Mucus Secretion and Calcium Mobilization in Airway Submucosal Gland Cell

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ABSTRACT

Airway mucus secretion from submucosal gland cells (SMGC) plays an important role in protecting the respiratory system from pathogens and particles. However, mucus hypersecretion is a common pathophysiological characteristic of many chronic inflammatory pulmonary diseases such as chronic obstructive pulmonary disease (COPD), cystic fibrosis, and asthma that can lead to airway obstruction and impaired gas exchange, and if serious enough even plug the airways, possibly leading to death. Both hyperplasias of submucosal glands and increased sensitivity of SMGC to various secretagogues such as neurotransmitters and hormones play roles in mucus hypersecretion. Acute increases in mucus release are the result of sensitization of SMGC. Therefore, understanding the mechanisms of mucus secretion and its sensitization will provide potential targets for therapies that would be beneficial in the treatment of chronic inflammatory lung diseases. The submucosal gland is comprised of serous cell and mucous cell ascini. Serous cells secrete water, electrolytes and some mucin, while mucous cells secrete mainly mucin but also some fluid. Mucin, a highly glycosylated protein, is the main macromolecular component of airway-secreted mucus. It forms high molecular weight chains that are packaged within SMGC in vesicles that are released by exocytosis upon activation of the SMGC by a stimulus. An increase in intracellular Ca²⁺ is required for ion movement and evoked mucin release stimulated by secretagogues such as acetylcholine (Ach), histamine, or ATP. In SMGC, membrane receptor activation induces Ca²⁺ release from internal stores and subsequent Ca²⁺ influx through store-operated Ca²⁺ channels (SOC) or receptor-operated channels (ROC).

Keywords: Submucosal gland cell, mucus, calcium,
1. Physiology of Mucus secretion

1) Surface epithelium and secretory cells

In humans and other mammals, the surface epithelium lines all airways (nose to alveolus), which is continuous with that forming the tubulo-acinar submucosal glands [1]. Several different epithelial cell types are recognized in airway epithelium including ciliated, columnar, undifferentiated, secretory and basal cells [2]. In human airway, submucosal glands are distributed primarily in the cartilaginous airway [3]. Submucosal glands consist primarily of mucous and serous cells. The distal serous acini produce relatively watery secretions that contain antibacterial enzymes (lysozyme, lactoferrin). It has been proposed that they wash over the more viscous mucin secretions produced by the more proximal mucous acini and flush them into the collecting duct. The normal density of mucous cells is estimated at 6,000-7,000 cells/mm² surface epithelium [4]. Most mucous cells contain high molecular weight glycoprotein, which is acidic due to sialic acid or sulphate groups located at the ends of the oligosaccharide side-chains [5]. Secretion of the correct amount of mucus with an optimum viscoelastic profile is important to maintain normal mucociliary clearance [6].

Serous cells have an electron-dense cytoplasm, rough endoplasmic reticulum and, in contrast to mucous cells, discrete electron-dense granules of about 600 nm diameter. Morphologically, serous cells of the surface epithelium resemble those present in the submucosal glands. They have been described in surface epithelium only in the rat, cat, young hamster and human foetus [7, 8]. They have also been described in human small bronchi and bronchioles [9]. Many contain neutral mucin, and there is evidence that some may also contain a nonmucoid substance, probably lipid [10]. Mucous cells and serous cells can be differentiated by using Periodic acid-Schiff and alcian blue staining methods: mucous cells stain blue and serous cells stain neutral red [11, 12].

The two major sources of secreted gel-forming mucins in the respiratory tract are the surface epithelial goblet cells that produce and secrete predominantly MUC5AC and some MUC5B, and the mucous cells of the submucosal glands that secrete mainly MUC5AC and MUC5B [13, 14]. In health, goblet cells are present in human large airways, with few or none being found in the small airways [1]. Submucosal glands are distributed in large airways, and their density decreases with airway diameter [1]. Submucosal glands are complex organs that rapidly produce abundant mucus in response to neural signals or inflammatory factors [15]. In healthy humans, submucosal glands are estimated to provide more than 95% of upper airway mucus [16].

2) Airway mucin genes

Mature mucin can be either membrane associated or secreted and released. Membrane associated mucin present at plasma membrane surfaces provides structural support for cilia and the extracellular matrix. Secreted mucin is stored in apically localized secretory granules and is released by exocytosis. Four types of secreted mucins are found in human airway mucin: MUC2, MUC5AC, MUC5B, and MUC6 [17, 18, 19, 20]. MUC5AC and MUC5B are major mucins secreted into the airway [21]. In humans, MUC5AC localizes to airway ep-
3) Ion transport and fluid secretion in SMGC

A thin layer of airway surface liquid (ASL) is maintained over the highly water-permeable airway epithelial lining by active ion transport processes that control the mass of salt (NaCl) on airway surface, with water following passively by osmosis [23]. The apical secretion is primarily linked to the efflux of Cl− and HCO3− through Cl− channels in the apical membrane [24]. There is also an absorptive pathway that is present in some cells to take up Na+ into the cells from the ASL through ENaC, the epithelial sodium channel.

