Yong-Xiao Wang *Editor*

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Preface

Smooth muscle cells (SMCs) are the major functional and structural components of the airway. In addition to maintaining air flow, these cells can proliferate, migrate, and induce cytokines, extracellular matrix proteins, growth factors, and other molecules. Airway SMCs are also key players in the development of asthma and asthma attacks by causing airflow obstruction secondary to contractile hyperresponsiveness, remodeling (e.g., hyperplasia and hypertrophy), and inflammatory responses. These physiological and pathological functions of airway SMCs are, in fact, precisely controlled and regulated by calcium signaling, which may result from extracellular calcium influx or intracellular calcium release as a result of activation or inactivation of ion channels, exchangers, and transporters on the cell plasma membrane and sarcoplasmic reticulum membrane. Scientific research in calcium signaling in airway SMCs is growing; however, to date, there has not been a comprehensive book compiling and detailing our state-of-the-art advances.

The major objective of this book is to create a valuable platform in which numerous, well-established, and emerging pioneers are able to report their recent, inspiring findings from basic, translational, and clinical research, particularly focusing on genesis, networks, microdomains, regulation, functions, and therapies of calcium signaling in airway SMCs. We also strive to include data from clinical trials exploring interventions of calcium signaling in the treatment of asthma and other related diseases. The innovative and ample contents presented will update our understanding of the role of calcium signaling and help direct future research in the field.

This book offers a broad and detailed overview for academic and industrial scientists, postdoctoral fellows, and graduate students engaged in studies of calcium signaling in airway smooth muscle and other cells, particularly in the fields of molecular biology, cell biology, biochemistry, physiology, and pharmacology. In addition, the book may also serve as a useful reference tool for clinicians, medical students, and allied health professionals.

Many of the authors of this book have played dual roles as reviewers. I would like to express my wholehearted appreciation to all of the contributors for their dedication and hard work. I also wish to thank Ms. Aleta Kalkstein, Editor of Cell Biology, and Ms. Rita Beck, Assistant Editor of Food Science, at Springer Science +Business Media for their patience and assistance.

Albany, NY, USA Yong-Xiao Wang

Contents

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Ryanodine and Inositol Trisphosphate Receptors/Ca^{2+} Release Channels in Airway Smooth Muscle Cells

Lin Mei, Yun-Min Zheng, and Yong-Xiao Wang

Contents

Abstract Ryanodine receptors (RyRs) and inositol 1,4,5-trisphosphate receptors (IP_3Rs) are the most important Ca^{2+} release channels on the sarcoplasmic (or endoplasmic) reticulum (SR) in almost all types of cells. In the past several decades, the studies of RyRs and IP_3Rs have greatly facilitated our understanding of the physiological functions and pathological mechanisms for various diseases including heart failure, arrhythmias, myopathy, and seizure. Similarly, their important roles have been explored in airway smooth muscle cells (SMCs). These two receptors control intracellular Ca^{2+} release and modulate extracellular Ca^{2+} influx, thereby playing an essential role in cell contraction, relaxation, proliferation,

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migration, metabolism, and, ultimately, cell fate. The abnormality of Ca^{2+} signaling in airway SMCs may contribute to the development of multiple lung diseases, notably asthma. Concomitantly, many regulators, including Ca^{2+} itself, calmodulin, protein kinases, FK506-binding protein 12.6 (FKBP12.6), cyclic adenosine diphosphate ribose (cADPR), and redox status, are involved in the regulation of Ca^{2+} signaling and, thus, the physiological function and pathological alterations. The two SR Ca^{2+} release channels may also directly or indirectly interact with plasmalemmal and mitochondrial ion channels such as transient receptor potential cation, big-conductance Ca^{2+}-activated K^+, Ca^{2+}-activated Cl^-, and other channels, providing positive or negative feedback mechanisms to control Ca^{2+} signaling and cellular functions.

Keywords Ryanodine receptor • Inositol 1,4,5-trisphosphate receptor • Intracellular Ca^{2+} release • Extracellular Ca^{2+} influx • Big-conductance Ca^{2+}-activated K^+ channel • Ca^{2+}-activated Cl^- channel

Abbreviations

2-APB	2-aminoethoxy-diphenylborate
ACh	Acetylcholine
BK_{Ca}	Big-conductance Ca^{2+}-activated K^+ channel
cADPR	Cyclic adenosine diphosphate ribose
CaMKII	Calmodulin-dependent protein kinase II
CICR	Ca^{2+}-induced Ca^{2+} release
Cl_{Ca}	Ca^{2+}-activated Cl^- channel
CPVT	Catecholaminergic polymorphic ventricular tachycardia
DAG	Diacylglycerol
FKBP12.6	FK506-binding protein 12.6
GPX1	Glutathione peroxidase-1
H_2O_2	Hydrogen peroxide
IL-13	Interleukin-13
IP_3Rs	Inositol 1,4,5-trisphosphate receptors
IRAG	cGMP kinase substrate
LTCCs	L-type voltage-gated Ca^{2+} channels
LTD4	Leukotriene D4
M_3R	Muscarinic M_3 receptor
mACh	Methacholine
MLC	Myosin light chain
MLCK	Myosin light chain kinase
MLCP	Myosin light chain phosphatase
mTOR	Mammalian target of rapamycin
NCX	Na^+/Ca^{2+} exchanger
NO	Nitric oxide

