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Ca^{2+} handling and sensitivity in airway smooth muscle: emerging concepts for mechanistic understanding and therapeutic targeting

Review manuscript

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Abstract

Free calcium ions within the cytosol serve as a key secondary messenger system for a diverse range of cellular processes. Dysregulation of cytosolic Ca(2+) handling in airway smooth muscle (ASM) has been implicated in asthma, and it has been hypothesised that this leads, at least in part, to associated changes in both the architecture and function of the lung. Significant research is therefore directed towards furthering our understanding of the mechanisms which control ASM cytosolic calcium, in addition to those regulating the sensitivity of its downstream effector targets to calcium. Key aspects of the recent developments in this field were discussed at the 8th Young Investigators' Symposium on Smooth Muscle (2013, Groningen, The Netherlands), and are outlined in this review.

Keywords

Asthma, airway smooth muscle, calcium, sensitisation, WNT, contraction

Introduction

The concentration of free calcium ions within the cytosol ($[Ca^{2+}]_i$) of airway smooth muscle acts as a crucial secondary messenger for many cellular processes such as contraction, proliferation, gene transcription and secretion of signalling mediators [1]. There are two primary levels of control regarding Ca^{2+} signalling: the regulation of $[Ca^{2+}]_i$ and the sensitivity of "effector" proteins to changes in $[Ca^{2+}]_i$. Understanding the molecular mechanisms underlying these pathways is essential to gain insights into various respiratory diseases including asthma, chronic obstructive pulmonary disease (COPD) and cystic fibrosis (CF) [2], and is the focus of the current review.

The ability of diverse changes in $[Ca^{2+}]_i$ to mediate such a plethora of functions relies on tightly regulated spatial and temporal signalling patterns, achieved primarily through intracellular signalling microdomains and Ca^{2+} oscillations [1]. The latter are now believed to encode Ca^{2+} signals in airway smooth muscle (ASM) cells, and arise as periodic increases in $[Ca^{2+}]_i$ with a time course in seconds. This temporal variation in $[Ca^{2+}]_i$ may be important, because sustained elevations in $[Ca^{2+}]_i$ can be harmful to many cell types. There are numerous cellular mechanisms that potentially contribute to or modulate the generation of Ca^{2+} oscillations, including but not limited to store operated calcium entry (SOCE), receptor operated calcium entry (ROCE), the sarcoendoplasmic reticulum Ca^{2+} ATPase (SERCA), non-canonical WNT (wingless-integrase-1) signalling and kinases such as protein kinase C (PKC). In addition, various factors which modulate and are influenced by calcium signalling are outlined with regard to their recent developments and potential for therapeutic targeting.

Ca^{2+} oscillations in airway smooth muscle

Ca^{2+} oscillations in ASM cells can be generated endogenously and experimentally by stimulation with contractile agonists such as muscarinic, histamine, cysteinyl leukotrienes and purinergic agonists activating G-protein coupled receptors (GPCR) associated with Gαq, as well as membrane depolarising agents such as buffers containing high KCl concentration [3–5]. Activation of GPCR causes Gαq to dissociate from Gβγ, and bind to and activate membrane-bound phospholipase-Cβ (PLCβ) [6,7] (Fig. 1). PLCβ generates inositol 1,4,5-trisphosphate

(IP_3) and diacylglycerol (DAG) from the hydrolysis of phosphatidylinositol 4,5-bisphosphate [8–10]. IP_3 activates the IP_3 receptor (IP3R) expressed on the SR membrane, causing release of SR Ca^{2+} and initiating the process.