Cl− and HCO3− transport occur through a cAMP-activated channel, the cystic fibrosis conductance regulator (CFTR) and through Ca2+-activated Cl− channels (ClCa) both located in the apical membrane [24]. There are abundant CFTR expressed in serous cells [25, 26, 27]. CF results from a spectrum of more than 1,400 mutations in the (CFTR) gene. The most common mutation, that occurs in 70% of American CF patients, is deletion of phenylalanine at position 508 of the CFTR protein, that has been designated as ΔF508 [28]. Mucous cells express few CFTR but rather express ClCa in the apical membrane. Significant Ca2+-activated K+ (KCa) activity is also found in mucous cells in the serosal membrane [29]. K+ channels including cAMP-activated and Ca2+-activated are expressed in the serosal membrane of SMGC. Activation of potassium channels are important to hyperpolarize the cell to maintain the anion flux necessary for sustained water secretion [30, 31].

2. The signal transduction for ACh-induced Ca2+ mobilization in SMGC

1) Ca2+ release

The IP3/Ca2+ pathway in gland cells

Elevation of intracellular free Ca2+ is one of the key signals in triggering cellular activity in various kinds of cells, including lymphocytes, smooth muscle cells, neurons, and submucosal mucous gland cells. In exocrine cells, such as mucous cells, increases in intracellular Ca2+ play a key role in electrolyte- and mucus-secretion. Generally, Ca2+-elevating agonists, such as ACh, histamine and ATP bind to Gs-coupled receptors activating phospholipase C (PLC) at the cell membrane, to produce inositol 1,4,5-triphosphate (IP3) and diacylglycerol (DAG). IP3 mobilizes Ca2+ from internal stores by binding to IP3 receptors in the endoplasmic reticulum (ER), Ca2+-release, the released Ca2+ activating calcium-dependent K+ channel (KCa) and calcium-dependent Cl− channel (ClCa) [32, 33] and mucins. In serous cells, fluid containing bacteriostatic and non-glycosylated proteins are secreted, but in mucous cells, mucin, consisting of large, heavily glycosylated proteins, is secreted.

Serous cells have a large number of cystic fibrosis transmembrane conductance regulator
chloride channels (CFTR) in the apical membrane. Anions such as Cl$^-$ and HCO$_3^-$ can pass through CFTR. Water secretion follows anion secretion [24]. CFTR activation is critical for the majority of fluid secretion by serous cells. Defective CFTR activity in cystic fibrosis decreases fluid secretion into the airway resulting in drying of the airways and thickening of mucus secretions [34, 35]. However, Joo et al. found that the muscarinic activation-induced gland secretion is intact in cystic fibrosis patients [36]. This can occur because although cAMP-mediated CFTR is lost in cystic fibrosis, Cl$_{Ca}$-activated secretion is not lost [37, 38]. In mucous cells, there are few CFTR but abundant of K$_{Ca}$ and Cl$_{Ca}$ [29]. Iwase et al. (2002) found that mucus cells have dominant K$_{Ca}$ activities compared to Cl$_{Ca}$ in patch clamp studies [39]. Liu and Farley [40] also observed similar results using swine SMGC [41].

Interaction of IP$\_3$/Ca$^{2+}$ pathway and cAMP/PKA pathway in SMGC

There is evidence suggesting that cAMP-elevating agents enhance Ca$^{2+}$-mediated responses in various cell types. Choi et al. reported that VIP and carbachol acted synergistically to produce airway gland mucus secretion in both humans and pigs [42]. Hosoda et al. found that PGE$_2$ increases the Ca$^{2+}$-activated Cl$^-$ current induced by substance P in guinea pig distal colonic epithelia [43]. Also, the acute sensitization of SMGC ion channel responses to ACh after exposure of the cells to PGE$_2$ has also been observed [41]. Although Ca$^{2+}$ release and Ca$^{2+}$ influx are known to be required for mucous gland cell activation and enhanced Ca$^{2+}$ release and Ca$^{2+}$ influx have been linked to CAMP-elevating agents in various cells, the mechanism of the cAMP/PKA pathway modulation of Ca$^{2+}$ mobilization is not clear. The modulation of Ca$^{2+}$-mediated responses by cAMP/PKA is a potential mechanism for the modulation of secretory responses in both physiological and pathophysiological states. The mechanism of enhancement of Ca$^{2+}$ mobilization by cAMP/PKA is proposed to be studied.