NSCCs	Nonselective cation channels
PI3K	Phosphatidylinositol 3 kinases
PIP2	Phosphatidylinositol 4 5-bisphosphate
PKA	Protein kinase A
PKC	Protein kinase C
PKG	cGMP-dependent protein kinase
PLC	Phospholipase C
ROS	Reactive oxygen species
RyR	Ryanodine receptor
SERCA	Sarcoplasmic reticulum Ca^{2+} ATPase
SMC	Smooth muscle cell
SOCE	Store-operated calcium entry
SR	Sarcoplasmic reticulum
STIC	Spontaneous transient inward current
STOC	Spontaneous transient outward current
TNF-α	Tumor necrosis factor-α
TRP	Transient receptor potential channel
VICR	Voltage-induced Ca^{2+} release

1 Introduction

Ca^{2+} signals are precisely generated and regulated by both extracellular Ca^{2+} influx and intracellular Ca^{2+} release, which are controlled and regulated by ion channels, exchangers, and transporters. L-type voltage-gated Ca^{2+} channels (LTCCs), nonselective cation channels (NSCCs), and Na^+/Ca^{2+} exchangers (NCX) play an important role in mediating Ca^{2+} influx. The intracellular Ca^{2+} is primarily stored in the sarcoplasmic/endoplasmic reticulum (SR). In smooth muscle cells (SMCs), a Ca^{2+} signal can express in various forms, such as Ca^{2+} flashes, puffs, ripples, sparklets, sparks, waves, oscillations, and global changes in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$). These diverse Ca^{2+} signals regulate physiological and pathological functions in nearly all types of cells.

Ca^{2+} release channels on the SR can be divided into two categories: ryanodine receptors (RyRs) and inositol 1,4,5-trisphosphate receptors (IP_3Rs). RyRs were originally identified using the plant alkaloid ryanodine, in which ryanodine induced profound paralysis of cardiac and skeletal muscle cells and was bound to the SR membrane [1, 2]. Three subtypes of RyRs (RyR1, RyR2, and RyR3) have been identified. Each of them is encoded by a distinct gene in mammalian and human cells. The three subtypes of RyRs share approximately 70 % sequence homology. RyRs are the approximately 2,200 kDa homotetramer of four ~550 kDa subunits and mediate Ca^{2+} release from the SR. These Ca^{2+} release channels can be divided into three domains: N-terminal, central, and C-terminal. The C-terminal of RyRs contains the channel region; the N-terminal domain modulates the channel gating; and the central domain comprises a large cytosolic region that serves as a scaffold to interact with regulatory proteins to form a macromolecular signaling complex [3].

IP_3Rs on the SR are also tetramers with a monomeric molecular mass of approximately 300 kDa. Each subunit is composed of the N-terminal, central, and C-terminal domains. The N-terminal domain composes an IP_3-binding site, a suppressor region that inhibits IP_3 binding, and the regulatory region [4]. To date, three IP_3R subtypes (IP_3R1, IP_3R2, and IP_3R3) have been identified. Each subtype of IP_3Rs is encoded by a separate gene in mammalian and human cells. The full-length amino acid sequences of three IP_3R subtypes share 60–80 % homology overall.

Stimulation of neurotransmitter, hormone, growth factor, and other G protein-coupled receptors activates phospholipase C (PLC), which hydrolyzes the membrane lipid phosphatidylinositol 4, 5-biphosphate (PIP2) to generate diacylglycerol (DAG) and IP_3. The latter molecule can bind to its receptors and then induce Ca^{2+} release from the SR. To date, all three subtypes of IP_3Rs (IP_3R1, IP_3R2, and IP_3R3) have been discovered in SMCs; among them, IP_3R1 appears to be the most predominant subtype [5].

Depolarization of the cell membrane can cause the opening of LTCCs and extracellular Ca^{2+} influx, which gives rise to an increase in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$). The increased $[Ca^{2+}]_i$ activates RyRs and possibly IP_3R to release Ca^{2+} from the SR, a process called Ca^{2+}-induced Ca^{2+} release (CICR). Subsequently, the elevated intracellular Ca^{2+} binds to calmodulin. This complex activates myosin light chain kinase (MLCK) to phosphorylate myosin light chain (MLC). In cooperation with actin, the phosphorylated MLC initiates cross-bridge cycling and contraction of the cell. The removal of the contractile stimulation or inhibition of related contractile molecules results in cell relaxation. Concomitantly, the relaxation process requires a decrease in $[Ca^{2+}]_i$, which is mediated by the SR Ca^{2+} ATPase (SERCA) to refill the Ca^{2+} store or by NCX to pump Ca^{2+} out of the cell. This process increases MLC phosphatase (MLCP) activity to dephosphorylate the MLCs [6].

In addition to contraction, Ca^{2+} signaling plays an important role in metabolism, transcription, migration, and proliferation in SMCs in a time-dependent manner. For example, Ca^{2+} influx triggers exocytosis within microseconds at the synaptic junction, whereas the Ca^{2+} signal drives gene transcription and cell proliferation over minutes and even hours [7]. An alteration in Ca^{2+} signaling might be an essential factor for many airway diseases, as exemplified by asthma.

2 Expression of RyRs and IP_3Rs

Among the three subtypes of RyRs, RyR1 is mainly expressed in skeletal muscle cells and serves as the major Ca^{2+} release channel required for cell contraction. In addition, a low level of RyR1 expression has been reported in SMCs, cerebellum, testis, adrenal gland, spleen, and ovary. It was recently shown that RyR1 is also expressed in B-lymphocytes [8]. RyR2 is the most abundant in the heart, lung, SMCs, and pancreatic β cells. This channel also shows a high expression level in Purkinje cells of cerebellum and cerebral cortex, but a low level in stomach, kidney,

adrenal glands, ovaries, thymus, and lungs [9]. RyR3 is found in the brain, spleen, heart, and testis [10]. Coronary vasculature, lung, kidney, ileum, jejunum, spleen, stomach, aorta, uterus, ureter, urinary bladder, and esophagus express RyR3 as well.