It has been suggested that ryanodine receptors (RyR) are crucial to the initiation but not maintenance of Ca^{2+} oscillations [11]. A proposed model is that at the start of the Ca^{2+} oscillation, when sarcoplasmic reticulum (SR) Ca^{2+} levels are sufficiently high, the open probability of RyR can be increased via calcium-induced calcium release (CICR) following activation of IP3R [5]; this leads to emptying of the SR store into the cytosol. The sarcoendoplasmic reticulum calcium ATPase (SERCA) then sequesters the Ca^{2+} back into the SR [12]. However, SERCA is unable to fully restore SR Ca^{2+} to basal levels during continual IP3R activation. This diminished SR Ca^{2+} concentration inhibits RyR opening but has less effect on the IP3R. Hence, momentarily after the initial Ca^{2+} oscillation, the periodic increases in $[Ca^{2+}]_i$ may be due entirely to Ca^{2+} flux through the IP3R. The importance of the RyR to the initiation of contractile agonist-induced Ca^{2+} oscillations has been suggested both experimentally and through mathematical modelling [5,11], although its role is still contentious and maybe agonist specific. Upon stimulation with histamine, ryanodine did not affect contraction or the propagation of Ca^{2+} oscillations however it did inhibit the slow oscillations induced by KCl [13]. Furthermore the RyR antagonist ruthenium red completely inhibited acetylcholine established oscillations in porcine tracheal smooth muscle cells [14]. Another proposal is that the endogenously produced nucleotide metabolite cyclic ADP ribose (cADPR), which is synthesised by CD38 and enhanced by inflammatory mediators, increases basal $[Ca^{2+}]_i$ and augments Ca^{2+} signals induced by muscarinic agonists [15,16]. Teasing out the relative contribution of both the IP3R and RyR experimentally is a complex matter due to their close interaction with each other, the non-specific effects of the pharmacological tools and the time-scale of events being measured.

IP3R contains Ca^{2+} binding sites which when bound to Ca^{2+} increase the IP3R open probability [17]. The result is an enhanced Ca^{2+} release and a further increase in $[Ca^{2+}]_i$ which initiates a propagating Ca^{2+} wave in the cell by stimulating the opening of adjacent IP3R via CICR. The IP3R also contains a second, lower affinity Ca^{2+} binding site, which reduces IP3R open probability

when $[Ca^{2+}]_i$ is sufficiently high [17,18]. This negative feedback and the diminished level of SR Ca^{2+} momentarily inactivates the IP3R, enabling SR Ca^{2+} levels to replenish via the actions SERCA, and $[Ca^{2+}]_i$ to decrease. However, as long as IP3 is continuously generated by activation of Gαq-coupled GPCRs, the IP3R will open once SR Ca^{2+} level replenishes and $[Ca^{2+}]_i$ decreases to a threshold which favours IP3R opening [19]. It is this Ca^{2+}-regulated opening and closing of the IP3R that gives rise to agonist-induced Ca^{2+} oscillations.

The replenishment of the SR by SERCA2 is important for the maintenance of Ca^{2+} oscillations as pharmacological inhibition of it diminishes them [20,21]. Phospholambin is the most studied regulator of SERCA2 activity and it has been shown to disrupt cardiac contractility by reducing its affinity with Ca^{2+} [22]. Human ASM appears to be unique in respect to SERCA2 regulation as phospholambin protein is not expressed and it has been suggested CaMKII may play a role [23]. If a unique regulatory mechanism of SERCA2b, the highest expressed isoform in ASM, is described it would putatively provide a new therapeutic target. Although SERCA actively pumps Ca^{2+} back into the SR during Ca^{2+} oscillations, some of the Ca^{2+} released into the cytosol is extruded out of the cells via the sodium–calcium exchanger (NCX) and plasma membrane calcium ATPase pump (PMCA). Hence, for Ca^{2+} oscillations to persist, Ca^{2+} in the SR must be replenished from extracellular sources. In addition to Ca^{2+} entry through DAG-activated receptor operated mechanisms (ROCE) [24,25], entry via store-operated Ca^{2+} channels (SOCE, see below) [26,27] replenishes the Ca^{2+} stores when SR Ca^{2+} levels decrease below a certain threshold, thus sustaining airway contraction during prolonged contractile agonist stimulation [4,27]. Very little is currently known about the role of the PMCA in ASM tissue and given its potential to shape calcium dynamics this must be addressed within the context of health and disease.

