Anoctamin 1 in secretory epithelia

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Fluid and electrolyte releasing from secretory epithelia are elaborately regulated by orchestrated activity of ion channels. The activity of chloride channel at the apical membrane decides on the direction and the rate of secretory fluid and electrolyte. Chloride-dependent secretion is conventionally associated with intracellular increases in two second messengers, cAMP and Ca2+, responding to luminal purinergic and basolateral adrenergic or cholinergic stimulation. While it is broadly regarded that cAMP-dependent Cl− secretion is regulated by cystic fibrosis transmembrane conductance regulator (CFTR), Ca2+-activated Cl− channel (CaCC) had been veiled for quite some time. Now, Anoctamin 1 (ANO1 or TMEM16A) confers Ca2+-activated Cl− currents. Ano 1 and its paralogs have been actively investigated for multiple functions underlying Ca2+-activated Cl− efflux and fluid secretion in a variety of secretory epithelial cells. In this review, we will discuss recent advances in the secretory function and signaling of ANO1 in the secretory epithelia, such as airways, intestines, and salivary glands.

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1. Introduction

Epithelium is a membranous tissue composed of one or more layers of cells that form the cavities or glands of the body. Epithelial cells act multiple functions, such as protection, transportation, absorption, and secretion across the boundary between cavity and surface [1–3]. Secretory epithelial cells release fluids or electrolytes that are necessary for various processes, including in digestion, protection, excretion of waste products, and metabolic regulation [1–3]. The total amount of secretions was drastically adjusted by their rate and direction in response to external stimuli. Chloride flow across the secretory epithelial cells is an important determinant to fluid and electrolyte secretion.

In most non-epithelial cells, the concentration of intracellular Cl− is close to its electrochemical equilibrium. However, in epithelial cells, Na+/K+/2Cl− co-transporter 1 (NKCC1) located in the basolateral membrane maintains electrochemical Cl− concentration as high as five-fold in secretory epithelial cells [4–6]. The driving force for Cl− uptake is provided by the Na+ gradient which is established by Na+/H+−ATPase exchanger. High Cl− concentration is a key factor for driving Cl− out to the lumen. Thus, the activation of Cl− channels induces the Cl− efflux from apical membrane into the lumen [4–6]. The Cl− efflux is electrically neutralized by the discharge of K+ via K+ channels [4–6]. This luminal accumulation of ions sets a transepithelial osmotic gradient to drive the movement of fluid [4–6]. Thus, the conductance of Cl− plays an active part in determining the rate and direction at which fluid and electrolyte secretion occurs.

Intracellular cAMP and Ca2+ function as second messengers to regulate chloride-dependent secretion [5]. cAMP-activated Cl− currents are mainly mediated by CFTR, an anion channel that belongs to ATP-binding cassette transporter gene family. In secretory epithelial cells, cAMP-activated CFTR is localized in the apical membrane, and its dysfunction in cystic fibrosis severely impairs the luminal fluid and composition [7]. Although CFTR is clearly related with fluid and electrolyte secretion in secretory epithelia, Cl− dependent secretion is still observed in the epithelia cells isolated from patients with cystic fibrosis and mice lacking Cfr [8,9]. This ‘remnant’ secretion appears to be mediated by intracellular Ca2+ [8,9]. In fact, the increase in intracellular Ca2+ using direct application of Ca2+ ionophore and the activation of purinergic receptors evokes Ca2+-activated Cl− currents in both normal and cystic fibrosis originated airway epithelial cells [10,11]. These Ca2+-activated Cl− currents are voltage dependent and inhibited by some Cl− channel blockers such as DIDS, niflumic acid, and NPPB [5,7,12]. The single channel conductance typically showed 1–15 pS in secretory epithelial cells, and have an anion selectivity sequence of I− > NO3− > Br− > Cl− [5,7,12,13].
Undiscovered for quite long time, CaCC was finally cloned. Yang et al. reported that TMEM16A confers CaCC currents. They renamed it as anoctamin 1 (ANO1) because it is an anion channel with octa-(8) transmembrane domains [14]. Coincidently, independent two other groups also reported that TMEM16A acts as a CaCC [15,16]. Because ANO1 shows similar biophysical property, pharmacological profile as well as expression pattern with those of CaCCs, it is now generally accepted that ANO1 is a candidate gene for CaCC. In this review, we will address recent advances in studying ANO1 in the secretory epithelial cells.

