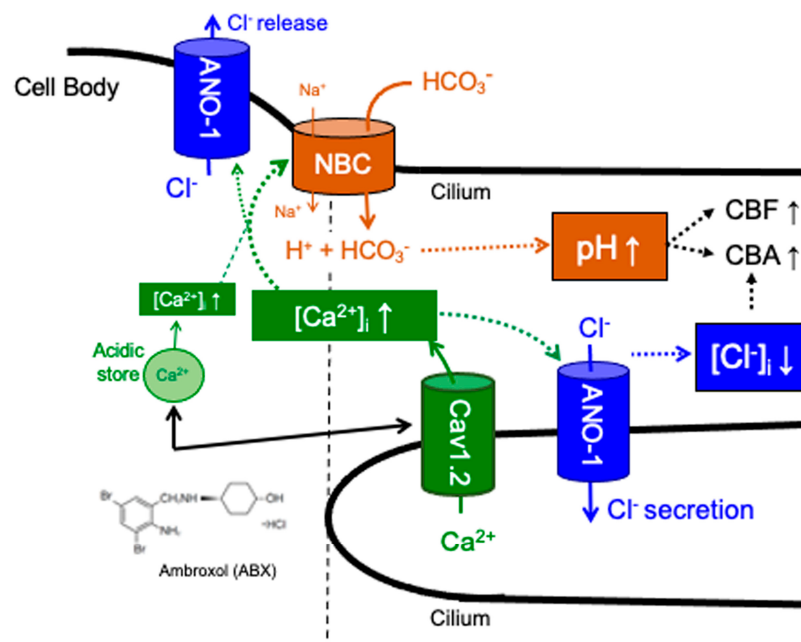


Figure 12. Schematic diagram of the ABX-stimulated c-LAECs. ABX stimulates the Ca^{2+} entry via Cav1.2 and increases $[Ca^{2+}]_i$. The $[Ca^{2+}]_i$ increase stimulates the NBC to accelerate HCO_3^- entry. The HCO_3^- entering via NBC binds H^+ to increase pH_i . The Ca^{2+} entering via Cav1.2 directly stimulates ANO1 in cilia to activate Cl^- secretion, which decreases $[Cl^-]_i$. The pH_i elevation enhances the CBF and CBA, and the $[Cl^-]_i$ decrease enhances the CBA. A small and transient Ca^{2+} release from the acidic stores increases $[Ca^{2+}]_i$ and activates NBC in ABX-stimulated c-LAECs.

4. Materials and Methods

4.1. Ethical Approval

The experiments were approved by the Committees for Animal Research of Kyoto Prefectural University of Medicine (No. 26-263, April 2017) and Ritsumeikan University (BKC-HM-2017-050). The animals were cared for and the experiments were carried out according to the guidelines of these committees. Female mice (C57BL/6J, 5 weeks of age) were purchased from Shimizu Experimental Animals (Kyoto, Japan), fed standard pellet food and water ad libitum and were used for experiments at 6–10 weeks of age. The mice were first anaesthetized using inhalational isoflurane (3%) and were further anesthetized using an intraperitoneal injection (ip) of pentobarbital sodium (70 mg/kg) and heparinized (1000 units/kg) for 15 min. Then, the mice were sacrificed with a high dose of pentobarbital sodium (100 mg/kg, ip).

4.2. Solutions and Chemicals

The CO_2/HCO_3^- -containing control solution (control solution) contained (in mM): NaCl, 121; KCl, 4.5; $NaHCO_3$, 25; $MgCl_2$, 1; $CaCl_2$, 1.5; Na-HEPES, 5; H-HEPES, 5 and glucose, 5. To prepare the CO_2/HCO_3^- -free solution, the HCO_3^- was replaced with Cl^- . To prepare the Cl^- -free NO_3^- solution, the Cl^- was replaced with NO_3^- . To prepare the Ca^{2+} -free solution, $CaCl_2$ was removed from the solution. The CO_2/HCO_3^- -containing solutions were aerated with 95% O_2 and 5% CO_2 and the CO_2/HCO_3^- -free solutions were aerated with 100% O_2 . The pH of the solutions was adjusted to 7.4 by adding 1 N HCl or 1 N HNO_3 , as appropriate. The experiments were carried out at 37 °C. The ABX,

nifedipine, NPPB, DIDS and dimethyl sulfoxide (DMSO) were purchased from Sigma (St. Louis, MO, USA), and the heparin, elastase and bovine serum albumin (BSA) were purchased from FUJIFILM Wako (Osaka, Japan). All reagents were dissolved in DMSO and prepared to their final concentrations immediately before the experiments. The DMSO, the concentration of which did not exceed 0.1%, had no effect on the CBF or CBA [6,9–11].

4.3. Cell Preparation

The ciliated LAECs were isolated from the lungs using an elastase treatment [6,9,11]. Following the elastase treatment, the lungs were minced using fine forceps in a control solution containing DNase I (0.02 mg/mL) and BSA (5%). The minced tissue was filtered through a nylon mesh (a sieve with 300 µm openings). The isolated cells were washed and then suspended in the control solution. The cell suspension was stored at 4 °C and the cells were used within 5 h after the isolation.