The frequency of Ca^{2+} oscillations is positively correlated with the strength of airway ASM cell contraction in mice [4,28,29], rat [30], and human airways [13]. The elevation of $[Ca^{2+}]_i$ that results from Ca^{2+} oscillations stimulate the myosin light chain kinase (MLCK) to phosphorylate the regulatory myosin light chain (rMLC) which is necessary for myosin-actin cross-bridge cycling and smooth muscle contraction [31]. The contractile machinery is the main target for Ca^{2+}

sensitisation pathways and is discussed later.

Store-operated Ca^{2+} entry in airway smooth muscle cells

The finite capacity of the SR necessitates the recruitment of Ca^{2+} from the extracellular environment in order to sustain the Ca^{2+} signals required to generate prolonged contraction of ASM cells. The gating of Ca^{2+} across the plasma membrane in response to the emptying of the SR was first proposed in 1986 by James Putney Jr. [32] and is now widely termed store-operated Ca^{2+} entry (SOCE). However, the molecular mechanism for sensing store depletion and transducing this into a Ca^{2+} influx remained elusive for almost 20 years after Putney's proposal. Finally, in 2005, two RNAi screens identified STIM (stromal-interacting molecule) proteins as the SR-localised Ca^{2+}-sensors [33,34]. A year later, the Orai family of proteins was identified, again from genome-wide RNAi screens, as the pore-forming subunits constituting the SOCE channels [12,35,36]. Fig. 2 illustrates the basic proposed mechanism underlying SOCE.

In ASM cells STIM1 [37] and Orai1 [38,39] appear to be the critical components of the SOCE response. Using siRNA-mediated knockdown of STIM1 and STIM2, Peel et al., demonstrated that STIM1 (but not STIM2) was involved in SOCE resulting from cyclopiazonic acid (CPA)-mediated store depletion. Although SOCE induced by histamine-mediated SR depletion was similarly dependent upon STIM1, SOCE following bradykinin treatment was much less STIM1-dependent [37], suggesting that multiple SOCE pathways may exist in ASM. The role of Orai1 in ASM SOCE has been convincingly demonstrated by siRNA-mediated depletion in two independent studies [38,39]. Peel et al., also demonstrated that while Orai2 had no role in SOCE, Orai3 depletion resulted in an attenuated store release and subsequent Ca^{2+} entry following CPA treatment, suggesting that Orai3 may be involved in regulating store release and/or basal Ca^{2+} levels [38].

Prior to the discovery of the Orai family, there was much interest in the potential for the canonical transient receptor potential (TRPC) family of ion channels to mediate SOCE [40]. Considerable evidence has accrued that they can interact with STIM and Orai proteins [41], perhaps generating multiple SOCE pathways all utilising STIM1 as a sensor of store depletion. This may be worthy of further investigation in ASM cells, where two distinct components of

store-operated current (one consistent with a classical Orai-dependent ICRAC current and another "ISOC-like" current, with properties more in line with a TRPC-mediated current) have been identified [38].

Functionally, SOCE has been implicated in ASM contraction [42] through the maintenance of Ca^{2+} oscillations [43] and in regulating migration and proliferation of ASM cells. SOCE is elevated in proliferating ASM cells, relative to quiescent cells [42,44] and Orai1 expression is also increased in proliferating cells [44]. PDGF and serum-mediated ASM cell migration has been shown to be dependent upon STIM1 and Orai1 expression, using siRNA approaches [45,46]. SOCE is also likely to be involved in the recent links drawn between sustained contraction inducing remodelling via TGF-β release from the extracellular matrix (ECM) [47,48]. Given the potential role of proliferation and migration in airway remodelling, these findings suggest that SOCE could contribute to the structural changes occurring in inflammatory, obstructive airway disease.