2. Cl− channels in airway epithelium

The airway epithelium serves a dynamic host barrier designed to protect from toxic and infectious materials in inhaled air. Besides the physical barrier of epithelial cells, their viscoelastic resistance exerted effectively by the airway surface layer (ASL) has a protection from pathogens. ASL is composed of soft elastic solid and viscous fluid secreted by airway epithelial cells. Due to their viscous nature, penetrated pathogens are trapped and removed from the lung through ciliary beatings and coughs.

According to unique morphology and functions, airway epithelial cells are broadly separated into basal, secretory and ciliated epithelial cells (Fig. 1). The basal cells are populated beneath secretory and/or ciliated cells and attached on the top of the basement membrane, which helps to anchor the epithelium to the matrix. Moreover, basal cells have been reported to possess stem cell like properties so that they can differentiate into secretory or ciliated cells after epithelial injury [17]. While basal cells have a role in connecting and supporting other epithelial cells, secretory and ciliated cells take part in the formation and regulation of ASL to trap and remove pathogens. The secretory cells are further divided into subtypes such as goblet (or mucous) and serous cells, which predominantly produce mucin proteins that impart the properties of sticky gel in the airway. Besides mucin proteins, these secretory cells have been known to secrete a variety of antimicrobial peptides (β-defensin and lysozymes), immunomodulatory molecules (chemokines and cytokines), and protective molecules such as growth factors into mucus [18–21].

The viscoelastic mucus layer containing ASL is composed of water, proteins (mainly mucins), salts, and lipids, which is generally maintained in 97% solvent and 3% solids in the normal mucus layer [1]. This mucous property is maintained by well controlled secretion of electrolytes and water. Electrolyte secretion is associated with Cl− secretion through CFTR and CaCCs in airway epithelial cells.

The stimulation of luminal purinergic receptors causes increase in intracellular cAMP and Ca2+. cAMP induces Cl− secretion through the activation of CFTR in the luminal side whereas intracellular Ca2+ causes Cl− secretion through the activation of ANO1. Occasionally, a cellular crosstalk between CFTR and ANO1-dependent secretions regulates the secretory signaling in the airway epithelium.

3. ANO1 as a candidate for CaCC

In search of a CaCC candidate gene, many genes such as CLCA, CICs, Tweety, and bestrophins were introduced [26]. However, none of them satisfied the hallmark property of CaCC. CaCCs are
known to have unique biophysical and pharmacological properties. CaCCs are activated by micromolar intracellular Ca\(^{2+}\) [13,27]. CaCCs are also activated by voltage with outwardly rectifying \(I-V\) relationship [5,13]. The \(I-V\) sensitivity is voltage dependent because Ca\(^{2+}\) affinity is greater at depolarization than at hyperpolarization [26,27]. CaCCs are non-selective anion channels permeable to various halides. The permeation sequence is \(I>F>Br^{-}>Cl^{-}>E^{-}\) [5,13]. Single channel conductance is small [28,29]. CaCCs currents are blocked by DIDS, NPPB, niflumic acid, flufenamic acid, and tamoxifen [26]. More importantly, CaCCs are activated by important physiological ligands such as ATP, acetylcholine, endothelin 1, angiotensin II, and histamine that mobilize intracellular Ca\(^{2+}\) from ER via the PLC/IP\(_{3}\) pathway [26]. As expected from its major role in Cl\(^-\) transport in secretory epithelia, CaCC currents are found in secretory epithelia [5].