Airway SMCs express all three subtypes of RyRs; however, RyR2 is likely to be the predominant subtype. In these cells, RyR1 is localized to the periphery near the cell membrane, whereas RyR3 is more centrally localized [11]. The heterogeneous distribution of RyR1 and RyR3 may endow their multiple functions in airway SMCs.

In mammals, IP_3R is ubiquitously expressed in almost all cell types. IP_3R1 is predominantly expressed in Purkinje cells, IP_3R2 primarily in cardiac myocytes, and IP_3R3 mainly in pancreatic β cells [12]. IP_3R1 knockout mice show neurological defect and early death [13], which is consistent with its primary expression in brain. Interestingly, IP_3R2 null mice have the abolished positive ionotropic effect in atrial myocytes of the heart, revealing the potential importance of this Ca^{2+} release channel in cardiac cells [14].

SMCs express all three IP_3R subtypes. The density of IP_3Rs is approximately 100 times less in SMCs than in brain. It is intriguing to point out that the overall stoichiometric ratio of IP_3Rs to RyRs is approximately 10–12:1 in visceral SMCs but 3–4:1 in vascular SMCs [15]. IP_3R1 is the predominant subtype expressed in vascular SMCs, including aortic, cerebral artery, mesenteric artery, and portal vein myocytes [5]. IP_3R2 and IP_3R3 expression levels have been found to be higher in proliferating, neonatal, and cultured aortic SMCs [16]. IP_3R1 is also predominantly expressed in intestinal SMCs.

Western blot analysis reveals that IP_3R1, IP_3R2, and IP_3R3 are all expressed in airway SMCs [17]. The heterogeneity of tissue-dependent expression levels of IP_3R subtypes may significantly contribute to the diversities of Ca^{2+} signaling and cellular functions in different types of SMC.

3 RyR- and IP_3R-Mediated Ca^{2+} Release

RyR-mediated Ca^{2+} release is required for excitation-contraction coupling in skeletal, cardiac, and some SMCs. RyRs on the SR can be functionally coupled to LTCCs (also known as dihydropyridine receptors, DHPRs) on the plasmalemma, by which Ca^{2+} influx through LTCCs during membrane depolarization opens RyRs to induce Ca^{2+} release from the SR, i.e., CICR. RyRs may also be physically coupled to LTCCs; as such, a conformational change in LTCCs following membrane depolarization (voltage stimulation) causes the opening of associated RyRs and Ca^{2+} release, termed voltage-induced Ca^{2+} release (VICR) process. The three subtypes of RyR exhibit differences in Ca^{2+}-dependent activation and deactivation. Usually, RyR activation occurs when the concentration of Ca^{2+} in the cytosol is within approximately 0.3–10 μM, and inhibition occurs at concentrations of approximately 1 mM Ca^{2+} or higher [18].

Following stimulation of G protein-coupled receptors (GPCRs), IP_3 is produced from PIP2 by PLC. IP_3 can bind to and activate its receptors to induce SR Ca^{2+}

release and increase $[Ca^{2+}]_i$. The increased $[Ca^{2+}]_i$ may promote the binding of Ca^{2+} to IP_3Rs, which in turn forms a positive feedback to initiate the propagation of a Ca^{2+} wave through stimulation of neighboring IP_3Rs and RyRs. However, at high $[Ca^{2+}]_i$, binding of Ca^{2+} to a low-affinity site on the IP_3Rs reduces the channel open probability. This, together with a localized decrease in the SR Ca^{2+}, leads to the termination of further Ca^{2+} release and wave propagation [19].

IP_3Rs and RyRs can both mediate local and global Ca^{2+} release in SMCs. Spontaneous transient local Ca^{2+} release events, termed Ca^{2+} sparks, are due to the concerted opening of several localized RyRs. A Ca^{2+} spark has been described in SMCs of numerous tissues including the artery, vein, stomach, trachea, and bladder [20]. Interestingly, IP_3Rs are involved in RyR-mediated Ca^{2+} sparks in airway SMCs [21, 22]. Similar findings have been made in portal vein myocytes [23]. However, one study showed that IP_3R inhibition has no effect on the frequency or amplitude of spontaneous Ca^{2+} sparks in rat pulmonary artery SMCs [24]. Therefore, IP_3Rs may participate in the generation of Ca^{2+} sparks in some, but not all, types of SMCs.

Besides global and local Ca^{2+} release, RyRs and IP_3Rs generate other forms of Ca^{2+} signals in SMCs. For example, spontaneous or agonist-induced synchronous opening of approximately 30 IP_3Rs clustered within an approximately 400 nm diameter region produces a Ca^{2+} puff. Ca^{2+} flash, a rapid increase in local $[Ca^{2+}]_i$, has been observed in gallbladder SMCs [25]. This local Ca^{2+} signal is likely to be mediated by LTCCs, IP_3Rs, and RyRs. Ca^{2+} oscillation is a repetitive, non-propagating elevation in global $[Ca^{2+}]_i$ that results from periodic, pulsatile release of SR Ca^{2+} in SMCs. Ca^{2+} ripple is an IP_3R-mediated spontaneous, pro-pagating Ca^{2+} signal with a low amplitude. The activation of IP_3Rs, RyRs, or both can induce a Ca^{2+} wave, a propagating elevation in global $[Ca^{2+}]_i$ in SMCs [5]. Presumably, the tissue-specific variability of IP_3R- and RyR-mediated Ca^{2+} signal strengths and patterns are required for multiple and different physiological functions in SMCs.