**IP$\_3$, receptors**

Activation of the PLC pathway by various cytokines, hormones, and neurotransmitters generates an important second messenger IP$\_3$. IP$\_3$ binds to IP$\_3$ receptors, a type of Ca$^{2+}$ release channel located in the endoplasmic reticulum (ER). The activation of these channels results in Ca$^{2+}$ release from the ER that then induces many cellular effects, such as evoking Cl$^-$ and K$^+$ channel activity mucous gland cells. Three subtypes of IP$\_3$ receptors (IP$\_3$R1, IP$\_3$R2, and IP$\_3$R3) share basic properties, such as primary structure and regulatory domains [44, 45]. Wojcikiewicz purified each IP$\_3$R subtype protein from cell lines and used them as standards in various immunoblotting experiments [46]. Taylor et al. made an antiserum that recognizes all three IP$\_3$R almost equally and this was used to study IP$\_3$R protein [47, 48, 49]. All three subtypes of IP$\_3$R are regulated by intracellular Ca$^{2+}$ concentration in a biphasic manner. Ca$^{2+}$ stimulates the receptors at low concentration but inhibits at higher concentrations [50]. Intracellular Ca$^{2+}$ oscillations reflect this biphasic regulation. But Miyakawa et al. reported that IP$\_3$R1 mediates irregular Ca$^{2+}$ oscillations, that activation of IP$\_3$R2 produces long-lasting and relative regular Ca$^{2+}$ oscillations, and that activation of IP$\_3$R3 generates monophasic Ca$^{2+}$ transients [51]. Furthermore, although the three subtypes have the same IP$\_3$ binding site, same Ca$^{2+}$ gating property
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and similar ionic conductance [51, 52], they differ in ligand binding sensitivity: IP$_3$R1 has medium IP$_3$-affinity and low Ca$^{2+}$-affinity; IP$_3$R2 has high IP$_3$-affinity and medium Ca$^{2+}$-affinity; IP$_3$R3 has low IP$_3$-affinity and high Ca$^{2+}$-affinity [51, 52].

IP$_3$R1 is the dominant subtype in the central nervous system and Purkinje cells [53]. IP$_3$R1 displays bell-shaped dependence on cytosolic Ca$^{2+}$ concentration [54]. There are five domains (I-V) in IP$_3$R1 separated by linker regions [55]. Domain I (aa 1-345) contains IP$_3$-binding suppressor domain [54]. Domain II (aa 346-923) contains the IP$_3$-binding domain [56]. Domain III (aa 924-1583) contains several domains, including carbonic anhydrase related protein-binding site, GAPDH-binding site, and Ca$^{2+}$-CaM binding motif 50, 57, 58, 59]. Domain IV (aa 1584-1932) contains two PKA/PKG phosphorylation sites, S1589 and S1755 [60, 61, 62, 63]. This domain also contains several other domains, such as SII alternative splicing site, ATP-binding site, and caspase-3 cleavage site [60, 61, 62, 63]. Sensitivity of IP$_3$R1 to IP$_3$ is enhanced by phosphorylation of the receptors by PKA [68, 69, 70, 71, 72, 73]. Recently, deSouza et al. found that in lymphocytes, phosphorylation of IP$_3$R1 by tyrosine kinase increased sensitivity of the receptors to IP$_3$ and decreased the inhibitory effect of high concentration Ca$^{2+}$ on the receptors. They also found that this phosphorylation results in increased IP$_3$R1 open probability and prolongation of Ca$^{2+}$ release [74]. Domain V could be divided into three subdomains. The most important subdomain is a Ca$^{2+}$ sensor region responsible for IP$_3$R1 modulation by Ca$^{2+}$.

Much less is known about IP$_3$R2 and IP$_3$R3 than IP$_3$R1. Sequence analysis shows that the three IP$_3$R isoforms share similar IP$_3$-binding, Ca$^{2+}$-gating, and ion conductance properties but may differ in modulation [55, 75, 76]. For example, IP$_3$R1 and IP$_3$R3 are both ATP-dependent, and IP$_3$R2 is ATP-independent, although it has two ATP-binding sites [48, 77, 78, 79, 80]. IP$_3$R2 in rat has one PKA phosphorylation site S1687, and IP$_3$R3 has three consensus sites: S934, S1133, and S1460 [44]. Wojcikiewicz found that PKA phosphorylated IP$_3$R2 and IP$_3$R3 very inefficiently [46]. Despite this inefficient phosphorylation, IP$_3$-induced Ca$^{2+}$ release was potentiated by PKA [73].

Despite the important role of IP$_3$R in Ca$^{2+}$-mediated ion secretion, little information is available about the subtype(s) of IP$_3$R expressed and details of their phosphorylation state in airway SMGC. Therefore, we propose to characterize the subtype(s) of IP$_3$ in mucous gland cells and their phosphorylation states.