If ANO1 is a candidate gene for ANO1, it should show all the hallmarks of CaCCs. Indeed, ANO1 retains the hallmark of CaCCs. Firstly, ANO1 is activated by intracellular Ca\(^{2+}\) with the half-maximal concentration of 2.6 \(\mu\)M at \(-60\) mV of membrane potential [14]. ANO1 is also activated by voltage. The time courses of ANO1 currents activated by voltage pulses are similar to those of endogenous CaCCs: CaCC currents are slowly activated by voltage at low Ca\(^{2+}\). Similarly, ANO1 currents are also activated by depolarization at low Ca\(^{2+}\) with outwardly rectifying \(I-V\) relationship [14,30]. The pharmacological profile of ANO1 also resembles that of native CaCCs because ANO1 currents activated by Ca\(^{2+}\) is blocked by DIDS, NPPB, niflumic acid, flufenamic acid, and tamoxifen [14]. ANO1 is activated by various physiological ligands such as ATP, endothelin 1, angiotensin II, carbobal and bradykinin in vitro as well as in vivo [14,31,32]. Numerous reports now confirmed that ANO1 is expressed in various secretory epithelial cells as well as other tissues where CaCCs were reported [14,30,33–36]. Thus, judging from these biophysical, pharmacological and expression characteristics, ANO1 is now considered as a candidate gene for CaCC.

### 4. ANO1 functions in the airway epithelium

As identified ANO1 as a CaCC, its roles have been elucidated in the airway. The presence of ANO1 in the airway was firstly shown in staining with antibody [14,37]. Then in situ hybridization analysis, dense mRNA granules of ANO1 were observed in the epithelial cells in submucosal glands of the airway that contributes to the secretion of mucus and liquid [14,37]. In primary cultures of porcine submucosal gland serous cells, the stimulation with ATP or carbobal caused transient intracellular Ca\(^{2+}\) with a concomitant Cl\(^-\) secretion and cell shrinkage. ANO1 is expressed on the apical membrane of serous cells [38]. The carbobal-induced Ca\(^{2+}\) increase in these cells was reduced with niflumic acid. Furthermore, Fischer et al. measured mRNA levels of CTR and ANO1 from primary cultures of in serous and mucous cells of human airway gland [39]. ANO1 transcript levels are about 10 and 100 times more abundant than those for CTR in the serous and mucous cells, respectively [39]. In addition, ionomycin-induced currents were much greater than CAMP-induced currents in both types of cells of human airway glands.

Useful data on the roles of ANO1 in the airway would be expected if Ano1-deficient mice were generated. However, systemic knock-out of Ano1 in mice leads to death within a few weeks after birth, mainly with tracheomalacia, a severe mal-formation of trachea [40]. Rock et al. observed severe mucus accumulation in the lumen in the trachea of Ano1\(^{-/-}\) mice, suggesting inadequate hydration in the airway [25]. In addition, UTP-induced transepithelial currents were also markedly reduced in the trachea of Ano1\(^{-/-}\) mice [25]. Ousingsawat et al. also observed similar results in the trachea of Ano1\(^{-/-}\) mice [37]. The transepithelial voltage responding to carbobal, UTP, or ATP was significantly reduced in the Ano1\(^{-/-}\) trachea [37].

ANO1 expression is increased in the epithelial cells of asthmatic human patients and asthma rodent models [33]. In particular, ANO1 is highly expressed in Muc5AC-positive secretory mucous cells but not ciliated cells in ovalbumin-treated asthma mouse model [33]. Similarly, the application of a cytokine, interleukin-4 to primary human bronchial epithelia cells up-regulates the expression of ANO1 in apical membrane of Muc5AC-positive mucous goblet cells, not in ciliated cells [36]. Therefore, ANO1 is considered to be required for hypersecretion ensuring the host defense in inflammatory condition.