The first evidence of the involvement of RyRs in Ca^{2+} signaling in airway SMCs came from the findings that the RyR antagonist ryanodine (at a high concentration) and ruthenium red could inhibit acetylcholine (ACh)-induced Ca^{2+} oscillations in porcine tracheal SMCs [26, 27]. The inhibition of RyRs by procaine and tetracaine also blocks Ca^{2+} oscillations. Similar results have been reported in human airway SMCs [28]. Exogenous application of IP_3, similar to ACh, also induces Ca^{2+} oscillations in porcine tracheal SMCs. ACh- or IP_3-induced Ca^{2+} oscillations can be inhibited by IP_3R antibodies and antagonists (e.g., heparin and 2-aminoethoxydiphenyl borate) [21, 29]. RyR inhibitors also attenuate norepinephrine- or phenylephrine-induced, IP_3R-dependent Ca^{2+} release in renal artery SMCs [30]. Thus, it appears that the initiation of Ca^{2+} oscillations is mediated by IP_3Rs, whereas the maintenance of these Ca^{2+} signals are required for the participation of RyRs.

It has been demonstrated that RyRs and IP_3Rs can localize on the same SR (Ca^{2+} store) in mesenteric and small pulmonary artery as well as portal vein SMCs. However, other studies suggest that these two Ca^{2+} release channels are present

at discrete regions of the SR in mesenteric, large pulmonary artery as well as ureter myocytes [5]. This discrepancy may indicate a complicated interaction between IP_3Rs and RyRs.

The localization of RyRs and IP_3Rs on the same SR has also been observed in airway SMCs. This unique structural feature renders a distinctive platform for the synergistic role of both Ca^{2+} release channels in the accurate control of cellular functions in airway SMCs. Indeed, Ca^{2+} released from IP_3Rs readily causes the opening of RyRs and further Ca^{2+} release from the SR in airway SMCs [21, 22]. Our further studies demonstrated that this local IP_3R-mediated, RyR-dependent local CICR process is specifically attributable to the opening of RyR2 [22].

4 Interaction of RyRs and IP_3Rs with Other Ion Channels

In SMCs, IP_3Rs and RyRs may communicate with several SR and plasma membrane localized ion channels in order to regulate $[Ca^{2+}]_i$ and cellular functions.

4.1 Transient Receptor Potential (TRP) Channels

TRP channels play a major role in controlling extracellular Ca^{2+} influx in SMCs [31]. TRP channels are classified into six different subfamilies based on their activation stimuli and homology: canonical, vanilloid, melastatin, polycystin, mucolipin, and ankyrin TRP (TRPC, TRPV, TRPM, TRPP, TRPML, and TRPA) channels. The TRPC channel subfamily, which comprises seven members that are designated TRPC1-7, is perhaps the one most studied in airway SMCs. It has been shown that TRPC3 is a major member of nonselective cation channels. TRPC3 contributes to the resting $[Ca^{2+}]_i$ and is also involved in muscarinic increases in $[Ca^{2+}]_i$ in freshly isolated airway SMCs [32, 33]. Our group has also reported that the expression and activity of TRPC3 channels are significantly increased in asthmatic airway myocytes, whereas TRPC1 channel activity is also augmented but its expression remains unchanged [34].

IP_3Rs and RyRs may directly interact with TRPC channels. Studies have shown that there is a physical coupling between the IP_3R1 N-terminal and TRPC3 channel C-terminal in cerebral artery SMCs [35]. It is unclear whether TRPC1 also has a physical interaction with IP_3Rs; however, gene silencing of TRPC1 inhibits the increased $[Ca^{2+}]_i$ following stimulation of IP_3Rs with endothelin-1 in aortic SMCs [36]. TRPC6 channels in cerebral artery SMCs contain the IP_3R binding domain but do not interact physically with IP_3R1 [37]. It is also interesting to note that in the absence of IP_3 or functional IP_3Rs, TRPC channels can be activated by PLC, PIP2, and DAG in SMCs.

4.2 Big-Conductance Ca^{2+}-Activated K^+ Channels (BK_{Ca})

BK_{Ca} channels are voltage- and Ca^{2+}-sensitive and have a large single-channel conductance (100–300 pS under symmetrical extracellular and intracellular K^+ conditions). BK_{Ca} channels are formed by α-subunits and accessory β-subunits. The β-subunits play an important role in the regulation of the physiological properties of BK_{Ca} channels. The lack of β1-subunits decreases the Ca^{2+} sensitivity of BK_{Ca}, reducing the extent of plasma membrane hyperpolarization [38]. BK_{Ca} channels are acknowledged to be activated by Ca^{2+} sparks as a result of the opening of RyRs, which generates spontaneous transient outward currents (STOCs). IP_3R1 activation increases the Ca^{2+} sensitivity of BK_{Ca} channels in cerebral artery SMCs, and both IP_3R1 and BK_{Ca} channels have been shown to be physically coupled [39]. This could oppose membrane depolarization- and IP_3-induced contraction of SMCs, acting as a negative feedback mechanism to protect SMCs from overexcitation. Recently, Lifschitz et al. reported that RyR1 and RyR2, but not RyR3, formed clusters with BK_{Ca} channels and were localized near the plasmalemma of airway SMCs. Analyzing the spatial relationship between RyR2 and BK_{Ca} channels, they estimated that an average Ca^{2+} spark caused by the opening of clusters of RyR1 or RyR2 resulted in activation of two to three clusters of BK_{Ca} channels that were randomly distributed within an approximately 600 nm radius of the RyRs. Approximately eight RyRs opened to give rise to a Ca^{2+} spark, which activated approximately 15 BK_{Ca} channels to generate a STOC at 0 mV [40]. These results further support the close relationship between BK_{Ca} channels and RyRs.