In support of this notion, a number of studies have found that SOCE (or the molecular components that constitute it) is altered under inflammatory conditions. Inflammatory mediators including TNFα, IL-13 and TGFβ have been reported to elevate SOCE and/or STIM1/Orai1 expression in ASM cells [39,49–53]. In many cases, increased SOCE appears to reflect over-expression of STIM1/Orai1, but Jia et al., found that treatment of ASM cells with either TNFα or IL-13 enhanced STIM1 aggregation, suggesting that STIM1/Orai1 function may also be regulated under inflammatory conditions. Furthermore, in ovalbumin-challenged mice, both Orai1 and STIM1 protein levels were substantially elevated in ASM cells [45], indicating that SOCE might contribute to the development of asthma. Indeed, experimental studies in animal models of allergen-induced asthma have provided evidence that administration of SOCE blockers such as YM-58483 [54] and 3-fluoropyridine-4-carboxylic acid, FPCA [55] can have anti-inflammatory and bronchodilatory effects. Although some of these effects are likely to be mediated through inhibition of SOCE in non-ASM cells (e.g. immune cells, epithelial cells), bronchodilation is likely, at least in part, to be due to inhibition of Ca^{2+} entry into ASM cells. Both experimental [43] and mathematical modelling [27] approaches indicate that SOCE influences the frequency of Ca^{2+} oscillations in ASM cells, which in turn is responsi-

ble for setting the level of smooth muscle tone [19].

Receptor operated calcium entry

Receptor operated calcium entry (ROCE) also plays a role in maintaining Ca^{2+} oscillations and force generation in ASM cells [56,57]. Ca^{2+} flow mediated through receptor-operated calcium channels (ROCC) from extracellular spaces is not dependent on the Ca^{2+} status of internal stores. Activation of ROCCs occur either via extracellular ligand binding or indirectly via secondary messengers such as DAG [57] as shown in Fig. 2. Furthermore, ROCCs are insensitive to L-type voltage-gated calcium channel (VOCC) blockers [57].

Prominent members in this category include the TRP channels, a family of 28 proteins that function as homo or heteromeric non-selective Ca^{2+} permeable cation channels [58,59] and are present in ASM [59–63]. DAG stimulates Ca^{2+} entry through TRPC3/6/7 [64,65] possibly by increasing the open probability of the channel [66], however the exact interaction is yet to be described. Increased protein expression of TRPC3 following TNF-α treatment in human ASM cells is thought to be accompanied by a switch from ROCE to SOCE [53]. Furthermore, blocking TRPC3 with antibodies prevented membrane depolarisation and methacholine-induced cytosolic Ca^{2+} flux in ASM cells [66]. That is not to say ROCE doesn't play a role in inflammation as $TRPC6^{-/-}$ mice, in an allergic model of asthma, displayed reduced inflammation but enhanced tracheal contractility [67]. Discerning the individual roles of TRPC3 and TRPC6 in ROCE and SOCE is very challenging due to the lack of pharmacological tools and their complementary compensation in knockout models. Future studies using airway tissues are essential to further understand TRPCs role in normal and diseased airways.

In the last decade, numerous novel non-TRPC and non-SOCE Ca^{2+} entry mechanisms have been proposed in ASM cells. A few notable examples include arachidonic-acid regulated (ARC) channels [68,69] and ATP-gated P2X receptors [70,71]. ARC channels are Ca^{2+} selective ion channels specifically stimulated by arachidonic acid, a secondary messenger released due to phospholipase A2 activation during low-agonist binding of GPCRs [68,69]. While ARC channel expression, distribution and function in ASM cells is still debatable, these receptors in unrelated cell types were shown to