Cellular crosstalk between CFTR and ANO1-dependent secretions might regulate secretory signaling in the airway epithelia. For instance, CFTR inhibits endogenous CaCC currents in Xenopus oocytes or mammalian cells [41–43]. Furthermore, Ousingsawat et al. showed that over-expression of CFTR inhibits ANO1 currents in the airway epithelial cells whereas over-expression of ANO1 inhibits CFTR currents in these cells [43]. In general, G-protein coupled receptors like purinergic receptors are often associated with Gs, for activating adenyl cyclase and with Gq for stimulating phospholipase C. Thus, the adenyl cyclase-induced cAMP pathway and the phospholipase C-induced Ca\(^{2+}\) pathway stimulate CFTR and ANO1, respectively. The two pathways have a crosstalk. Intracellular Ca\(^{2+}\) modulates adenylate cyclase activity via changing the cAMP level whereas CAMP regulates intracellular Ca\(^{2+}\) acting on endoplasmic reticulum Ca\(^{2+}\)-ATPase SERCA or IP\(_{3}\) receptors [44,45]. Therefore, functional links between CFTR and ANO1 are present in Cl\(^-\) secretion.

### 5. Intestinal epithelium

The intestinal epithelium is a columnar, non-ciliated epithelium that covers the small and large intestine, which is responsible for the secretion of digestive enzyme and absorption of ingested food [46,47]. The epithelium of small intestine has a numerous finger-like protrusions, known as villi, toward lumen to maximize the surface area available for absorption [46,47]. The villi are in line with invaginations called crypts that consisted mainly of secretory cells and intestinal stem cells [46–48]. The small intestine is divided into three parts: duodenum, jejunum, and ileum. The duodenum is a receiving area for partially digested food from the stomach. Most of the nutrients are absorbed into blood in the jejunum. The remained nutrients are re-absorbed in the ileum prior to transfer into the large intestine. Meanwhile, the colon has a simple columnar epithelium alternating the flat surface and crypts without villi (Fig. 2) that absorb mainly water from the indigestible food.

In human, the gastrointestinal tract releases about 8–101 per day of fluid in the face of ingested food, which is reabsorbed by the small intestine and large intestine in gastrointestinal tract [49]. The absorptive and secretory roles of the fluid are performed by columnar cells (enterocytes in the small bowel and colonocytes in the colon) that are most abundant in the intestinal epithelium [50]. Moreover, intestinal goblet cells secrete mucin to protect and lubricate the surface of epithelium [51]. Endocrine cells discharge peptide hormones with endocrine or paracrine manner [47]. Besides, paneth cells are largely limited to the small intestine, which secrete a variety of proteins, including lysozyme, to preserve a sterile condition in the crypts [50].

This secretion and absorption of fluid is made possible by moving of Na\(^+\) and Cl\(^-\) across intestinal epithelial cells. Fine tuning of this process is essential for sustaining an adequate balance. This delicate ionic movement is achieved by the action of specialized transporters and channels. Basolateral membrane-bounded Na\(^+\)/K\(^+\) ATPase forms chemical and electrical gradient; pumping out
three Na\(^+\) ions outside the basolateral membrane in exchange for pumping in two K\(^+\) ions. Meanwhile, Na\(^+\) influx from the lumen into cytosol through Na\(^+\)/H\(^+\) exchanger 3 or ENaC induces the absorption of water from the lumen, whereas Cl\(^-\) efflux through Cl\(^-\) channel located at the apical membrane causes the secretion of water to lumen. Similar to airway epithelial cells, CFTR is mainly localized on the apical side in the crypt and functions as an anion channel in response to intracellular cAMP [52]. The infants suffering from Cystic Fibrosis (CF) sometimes show a meconium ileus, an obstruction with neonatal viscous stool resulting from impaired intestinal fluid secretion [53]. Moreover, subpopulation of CF patients has been suffering from chronic constipation [53].