4.3 Ca^{2+}-Activated Cl^- (Cl_{Ca}) Channels

Cl_{Ca} channels possibly contribute to the control of smooth muscle tone through their activation following an elevation in $[Ca^{2+}]_i$ to produce Cl^- efflux and inward membrane currents, which cause membrane depolarization. This depolarization promotes extracellular Ca^{2+} influx via LTCCs to increase muscle contraction. The properties of Cl_{Ca} channels are not completely understood. Ca^{2+} binding to the channel may directly activate the channel; however, in some tissues, activation may be involved by calmodulin-dependent protein kinase II (CaMKII) [41]. Spontaneous transient inward currents (STICs) due to the opening of Cl_{Ca} channels were first observed in tracheal myocytes [42]. The characteristics of STICs are consistent with Ca^{2+} sparks because they are abolished by chloride channel blockers in airway SMCs [43], and it has been shown that they are tightly controlled by RyR-generated Ca^{2+} sparks [44]. Recent evidence indicates that TMEM16A and TMEM16B are Cl_{Ca} channels [45]. TMEM16A is expressed in airway epithelial cells and SMCs, and knocking it out decreases Cl^- secretion in response to Ca^{2+}-dependent agonists and SMC contraction [46]. In asthmatic patients and in a mouse model of asthma, the expression of TMEM16A is upregulated in airway epithelial cells. TMEM16A

inhibitors negatively regulate both epithelial mucin secretion and airway SMC contraction [47]. Moreover, Zhang et al. reported that TMEM16A, but not TMEM16B, encods Cl_{Ca} channels in airway SMCs and contributes to agonist-induced contraction. Pharmacological blockade of TMEM16A-encoded channels prevents airway hyperresponsiveness [48].

5 Regulation of RyRs and IP_3Rs

5.1 Regulators of RyRs

RyRs can form a macromolecular complex with multiple regulators and thus cause adequate cellular responses. For example, calmodulin is an ubiquitously expressed Ca^{2+}-binding protein that inhibits cardiac RyR2 activity at any level of $[Ca^{2+}]_i$. Reduced affinity for calmodulin binding to RyR2 due to phosphorylation of RyR2 by protein kinase A (PKA) is found in catecholaminergic polymorphic ventricular tachycardia (CPVT)-associated lethal arrhythmia [49].

FK506-binding protein 12 (FKBP12) and FKBP12.6 function as RyR stabilizers in several different types of cells. Quantitatively, each RyR is bound by four FKBP12 or 12.6 proteins, with a stoichiometry of one per subunit. RyR1 and RyR3 exhibit much greater affinity for FKBP12 than FKBP12.6, while RyR2 exhibits greater affinity for FKBP12.6 [50]. Dissociation of FKBP12/12.6 from RyRs increases the channel open probability, which causes Ca^{2+} leak from the SR and altered $[Ca^{2+}]_i$ in pulmonary SMCs [51]. It is well known that Ca^{2+} leak from the SR contributes to various diseases, including heart failure [52], arrhythmia [53], and aging [54].

LTCCs (Cav1.1 in skeletal muscle cells and Cav1.2 in cardiac myocytes) and RyRs play an important role in muscle excitation-contraction coupling. In skeletal muscle cells, every RyR1 channel in the junctional membrane is physically coupled to a tetrad of four Cav1.1 channels in the T-tubule membrane; as such, membrane depolarization causes activation of Cav1.1 channels and RyR1 to mediate VICR. In cardiac myocytes, RyR2-mediated Ca^{2+} release is initiated by Ca^{2+} influx via Cav1.2, i.e., CICR. These Ca^{2+} release channels are redox sensitive, and redox modifications can result in either activation or deactivation of the Cav1.2 channel. RyRs have approximately 100 cysteines per subunit and are also susceptible to redox modification by oxidation, nitrosylation, or alkylation [9]. Redox-mediated RyR modifications have been implicated in muscular dystrophy, malignant hyperthermic crisis, and heart failure. For example, our group has reported that oxidation of RyR2 causes dissociation of FKBP12.6 from RyR2, leading to the increased activity of RyR2 in pulmonary arterial SMCs. Overexpression of glutathione peroxidase-1 (GPX1), which blocks the generation of reactive oxygen species (ROSs), prevents dissociation [51]. A similar mechanism has been reported in the development of muscle weakness in aging [54] and also in a heat-induced sudden

death model in RyR1 mutant mice [55]. In addition to oxidation, nitrosylation of RyR1 can also decrease the affinity of FKBP12 to RyRs, causing an SR Ca^{2+} leak that is implicated in the muscle weakness of patients with muscular dystrophy [56].

5.2 *Regulators of IP_3R*

Multiple protein kinases, regulatory proteins, and other modulators influence IP_3R activity in SMCs. cGMP-dependent protein kinase (PKG) may inhibit the activity of IP_3Rs via phosphorylation. PKG phosphorylates IP_3Rs at serine 1755 and inhibits IP_3-induced Ca^{2+} release in aortic SMCs [57]. PKG also phosphorylates IP_3Rs via IP_3-associated cGMP kinase substrate (IRAG) in tracheal SMCs, suggesting that PKG/IRAG regulation of IP_3R phosphorylation modulates airway SMC contractility [58].

Evidence indicates that FKBP12 also modulates IP_3R activity in addition to that of the RyRs. It may occur through three effector proteins: calcineurin, FK506, or mammalian target of rapamycin (mTOR). FKBP12 enhances IP_3R activity via mTOR and inhibits IP_3R activity through calcineurin in colonic SMCs, though this dual effect is tissue specific [59].