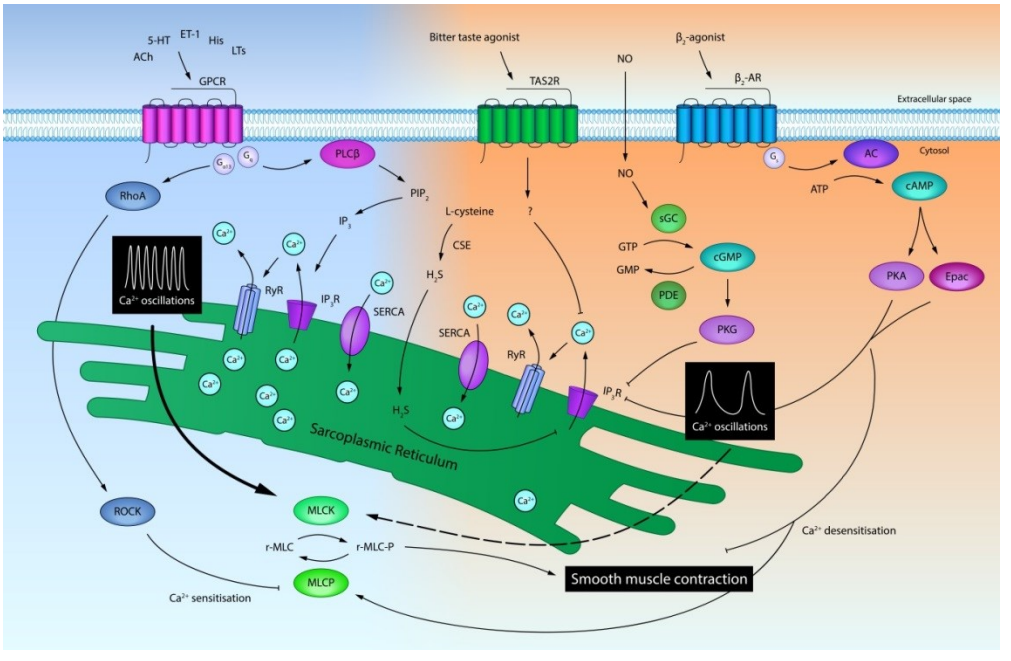

Figure 1. Illustration of our current view of the main intracellular pathways that regulate contraction. These are thought to be of primary importance in small intrapulmonary airways and the proposed mechanisms are supported by recent experimental evidence obtained by examining mouse and/or human lung slices with customised imaging techniques.

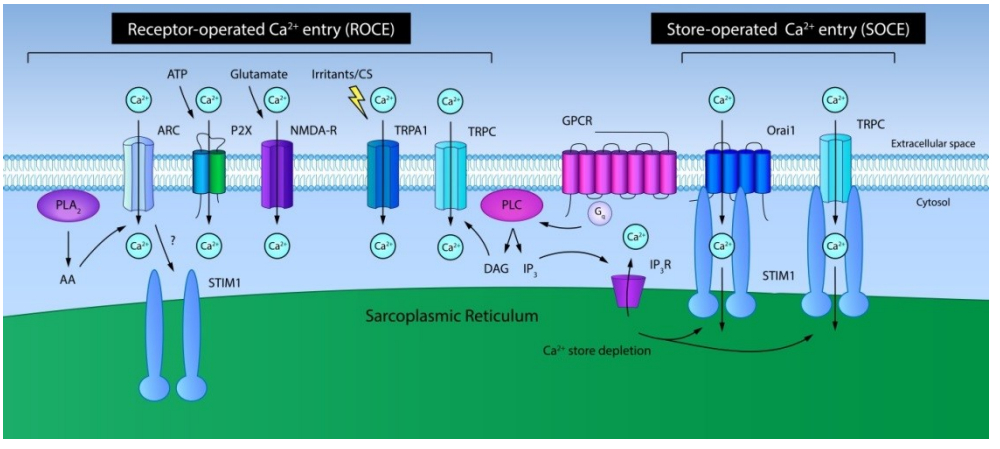

Figure 2. Activation of G protein-coupled receptors (GPCRs) by bronchoconstrictors initiates an intracellular signalling cascade generating DAG and a burst of IP3 leading to the release of Ca^{2+} from the SR. Depletion of the SR Ca^{2+} store leads to oligomerisation of STIM1 molecules and translocation into regions of the SR in close proximity to the plasma membrane. There, they can engage with Orai1 or TRPC channels, leading to an influx of Ca^{2+}, termed store-operated Ca^{2+} entry (SOCE). DAG generated by GPCR activation, along with PIP2 binds to TRPC channels and induces Ca^{2+} influx from extracellular spaces, termed receptor-operated calcium entry (ROCE). Further ROCE mechanisms involve TRPA1, P2X and NMDA-R. G protein stimulation also causes release of arachidonic acid (AA) which activates arachidonic acid regulated channels (ARC). ARC is suggested to regulate STIM1 in a store-independent manner. However, all the underlying mechanisms are still under investigation.

mobilise Ca^{2+} entry via STIM1, independently of intracellular stores [68,69,72]. While these observations propose the dual role of STIM1 as a SOCE and ROCE regulator, depending on the localisation [73,74], it still needs to be verified.