Exogenous substances, such as bacterial toxins, ingested food, and drugs evoke the intracellular signaling pathways to regulate of ion transport. Cholera toxin, a protein secreted by the bacterium *Vibrio cholera*, modifies the alpha subunit of G protein and causes the hyper-activation of adenylate cyclase, producing abundant amounts of cAMP. The augmented cAMP leads to continuous CFTR-dependent chloride and water secretion into the intestinal lumen, inducing the diarrhea. In addition, dextran sulphate sodium (DSS) is a polyanionic derivative of dextran that is a complex polymer of glucose synthesized by bacteria. The addition of DSS to drinking water of rodents causes the severe colitis, including diarrhea, weight loss, and impaired colon (crypt distortion, mucosal edema) [54]. Meanwhile, DSS–induced colitis enhances K\(^+\) secretion owing to up-regulation of apical BK channel, which also induces defective Na\(^+\) and Cl\(^-\) absorption [55].

The activity of ion transport molecules can be modulated by intracellular Ca\(^{2+}\) transient. Acetylcholine is a neurotransmitter that induces transient increase in intracellular Ca\(^{2+}\) through G-protein coupled muscarinic receptors on the basolateral membrane. Acetylcholine also causes Ca\(^{2+}\)-activated chloride secretion with water in the apical membrane [56]. This Ca\(^{2+}\)-dependent Cl\(^-\) secretion is balanced by the K\(^+\) efflux from basolateral side via Ca\(^{2+}\)-activated K\(^+\) channels [56]. It has been recently reported that colonic epithelial cells can synthesize the acetylcholine which is released on the basolateral side depending on the luminal stimulation with short chain fatty acid propionate, which causes the colonic Cl\(^-\) secretion into the lumen [57]. Vasoactive intestinal polypeptide, a peptide hormone, has been known to stimulate Cl\(^-\) secretion through the activation of adenylate cyclase. Moreover, several inflammatory mediators, including prostaglandin E\(_2\) (PGE\(_2\)) and substance P can modulate the Cl\(^-\) secretion [58]. Serosal application PGE\(_2\) in low concentration (<100 nM) to the distal colonic epithelium of a guinea pig stimulates K\(^+\) secretion through prostaglandin E\(_2\) receptor, whereas high concentration (>100 nM) induces the Cl\(^-\) secretion via prostaglandin D\(_2\) receptor with K\(^+\) efflux [59]. The exogenous addition of substance P induces the Ca\(^{2+}\)-dependent Cl\(^-\) secretion in colonic epithelial cells. Therefore, CaCC is suggested for the Ca\(^{2+}\)-activated Cl\(^-\) secretion in response to these intestinal neurotransmitters, inflammatory mediators, and exogenous substances. Unlike in the airway, the function of ANO1 in the intestine has not been fully identified due to limited number of reports. Several studies, however, proved an important role of ANO1 in the Cl\(^-\) secretion in the intestines.

In the large intestine, prominent ANO1 expression is observed in the distal colon while it is undetectable in the proximal colon [60]. In the neonatal colon, the stimulation with carbachol induces a transient Cl\(^-\) secretion, which is almost undetectable in *Ano1* knock-out mice [37]. The CFTR-dependent Cl\(^-\) secretion or amiloride-sensitive Na\(^+\) absorption, however, is not changed in *Ano1* knock-out mice [37]. Moreover, Mroz and Keely [61] suggested that acute exposure to epidermal growth factor in the colonic epithelial cells augments the capacity of Cl\(^-\) secretion with the potentiation of Ca\(^{2+}\)-activated Cl\(^-\) conductance. This shows that epidermal growth factor up-regulates the expression of ANO1. Rotavirus toxin NSP4 has been known to cause the severe diarrhea in infants, killing millions every year [62]. Interestingly, a synthetic NSP4 peptide induces the Ca\(^{2+}\)-dependent Cl\(^-\) secretion by the activation of ANO1 in addition with the suppression of Na\(^+\) absorption.
6. Salivary gland

In human, the exocrine salivary gland secretes watery saliva that composed of 99.5% water and 0.5% electrolyte (mucins, glycoproteins, enzymes, antibacterial substances), and typically releases more than a liter of saliva per a day [6]. Secreted saliva plays several roles in forming mucosal layer, aiding digestion and defending microbes in the oral cavity [6]. The release of saliva must be modulated by a highly regulated process, because saliva is secreted at relatively slow rate between meals and is almost not produced during sleep. In fact, the patients with salivary gland hyposecretion are suffering from oral pain, increased dental caries, and frequent infections [6].