In addition to ligands, kinases, and regulators, ROSs also modulate IP_3Rs in SMCs through two main processes: IP_3 generation and IP_3R affinity. Superoxide inhibits IP_3 hydrolysis, thereby enhancing IP_3-induced Ca^{2+} release [60]. Similarly, we have recently unveiled that hydrogen peroxide (H_2O_2) stimulates IP_3R-induced Ca^{2+} release, IP_3 generation, and IP_3R affinity in pulmonary artery SMCs, which suggests that ROSs are involved in IP_3R signaling in SMCs [61]. However, ROS regulation is multimodal and may also be tissue dependent.

6 Physiological and Pathological Functions of RyRs and IP_3Rs in Airway SMCs

6.1 *Role of RyRs*

We found that the basal activity of PLC is important for the activity of RyR-mediated, spontaneous Ca^{2+} sparks in airway SMCs. There are two distinct pathways for the role of PLC. One is mediated by PLC-dependent generation of IP_3 to induce SR Ca^{2+} release, which stimulates neighboring RyRs, leading to further Ca^{2+} release and Ca^{2+} sparks. The second pathway occurs via the PLC-mediated generation of DAG and subsequent activation of protein kinase C-ε (PKCε), which inhibits the activity of RyRs and attenuates Ca^{2+} spark generation [22]. The inhibitory effect of PKCε is attributed to its specific interaction with RyR1 [62].

Further studies in our laboratory demonstrate that membrane depolarization can induce RyR-mediated local Ca^{2+} release in airway SMCs. This membrane depolarization-mediated local Ca^{2+} release can occur independently of LTCCs; however, it is due to the direct activation of muscarinic M_3 receptors (M_3Rs) in the absence of exogenous agonists. The activation of M_3Rs causes an increase in the activity of PLC, generation of IP_3, opening of IP_3Rs, Ca^{2+} release, and then activation of RyR2, inducing further Ca^{2+} release [21].

It has been reported that interleukin-13 (IL-13) or IL-4 upregulates the expression of RyRs and IP_3Rs but decreases the expression of SERCA in human bronchial SMCs. Furthermore, the enlarged ACh-induced increase in $[Ca^{2+}]_i$ is reversed by blocking IP_3Rs [63]. It has been demonstrated that SERCA2 is reduced in human asthmatic subjects. Ca^{2+} release from the SR or inhibition of SERCA-mediated Ca^{2+} reuptake is attenuated in airway SMCs from asthmatics. Knockdown of SERCA2 mediated by siRNA in airway SMCs has been shown to cause the secretory and hyperproliferative phenotypes in asthma [64].

Failure of RyR inhibitors to affect agonist-induced increase in $[Ca^{2+}]_i$ or contraction has also been observed in airway SMCs. For example, exposure to IP_3R antagonist 2-aminoethoxydiphenyl borate (2-APB) inhibits Ca^{2+} oscillation and induces airway relaxation in a concentration-dependent manner. However, the RyR antagonist ryanodine has no significant effect [65]. Tetracaine, another RyR inhibitor, relaxes agonist-induced contraction and inhibits Ca^{2+} oscillation but has no effect on IP_3-induced Ca^{2+} release or wave propagation. Conversely, both ryanodine and tetracaine completely block slow Ca^{2+} oscillation induced by KCl [66]. However, Tazzeo et al. have reported that neither ryanodine nor chloroethyl phenol inhibits the responses to KCl, cholinergic agonists, or serotonin in airway SMCs. Airway relaxation induced by agonists including isoproterenol, salmeterol, and nitric oxide (NO) is also unaffected by ryanodine [67]. Ryanodine also fails to affect histamine-induced contraction or formoterol-induced relaxation in human airways [68]. In IL-13-pretreated airway SMCs, the RyR inhibitor ryanodine or ruthenium red partially blocks leukotriene D4 (LTD4)-induced Ca^{2+} oscillations. Ca^{2+} oscillations are almost completely inhibited by 2-APB at a concentration that dominantly blocks store-operated calcium entry (SOCE) [69]. Thus, further studies are needed to fully understand the complex regulation of Ca^{2+} signaling in airway SMCs.

6.2 *Role of IP_3Rs*

Enhanced IP_3 levels in airway SMCs lead to increased Ca^{2+} mobilization, airway hyperresponsiveness and remodeling, and, finally, asthma [70]. In spontaneously asthmatic rats, IP_3-5-phosphatase, which degrades IP3, is downregulated; therefore, the elevated $[IP_3]_i$ induces Ca^{2+} release, contributing to airway hyperresponsiveness [71]. IP_3Rs may also be involved in extracellular acidification-induced remodeling

of airway SMCs by increasing connective tissue growth factor production and extracellular matrix formation [70].

A recent study reported that knockdown of PIP5K1γ, which is the major source of PIP2 in airway SMCs, can prevent the development of asthma in mice. Instead, cell-permeable PIP2 increases airway contractility. These effects are mediated by integrin α9β1 and thus may lead to new treatment strategies for asthma [72].

Cytokines are known to be important factors in asthma, in which IL-13 plays a key role. It has been shown that IL-13 enhances LTD4-induced Ca^{2+} oscillation in human airway SMCs. This effect can be completely blocked by the specific IP_3R inhibitor xestospongin-C and the RyR inhibitor ryanodine or ruthenium red, which indicates that the role of IL-13 in the involvement of Ca^{2+} oscillation may be cooperatively modulated by IP_3Rs, RyRs, and, possibly, SOCE [69]. Incubation with IL-13 or IL-4 upregulates expression of RyRs, IP_3Rs, and SERCA2 and enhances ACh-induced Ca^{2+} transients in asthmatic airway SMCs. All of these effects could be reversed by inhibition of IP_3Rs [63].