Extracellular ATP (adenosine triphosphate) can mobilise intracellular Ca^{2+} in ASM via activating a family of ligand-gated purinergic ion channels, the P2X1–4 receptors [70,71,75]. ATP acting via these receptors potentiated methacholine-induced hyperresponsiveness through Rho kinase dependent Ca^{2+} sensitisation [70]. Furthermore, P2X receptors have been reported to activate the reverse mode of NCX [75,76] in a STIM1 dependent manner, thus putatively linking them to SOCE and the maintenance of Ca^{2+} oscillations [21,77]. Therefore ATP is potentially involved not just in contraction but in other $[Ca^{2+}]_i$ dependent pathophysiological processes as well [1].

The N-methyl-D-aspartate receptors (NMDA-R) and transient receptor potential ankyrin 1 (TRPA1) have been extensively studied in the neural system and are now thought to hold importance in ASM [78–80]. Using experimental animal models for allergic inflammation, the NMDA-R has been shown to be involved in airway hyperreactivity [81,82] and recently TRPA1 in non-neurogenic inflammation [80]. Recent research into TRPA1 and NMDA-Rs provide evidence of a unique cross-talk between neuronal and respiratory systems, which further adds to the diversity of ROCE mechanisms.

WNT signalling

The WNT (wingless-integrase-1) signalling pathway constitutes a family of secreted glycoproteins that are heavily modified before entering the extracellular space and typically involved in embryonic development and tissue homeostasis [83–85]. WNT ligands can activate the β-catenin dependent (canonical) pathway via the Frizzled receptor family in concerted action with the low-density-lipoprotein-related protein 5/6. Activation of this pathway regulates cellular differentiation, proliferation and self-renewal processes [86]. Alternatively, WNT ligands may activate β-catenin independent (non-canonical) pathways by either binding to Frizzled receptors alone or by interacting with the non-Frizzled tyrosine kinase receptors Ror2 [87,88], Ryk [89–92] and PTK7 [93]. Non-canonical signalling embodies at least two signalling branches that in some regards share certain characteris-

tics as shown in Fig. 3. The Planar Cell Polarity (PCP) pathway [94], defined by regulating directed migration and organised polarisation by shaping the actin cytoskeleton. Here, WNTs signal through Frizzled receptors and Dishevelled (DVL) via small GTPases and Jun-N terminal kinase (JNK) to regulate cell shape and polarity. The second non-canonical signalling branch is the WNT-Ca^{2+} pathway, named after the finding that some WNT ligands induce endoplasmic reticulum-mediated Ca^{2+} release [95,96]. Interestingly, all of the described non-canonical pathways include Ca^{2+} as a second messenger [97,98], where Ca^{2+} is either rapidly induced upon WNT stimulation, or directs a more delayed response [95].

The involvement of non-canonical WNT ligands with Ca^{2+} was first observed in Zebrafish embryos. Ectopic expression of WNT-5A enhanced the frequency of IP3-mediated cytosolic Ca^{2+} oscillations, whereas WNT-8, a canonical WNT, did not [99,100]. It was later shown that WNT-5A activates Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) and protein kinase C (PKC, discussed later) in Xenopus embryos [101,102]. Subsequent studies revealed that a downstream target is the pro-inflammatory Ca^{2+} dependent transcription factor Nuclear factor of activated T-cells (NFAT) [103]. Enhanced free cytosolic Ca^{2+} induced by WNT-5A directs NFAT from the cytosol to the nucleus, activating gene transcription and suppressing canonical signalling [104].