4.4. CBF and CBA Measurements

The cells were set in a micro perfusion chamber (20 µL) mounted on an inverted light microscope (ECLIPSE Ti, Nikon, Tokyo, Japan) connected to a high-speed camera (Photron Ltd., Tokyo, Japan) and video images were recorded for 2 s at 500 fps (frame per second) [6,9,11]. The stage of the microscope was heated to 37 °C [4]. After the experiments, the CBF and CBA were measured using an image analysis program (DippMotion 2D, Ditect, Tokyo, Japan) [6,9,11]. The normalized CBF and CBA values and CBF and CBA ratios (CBF_t/CBF_0 and CBA_t/CBA_0), calculated from 4 to 12 cells, were used to make a comparison among the experiments. Each experiment was carried out using 4–10 cover slips with cells obtained from 2 to 5 animals. “n” shows the number of cells.

4.5. Measurement of the Cell Volume

The outline of a c-LAEC was traced onto a video image, and the area of the cell (A) was measured using the image analysis program. The index of the cell volume ($V_t/V_0 = (A_t/A_0)^{1.5}$) was calculated [9]. Each experiment was carried out using 4–6 cover slips obtained from 2 to 3 animals. The V/V_0 values calculated from 4 to 6 cells were plotted and “n” shows the number of cells.

4.6. Measurement of pH_i , $[Cl^-]_i$ and $[Ca^{2+}]_i$

Changes in intracellular pH (pH_i) were monitored via SNARF1 fluorescence (a pH dye) at 37 °C and the cells were set on the heated stage (37 °C) of an inverted confocal laser microscope (model LSM 510-META, Carl Zeiss, Jena, Germany). The excitation was 515 nm and the emissions were 645 nm and 592 nm. The fluorescence ratio (F_{645}/F_{592}) was calculated to measure the pH_i . The pH_i of the c-LAECs was calculated using the calibration line.

Changes in $[Cl^-]_i$ were monitored via MQAE fluorescence (a chloride sensitive dye) [9,12]. The cells were incubated with 10 mM MQAE for 45 min at 37 °C. The MQAE was excited at 780 nm using a 2-photon excitation laser system (Mai Tai[®], Spectra-Physics, Santa Clara, CA, USA), and the emission was 510 nm. The ratio of MQAE fluorescence intensity (F_0/F_t) was calculated.

Changes in $[Ca^{2+}]_i$ were monitored via fura-2 fluorescence (a Ca^{2+} dye) as previously reported [6,8]. The ratio of fura-2 fluorescence (F_{340}/F_{380}) was calculated using an image analysis system (MetaFluor, Molecular Devices, San Jose, CA, USA) [6,8].

4.7. Western Blotting

The procedure for Western blotting has already been described in previous reports [6]. Protein (5–20 µg) obtained from the isolated lung cells was loaded into each lane. After blocking with 5% skim milk powder, the membrane with the protein was exposed to a primary anti-anoctamin-1 antibody (1:200) (ABN1669 Sigma-Aldrich Merck, Darmstadt, Germany) diluted with solution 1 (Can Get Signal, TOYOBO, Osaka, Japan), and the secondary antibody was applied to the membrane. Then, the antigen–antibody com-

plexes were visualized using a chemiluminescence system (Immobilon, Merck Millipore, Darmstadt, Germany).

4.8. Immunofluorescence Examination

The cell suspension containing the isolated lung cells (0.5 mL) was dropped and dried on the coverslip for attaching the cells [6]. After fixation with 4% paraformaldehyde, the cells were stained with an anoctamin-1 antibody and an anti-acetylated tubulin antibody (T6793, Sigma-Aldrich, St. Louis, MO, USA). The cells were washed with PBS containing 0.1% BSA to remove unbound antibodies. Finally, the cells were stained with Alexa 488-conjugated anti-rabbit antibody and Alexa 594-conjugated anti-mouse antibody (Invitrogen, Carlsbad, CA, USA). The cells were observed using confocal laser microscopy (FV10i, Olympus, Tokyo, Japan).

4.9. Statistical Analysis

Data are expressed as the means $\pm$ SEMs. Statistical significance was assessed using one-way analysis of variance (one-way ANOVA). Differences were considered significant at $p < 0.05$.

5. Conclusions

The Cav1.2 existing in the airway cilia plays an important role in the increases in the CBF and CBA in ABX-stimulated c-LAECs. An increase in $[Ca^{2+}]_i$ induced by the activation of Cav1.2 stimulates two signaling pathways: the pH_i pathway and Cl^- pathway. This is the first report to clarify the role of Cav1.2 in the activation of beating cilia in the airway. The Cav1.2 existing in cilia, which is activated by some chemicals and receptors, including ABX [6], may be a new target to prevent and improve respiratory problems.

Supplementary Materials: The following supporting information can be downloaded at <https://www.mdpi.com/article/10.3390/ijms242316976/s1>.

Author Contributions: C.S., S.T., S.H. and T.N. measured the ciliary activities, pH_i , $[Cl^-]_i$ and $[Ca^{2+}]_i$. K.K. and S.A. performed the WB and IHC. T.I. and Y.M. contributed to the data interpretation and discussion. T.N. contributed to the experimental design, data analysis and data interpretation and prepared the draft of the manuscript. All authors have read and approved the final version of the manuscript and agree to be accountable for all aspects of the work.

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Data Availability Statement: The data that support the findings of this study are in the paper itself, and no shared data are used in the paper.

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