Recently, Deshpande et al. reported that bitter taste receptors are expressed in human airway SMCs. Inhalation of bitter tastants decreases airway obstruction in a mouse model of asthma, which may be mediated via IP_3R-dependent localized Ca^{2+} release and activation of BK_{Ca} channels [73].

All of the investigations of the role of IP_3Rs in the regulation of intracellular Ca^{2+} homeostasis in airway SMCs show the great potential for providing new therapeutic options for the treatment of asthma.

6.3 Cyclic Adenosine Diphosphate Ribose (cADPR)/CD38

The second messenger cADPR was originally described in sea urchin eggs and can release Ca^{2+} from the SR via RyRs [74]. A similar mechanism has been reported in tracheal SMCs [75]. The levels of cADPR in airway SMCs are substantially increased by exposure to contractile agonists such as methacholine (mACh), bradykinin, endothelin, and histamine [76]. This response is blocked by RyR, but not IP_3R, inhibitors. cADPR may not only induce Ca^{2+} release [17] but also enhance the frequency of Ca^{2+} oscillation in airway SMCs [75]. 8-bromo-cADPR, an antagonist of cADPR, inhibits mACh-induced Ca^{2+} release. Furthermore, in the presence of 8-NH2-cADPR, another competitive antagonist of cADPR, the mACh-induced Ca^{2+} release in airway SMCs is blocked. In the presence of 8-bromo-cADPR, there is no further attenuation of Ca^{2+} response upon addition of ryanodine [77]. A similar effect is either mimicked or antagonized by tacrolimus (FK506), a compound that increases the activity of RyRs by dissociating its stabilizer FKBP12.6 [51]. In FKBP12.6 null mice, the ability of cADPR to induce Ca^{2+} release is lost. These results demonstrate the role of RyR2 in airway SMCs, providing a possible molecular basis for how cADPR activates RyRs and suggesting that FKBP12.6 may have a competing binding site for cADPR. It is possible that while IP_3Rs initiate Ca^{2+} oscillations in airway SMCs and determine the basal $[Ca^{2+}]_i$, cADPR and RyRs

may amplify or rectify Ca^{2+} homeostasis. However, the details of molecular mechanisms are incompletely elucidated. It should also be mentioned that cADPR can directly activate RyRs in coronary artery smooth muscle [78], and calmodulin can enhance cADPR-induced Ca^{2+} release in sea urchin eggs and pancreatic β-cells [79, 80].

CD38, an enzyme that converts nicotinamide adenine dinucleotide to cADPR, has been found on the membranes of airway SMCs [81]. In $CD38^{-/-}$ mice, the contractile responsiveness of airways to mACh and endothelin are lower than that in wildtype mice [82]. In addition, the Ca^{2+} response in $CD38^{-/-}$ airway SMCs is unaffected by 8-bromo-cADPR, indicating the exclusive role of CD38 in the cADPR-mediated Ca^{2+} signaling system [82]. Moreover, upregulation of CD38 has been associated with the stimulation of cytokines under inflammatory conditions, including asthma. For example, it has been shown that tumor necrosis factor-α (TNF-α), IL-1β, and IFN-γ all increase CD38 expression in cultured human airway SMCs [83]. Similarly, IL-13, which is a potent factor promoting airway remodeling, increases CD38 expression [84]. Further work by the same group has revealed that IL-13 fails to increase the response to mACh in $CD38^{-/-}$ mice [85]. However, although cADPR does appear to influence the initial peak of Ca^{2+} response, it is not clear how this transient response transforms into sustained contraction and remodeling of airway SMCs.

Apparently, various agonists can mobilize the release of Ca^{2+} from the SR through RyRs by increasing production of cADPR in airway SMCs. Supportably, the pro-proliferative effect of TNF-α on airway SMCs is inhibited by siRNA-mediated CD38 gene knockdown, while the enhanced SOCE induced by TNF-α is blunted by CD38 siRNAs and potentiated by CD38 overexpression [86]. These results indicate a critical role for CD38 in the TNF-α signaling pathway and associated SOCE. Further work has shown that TNF-α causes a greater induction of CD38 expression in asthmatic than in nonasthmatic human airway SMCs. This effect may stem from the reduced activation of JNK/MAPK and increased activation of ERK/p38 MAPKs [87] but is not associated with a change in NF-κB or AP-1 activation [87] or phosphatidylinositol 3 kinase (PI3K) [88].

7 Conclusion and Perspective

In this chapter, we focused on recent major progress in the studies of RyR and IP_3R Ca^{2+} signaling in airway SMCs. As illustrated in Fig. 1, RyRs exhibit spontaneous functional activity; as such, a cluster of approximately 10 RyRs is sufficient to generate Ca^{2+} sparks. These local Ca^{2+} release events may activate multiple plasmalemmal ion channels including Cl_{Ca}, TRPC, and BK_{Ca} channels. The activation of Cl_{Ca} and TRPC channels cause membrane depolarization, opening of LTCCs, and a further increase in $[Ca^{2+}]_i$. Extracellular Ca^{2+} influx through LTCCs may promote and increase the opening of RyRs, inducing Ca^{2+} release from the SR, also known as a CICR process. The increased $[Ca^{2+}]_i$ mediates contraction,