In the salivary gland, the primary isotonic fluid is secreted by salivary acinar cells [63]. This NaCl-rich fluid is subsequently modified by re-absorption of NaCl and excretion of K⁺ and HCO₃⁻, flowing across the duct (Fig. 3) [63]. Similar to airway and intestinal epithelial cells, the acinar cells in the salivary gland are polarized into the apical and basolateral membranes. The fluid and electrolytes are secreted into the lumen by coordinated regulation of ion transporters and channels [6].

The activation of muscarinic, adrenergic, or purinergic receptor that localized in the basolateral membrane of acinar cells triggers intracellular Ca²⁺ from the endoplasmic reticulum, which in turn induces the extracellular influx of Ca²⁺ through store operated channels, such as Orai and TRPC channels [63,64]. This coordinated regulation of intracellular and extracellular Ca²⁺ modulates optimal, temporal and spatial Ca²⁺ signals to secrete the fluid and electrolytes [64]. In order to secrete the fluid, this Ca²⁺ mobilization–responded effector is CaCC on the apical membrane. The activation of CaCC causes the efflux of Cl⁻ from the acinar cells due to the electrochemical driving force formed by NKCC1 and Na⁺/H⁺ exchanger on the basolateral membrane [6]. The subsequent activation of K⁺ channel is involved in maintaining the electrochemical driving force for Cl⁻ efflux. Consequently, the activation of these channels evokes a rapid loss of intracellular Cl⁻ and K⁺. The luminal accumulation of ions raises a transepithelial osmotic gradient that drives the movement of water.

In acinar cells, HCO₃⁻ is another anion possible to drive the fluid and electrolyte secretion [6,63]. Intracellular carbonic anhydrases produce HCO₃⁻ by catalyzing the reversible reaction of H₂O and CO₂ to form H₂CO₃⁻ and H⁺ [63]. This generated H⁺ was removed by the Na⁺/H⁺ exchanger. Since HCO₃⁻ is an abundant intracellular anion, the electrogenic gradient of HCO₃⁻ can efficiently induce the fluid secretion. The other effect of HCO₃⁻ is an intracellular CO₂/HCO₃⁻ buffering system to maintain a neutral intracellular pH. The activation of muscarinic receptor decreases intracellular pH owing to the efflux of HCO₃⁻ via CaCC or Na⁺/HCO₃⁻ co-transporter in the acinar cells [63,64]. This acidification is inhibited by the Cl⁻ channel blockers or HCO₃⁻ depletion [63,64]. Therefore, ANO1 is expected to play a role in the fluid secretion and intracellular acidification in the acinar cells.

In the submandibular salivary glands, ANO1 is highly expressed in the apical membrane, but much less with basolateral membrane in the acinar cells [14,37]. ANO1 knock down by siRNA treatment significantly reduced the rate of saliva production induced by pilocarpine [14]. Moreover, salivary acinar cells from the ANO1-deficient mice are hyperpolarized when compared with those from wild-type mice [37]. The carbacol, however, depolarizes the membrane voltage of acinar cells from wild-type mice, whereas hyperpolarizes the membrane voltage of those from ANO1-deficient mice [37]. Furthermore, Romanenko et al. [35]...
demonstrated a role of ANO1 in the salivary secretion. The CaCC currents and the rate of fluid secretion in the submandibular gland acinar cells from bestrophin 2-deficient mice are similar to those in wild-type mice, whereas acinar cells from ANO1-deficient mice displayed a complete absence in the CaCC-activated and carbachol-induced Cl− currents [35]. Interestingly, it has been recently reported that an intracellular high Ca2+ can increase the permeability of HCO3− through ANO1 in the submandibular gland acinar cells [67]. Therefore, ANO1 seems to be a critical component of the acinar Ca2+-activated Cl− flux that is indispensable for saliva production by the submandibular gland.

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