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Protons inhibit anoctamin 1 by competing with calcium

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ABSTRACT

Cl^- efflux through Ca^{2+}-activated Cl^- channels (CaCCs) in secretory epithelial cells plays a key role in the regulation of fluid secretion. The fluid and electrolyte secretion is closely related to intracellular pH. CaCCs have been known to be inhibited by intracellular acid. However, the molecular mechanism for the inhibition remains unknown. Anoctamin 1 (ANO1) is a Ca^{2+}-activated Cl^- channel that mediates numerous physiological functions including fluid secretion in secretory epithelia. However, little is known about whether ANO1 can be modulated by change of intracellular pH. Here, we demonstrate that Ca^{2+}-induced activation of ANO1 and its homolog ANO2 are strongly inhibited by intracellular acid. Intracellular acid caused a rightward shift of the concentration-response curve of Ca^{2+} in activating ANO1 and ANO2. To identify the location of the acid-induced inhibition, mutations were made on each of all histidine residues in cytoplasmic part of ANO1. However, none of the His-mutant showed the reduction in the acid-induced inhibition. Furthermore, mutation on Glu- or Asp-residues in the multiple acidic-amino acid regions was ineffective in blocking the acid-induced inhibition. Because the Ca^{2+}-binding site of a fungal anoctamin (*nh*TMEM16) was uncovered by crystallography, mutagenesis was performed in this region. Surprisingly, mutations at Glu, Asp or Asn residues in the hydrophobic core that are known to be essential for Ca^{2+}-induced activation of ANO1 blocked the acid-induced inhibition. These results suggest that protons interfere with Ca^{2+} at the Ca^{2+} binding site of ANO1. These findings provide a molecular mechanism underlying the acid-induced inhibition of ANO1, which may contribute to control fluid and electrolyte secretion in the secretory epithelia.

1. Introduction

Epithelial cells in the body have multiple functional roles such as fluid secretion and absorption. Fluid and electrolyte secretion across the epithelia is highly regulated by multiple processes and closely linked to Cl^- ion movements. Transepithelial Cl^- movement is dependent on various key transporters and channels. $Na^+/K^+/2Cl^-$ co-transporters located in the basolateral membrane of epithelial cells accumulate intracellular Cl^- resulting in high intracellular concentration above its electrochemical gradients [1–3]. The efflux of Cl^- by the opening of Cl^- channels in apical membrane induces luminal accumulation of Cl^-. The transepithelial Cl^- movement generates transepithelial osmotic gradient, which drives water movement.

Ca^{2+} and cAMP-dependent Cl^- channels are the two main channels that control the transepithelial Cl^- movement [4–6]. cAMP dependent Cl^- channel, cystic fibrosis transmembrane conductance regulator (CFTR) plays an important role in the Cl^- outflux in many secretory epithelia [6–8]. In addition, CaCCs also play a major role in secreting Cl^- through epithelial cells [5,9,10]. Notable action of CaCCs in salivary glands is secreting Cl^- in response to intracellular Ca^{2+}, which is induced by the stimulation of muscarinic receptors [1,9].

Similar to the Cl^- conduction pathway, the HCO_3^- efflux also regulates fluid and electrolyte secretion in acinar cells in salivary glands [1,9]. In acinar cells of salivary gland, carbonyl anhydrase catalyzes water and CO_2 to HCO_3^- and H^+. HCO_3^- thus generated by carbonyl anhydrase drives fluid secretion in acinar cells [1]. Inhibition of carbonic anhydrase reduces secretion in salivary cells [11,12]. HCO_3^- is known to be secreted through CaCCs, largely

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because blockers of CaCCs inhibit HCO_3^- secretion [13,14]. In addition, HCO_3^- buffers intracellular fluid to maintain it at neutral intracellular pH through the CO_2/HCO_3^- buffering system [5]. Agonists that increase intracellular Ca^{2+} evoke an acidification in acinar cells presumably [15]. This Ca^{2+} dependent intracellular acidification is inhibited by Cl^- channel blockers supporting the notion of the efflux of HCO_3^- through CaCCs [13,16]. Because CaCCs were permeable to HCO_3^-, it is plausible that intracellular acidity may affect CaCC activity, too. Indeed, the activity of CaCCs is inhibited by intracellular acid in parotid and lacrimal acinar cells, and human colon carcinoma T84 cell line [17,18].

Anoctamin 1 (ANO1, also known as TMEM16A) is a Cl^- channel activated by Ca^{2+} [19–21]. ANO1 mediates diverse functions in a variety of cells. ANO1 modulates mucus secretion in airway epithelial cells [22] and affects vascular contractility [23]. In addition, ANO1 also acts as a heat sensor in somatosensory neurons mediating thermal pain [24,25]. Most notably, as expected for a CaCC, ANO1 mediates fluid secretion in many types of secretory epithelia such as airway [22,26,27], colon [28,29], and salivary gland [19,30]. In salivary gland, knock-down or genetic disruption of Ano1 reduces salivary secretion [19,30]. Moreover, the permeability of HCO_3^- increases in submandibular gland cells through ANO1 activation by high intracellular Ca^{2+} [31]. Because ANO1 is involved in salivary secretion in acinar cells of the submandibular gland and permeable to HCO_3^-, these data suggest the possibility that intracellular pH regulates ANO1 activity.