2) Ca$^{2+}$ entry

Ca$^{2+}$ regulates the physiological function in a variety of non-excitable cell types, including airway SMGC. Receptor stimulation in most unexcitable cells results from an increase in cytosolic Ca$^{2+}$ concentration: the initial transient increase induced by IP$_3$-mediated release of Ca$^{2+}$ from ER, and a subsequent more prolonged Ca$^{2+}$ influx through store- or receptor-operated channels [81, 82, 83, 84, 85]. In SMGC, the increase in Ca$^{2+}$ influx is critical for the activation of various ion channels, such as K$_{Ca}$ and Cl$_{Ca}$, which result in the transport of fluid as well as the secretion of mucus [24, 41, 86, 87].

Dependent on the cell type there are several kinds of Ca$^{2+}$ channels that may mediate Ca$^{2+}$ influx, such as voltage-operated channels (VOC), second messenger-operated channels (SMOC),
store-operated channels (SOC), and receptor-operated channels (ROC). VOC are typically in excitable cells, like smooth muscle cells, nerve cells, and heart cells. Voltage-independent Ca\(^{2+}\) channels occur in many cells but dominate in unexcitable cells, such as SMGC. SOC are voltage-independent and a prominent Ca\(^{2+}\) channel in SMGC [83]. Store-operated Ca\(^{2+}\) entry is activated in response to depletion of intracellular Ca\(^{2+}\). SOC as a class consists of several types of channels: Ca\(^{2+}\)-release-activated channels (CRAC) and a broader class group of proteins termed non-selective cation channels. CRAC are Ca\(^{2+}\) selective and inward rectified, not voltage-activated, and activated by Ca\(^{2+}\) store depletion such as that induced by thapsigargin, IP\(_3\), or EGTA [88]. Non-selective cation channels have not been as well characterized as CRAC channels. Some are activated by store depletion and others are activated through signal transduction pathways via G-protein coupled receptors [89, 90].

TRPC channels are generally non-selective, Ca\(^{2+}\)-permeable cation channels that may be activated by stimulation of G protein-coupled and tyrosine phosphorylated receptors either directly or through ER Ca\(^{2+}\) release. Members of TRPC, such as TRPC1, TRPC3, TRPC4, and TRPC7 have been proposed as components of SOC channels in different cell types [83, 91, 92, 93, 94]. These findings have been further substantiated by studies showing that knockdown of TRPC1 reduces CRAC current (I\(_{\text{CRAC}}\)) in cell line A7r5 cell [95]. A store-operated mechanism for TRPC1 that has been recently been proposed involves interaction with Orai1 and STIM1. While CRACM1 (Orai1) has been shown to form tetramers that make up the essential pore component of the CRAC channel. STIM1 is a protein located in the ER membrane that monitors ER Ca\(^{2+}\) store concentration forms dynamic assembly with Orai1 and upon Ca\(^{2+}\) store depletion and Ca\(^{2+}\) entry occurs [96, 97, 98].

Recent reports have demonstrated in lymphocytes that two proteins, STIM1 and Orai1, are involved in stored-operated Ca\(^{2+}\) entry in some cells. STIM1 is a single-transmembrane protein localized in ER. STIM1 is thought to be an ER Ca\(^{2+}\) sensor [99, 100, 101, 102]. Upon release of Ca\(^{2+}\) from the ER, STIM1 migrates to a position in the ER membrane close to the plasma membrane where it can activate Orai1. Orai1, also known as CRACM1, is thought to be an important component of the CRAC channel [103, 104]. Although the SOC entry pathway is critical in Ca\(^{2+}\) influx after the initial transient Ca\(^{2+}\) release for sustained fluid and mucus secretion in SMGC, little information is available about SOC entry in airway SMGC. It is necessary to further examine the actions of pharmacological agents on Ca\(^{2+}\) entry, in particular the possible actions of macrolide antibiotics on this pathway.

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Figure 1. In swine tracheal submucosal gland cells (SMGCs), neurotransmitters such as acetylcholine (Ach) bind to transmembrane receptors, in this case, muscarinic receptors 3 (M3). Subsequently, the activation of M3 receptor induces intracellular inositol 1,4,5-triphosphate (IP3) formation, which binds to IP3 receptors (IP3-R) in the membrane of endoplasmic reticulum and open the calcium channel. Calcium (Ca2+) release from endoplasmic reticulum (ER) activates both store-operated calcium channels (SOC) in the cell membrane and ryanodine receptors (Ry-R) in the ER membrane, which induces further elevation of intracellular calcium concentration. This calcium mobilization eventually activate calcium-dependent Cl- channel (ClCa) and calcium-dependent K+ channel (KCa) in the cell membrane, and following mucus secretion from SMGCs in trachea.