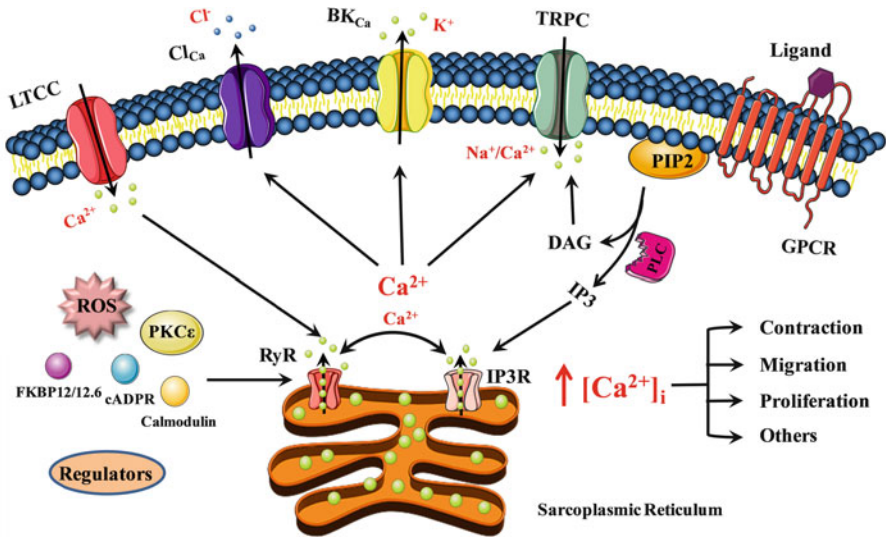

Fig. 1 *RyR and IP$_3$R Ca^{2+} signaling in airway SMCs*. Ryanodine receptors (RyRs) on the sarcoplasmic reticulum (SR) can generate spontaneous Ca^{2+} sparks in airway SMCs. These local Ca^{2+} release events activate multiple plasmalemmal ion channels including Ca^{2+}-activated Cl^- (Cl_{Ca}), big-conductance Ca^{2+}-activated K^+ (BK_{Ca}), and canonical transient receptor potential (TRPC) channels. The activation of Cl_{Ca} and TRPC channels causes membrane depolarization, opening of L-type voltage-gated Ca^{2+} channels (LTCCs), and extracellular Ca^{2+} influx, which promote the opening of RyRs and induce Ca^{2+} release from the SR, a process termed Ca^{2+}-induced Ca^{2+} release (CICR). The resulting increase in $[Ca^{2+}]_i$ mediates contraction, migration, proliferation, and other cellular responses. BK_{Ca} channels, if activated, lead to membrane hyperpolarization and closure of LTCCs. This provides a negative feedback mechanism to inhibit excessive Ca^{2+} signaling and associated cellular responses. RyRs can be colocalized with inositol 1,4,5-trisphosphate receptors (IP_3Rs). These two Ca^{2+} release channels may reciprocally activate each other to render a local CICR process. IP_3 is normally generated from phosphatidylinositol 4,5-bisphosphate (PIP_2) by phospholipase C (PLC), which is activated following stimulation of G protein-coupled receptors (GPCRs). In addition to IP_3, PLC also produces diacylglycerol (DAG) from PIP_2. DAG may directly activate TRPC channels. RyRs are also regulated by multiple intracellular signaling molecules such as FK506 binding protein 12.6 (FKBP12.6), cyclic adenosine diphosphate ribose (cADPR), calmodulin, protein kinase C-ε (PKC-ε), and reactive oxygen species in airway SMCs

migration, proliferation, and other cellular responses in airway SMCs. BK_{Ca} channels, if activated, lead to membrane hyperpolarization, closure of LTCCs, and a decrease in $[Ca^{2+}]_i$. This provides a negative feedback mechanism to inhibit excessive Ca^{2+} signaling and associated cellular responses.

RyRs can colocalize with IP_3Rs, by which these two colocalized Ca^{2+} release channels may reciprocally activate each other to render a local CICR process. IP_3 is normally generated from PIP_2 by PLC, which is activated following stimulation of GPCRs. In addition to IP_3, PLC also produces DAG by catalyzing PIP_2. DAG may directly activate TRPC channels, contributing to Ca^{2+} signaling in airway SMCs. RyRs are also regulated by multiple intracellular molecules such as FKBP12.6, cADPR, calmodulin, PKC-ε, and ROS in airway SMCs.

The Ca^{2+} signaling generated and regulated by RyRs and IP_3Rs is an important and sophisticated network in the initiation and maintenance of numerous physiological cellular responses in airway SMCs. On the one hand, overactivity of this Ca^{2+} signaling network has been shown to contribute to the development of asthma and, potentially, other respiratory diseases. On the other hand, the current understanding of the molecular genesis, regulatory mechanisms, and functional roles of RyRs and IP_3Rs still remain limited in airway SMCs. Indeed, significant advancements have been made in other types of cells, particularly in cardiac, skeletal, and even vascular SMCs. However, each organ or tissue has, to a greater or lesser extent, its own unique structural, functional, and molecular nature. One of the typical examples is that LTCC blockers are effective in the treatment of hypertension and other cardiovascular diseases but ineffective for asthma. Clearly, more efforts in the studies of these potentially important Ca^{2+} release channels are very necessary in the field.

It is also conceivable that the use of fluorescence resonance energy transfer, RNA interference, and other new techniques will allow us to fully elucidate the molecular mechanisms of both the RyR- and IP_3R-mediated Ca^{2+} signaling network in airway SMCs. Additionally, new transgenic animals and disease models will be valuable to future studies in this field. We believe that progress will generate novel and important findings, which might not only enhance our understanding of the functional importance of RyRs and IP_3Rs in airway SMCs but also significantly aid in the production of innovative and effective therapeutic targets for asthma, chronic obstructive pulmonary disease, and other respiratory diseases.

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