Thus, this study aimed to determine whether intracellular pH change can modulate ANO1 activity. We found that a slight decrease in the pH of intracellular solution profoundly blocked the Ca^{2+}-activated ANO1 currents. In addition, molecular mechanisms underlying the acid-induced inhibition of ANO1 were explored.

2. Materials and methods

2.1. Construction of ANO1 mutants

All mutants were generated from the wild-type mouse ANO1 construct (pEGFP-N1-mANO1). We used the transcript ***a,c*** variant of mouse ANO1 (NM_178642.5) [32,33]. Deletion or amino-acid substitution mutants were constructed using a site-directed mutagenesis kit (Muta-direct, iNtRON Biotech) or by using the overlap-PCR method as previously used [33]. Construction of mutants was verified with DNA sequencing.

2.2. Cell culture and the functional expression of ANO1 or mutants in HEK293T cells

HEK 293T cells were maintained at 5% CO_2, 37 °C incubator in DMEM supplemented with 10% FBS, 10 units/mL penicillin, 10 μg/mL streptomycin. Cells were transiently transfected with FugeneHD according to the manufacturer's protocol (Promega) and used for patch clamp experiments 24–48 h after transfection. Successful transfection was confirmed by EGFP fluorescence.

2.3. Electrophysiology

Patch-clamp experiments were performed in inside-out or whole-cell configurations at room temperature (20–25 °C). Borosilicate glasses (World Precision Instruments) were pulled with a puller P-97 (Sutter instruments) to make patch pipettes. The resistance of pipettes was adjusted to 3–5 MΩ after polishing tips with a microforge (MF830, Narishige, Japan). Gentle suctions were applied to cells expressing GFP to form gigaseals. To make an inside-out patch, the patch membrane attached to pipette was excised rapidly. To form whole cells, gentle suction was applied to rupture the membrane under the pipette. Currents were recorded with Axopatch 200B amplifier (Molecular Devices). Output of the amplifier was filtered at 5 kHz and fed to Digidata 1440A (Molecular Probes) and stored on a computer for analysis.

For inside-out patch recording, the pipette solution contained 140 mM NMDG, 2 mM $MgCl_2$, 10 mM HEPES, 10 mM EGTA adjusted to pH 7.2 with HCl. The control bath solution contained 140 mM NMDG, 2 mM $MgCl_2$, 10 mM HEPES for pH 6.8–8.0 or MES for pH 6.0–6.4, 10 mM EGTA, HEDTA or NTA adjusted to expected pH with HCl. Calcium chelator (EGTA, HEDTA, NTA) was selected appropriately according to their chelating capacity. To make 0.1–1, 3–30, and 100–1000 mM Ca^{2+} solution, 10 mM EGTA, HEDTA, and NTA were added to the solution, respectively. Free Ca^{2+} was calculated with WEBMAXC (http://www.stanford.edu/~cpatton/webmaxcS.htm). For the whole-cell current recording, the pipette solution contained 140 mM NMDG, 2 mM $MgCl_2$, 10 mM HEPES, 2 mM ATP, and 300 μM GTP adjusted to pH 7.2 with HCl.

All chemicals for electrophysiology experiments were purchased from Sigma. E_{act} was provided by Prof. Namkung Wan in the Yonsei University, Korea.

2.4. Structure modeling

To extract a putative structure of mouse ANO1 in α6–α8 helix region, the crystal structure of *nh*TMEM16 was used as a template. Model structure of mouse ANO1 was generated by MODWEB (https://modbase.compbio.ucsf.edu/modweb/), a web server for automated comparative protein structure modeling. Crystal structure and model structure were edited by PyMol.

2.5. Data analysis

Dose response relationships were fitted to the Hill equation, $I/I_{max} = 1/(1+[Ca^{2+}]/EC_{50})^n$, where I_{max} is the maximum current of each patches. $[Ca^{2+}]$ is Ca^{2+} concentration. EC_{50} is the Ca^{2+} concentration required for reaching the half-maximal activation. n denotes the Hill coefficient.

For G–V curves, the data was fit by the Boltzmann equation,

$$\frac{G}{G_{max}} = \frac{1}{1 + e^{(V_m - V_{1/2})\frac{zF}{RT}}}$$

$V_{1/2}$ is the membrane potential producing half-maximal activation, z is the equivalent gating charge associated with voltage-dependent channel opening. F is the Faraday constant, R is the gas constant, and the T is the absolute temperature. G, the conductance, was calculated from tail current at 100–200 μs after voltage pulses. G_{max} was determined by a fit of averaged G.

2.6. Statistics

All results are presented as mean ± SEM. Statistical significance was determined using unpaired Student t tests for comparison of two groups and one-way ANOVA followed by Tukey's post hoc test for multiple comparison.

3. Results

3.1. Intracellular acid inhibits ANO1

In order to investigate the modulation of ANO1 channels by shift in intracellular pH, we measured Ca^{2+}-evoked ANO1 currents at different intracellular pHs. After inside-out membrane patches of HEK293T cells transfected with mouse ANO1 were formed, 10 μM Ca^{2+} with various ranges of intracellular pH were applied to the bath. Cl^- was the main carrier ion because the pipette and bath solutions contained 140 mM NMDG-Cl. Membrane potential

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