The available evidence showing direct involvement of the WNT-Ca^{2+} in smooth muscle is limited, although many studies have been conducted that suggest that pathways are relevant in this cell type. NFATc1 is differentially expressed in vascular smooth muscle in response to various stimuli [105,106] such as driving migratory processes [107] or in disease [108]. Interestingly, overexpression of a constitutively active GSK3β, a canonical mediator, significantly delayed the nuclear translocation of NFATc1 [107]. Another line of research has shown that CaMKII is actively expressed in vascular smooth muscle [109] and is an essential component in driving vascular smooth muscle migration [110]. A recent publication demonstrated the active involvement of the WNT-Ca^{2+} pathway in an ASM cell line; treatment with WNT-5A-conditioned medium induced rapid nuclear translocation of NFATc1 and a Ca^{2+} dependent increase in fibronectin and collagen I expression [111]. The authors attributed a role for TGF-β in regulating this process, as TGF-β

strongly increased the expression of WNT-5A mRNA. Furthermore, in human pulmonary fibroblasts SERCA2 and RyR inhibition can effectively block TGF-β-induced fibronectin and collagen I mRNA expression [112]. The findings in these studies may point towards a general mechanism by which TGF-β regulates ECM production through activation of the WNT-Ca^{2+} pathway. Considering the aforementioned, it may well be speculated that non-canonical WNTs such as WNT-5A regulate ECM dynamics by enhancing free cytosolic Ca^{2+} and subsequently activating Ca^{2+} dependent processes.

While non-canonical WNTs such as WNT-5A may regulate the production of ECM proteins in ASM through elevation of cytosolic Ca^{2+}, an opposite relation has also been described. It was shown that fibronectin binding to α4β1-integrin activates the WNT-PCP pathway in vascular smooth muscle to enhance cellular migration through RhoA and Rac1 in a Dishevelled (DVL) dependent matter [113]. While the role of Ca^{2+} in the WNT-PCP pathway is less definitive, Ca^{2+} may very well play a role in this process, as small GTPases have been argued to function in a Ca^{2+}-dependent manner. For example, WNT-5A inhibits convergent/extension movements by decreasing the Ca^{2+}-dependent cell adhesiveness of Xenopus blastomeres [114] which could be overcome by overexpression of a dominant-negative small GTPase Cdc42. Furthermore, treatment with the dominant-negative Cdc42 could rescue the inhibitory effects of PKC-α on convergent/extension movements [115]. The authors hypothesised that Cdc42 normally functions downstream of WNT-Ca^{2+}-mediated PKC signalling. A recent paper provided evidence for the role of Ca^{2+} in the WNT-PCP pathway. The authors showed that WNT-5A regulates directional movement in migrating cells (via the WNT-PCP pathway) by recruiting and redistributing proteins into an intracellular polarised structure termed the WNT-5A-mediated receptor-actin-myosin polarity structure (WRAMP) [116]. Furthermore, membrane retraction directed by WNT-5A mediated WRAMP assembly was dependent on elevating free Ca^{2+} levels at the trailing edge of the cell [117]. The authors hypothesised that during membrane retraction, assembly of the WRAMP structure recruits the ER, which triggers Ca^{2+} release activating localised Ca^{2+} dependent enzymes, allowing substrate detachment and actomyosin contraction. WNT-5A treatment increased the abundance of calpain-2 and phosphorylated myosin light chain in the proximi-

ty of WRAMP. Although the findings from this study were performed in WM239A melanoma cell lines, they reported similar WRAMP structures in the mouse myoblast C2C12 cell line [117]. It is therefore plausible that the described mechanisms are of relevance in smooth muscle biology.

Contraction

Ca^{2+} drives contraction through calmodulin-dependent activation of myosin light chain kinase (MLCK) to phosphorylate the regulatory subunit of myosin light chain (MLC20) and initiate cross-bridge cycling (Fig. 1) [118,119]. Actin polymerisation is also a key aspect, as if it is blocked with latrunculin or actin decay peptides there is a profound suppression of smooth muscle contraction [120–122]. Using an allergic asthma model in rats it was found that force generation increases despite a reduction in smooth muscle myosin heavy chain (smMHC) [123]. The counter-intuitive result of this study was later explained by showing elevated F-actin polymerisation [124]. Such a change in contractile composition may relate, in part, to the AHR observed in asthma.

Cytoskeleton

Ca^{2+} regulation of actin filament cross-linking is essential for normal function and dynamics of the actin cytoskeleton, and is important for cell structure and growth [125]. By associating with membrane adhesion complexes the actin/cytoskeleton network can respond to mechanical or contractile stimuli and modulate cell stiffness through integrin interactions with the ECM, determining airway compliance and responsiveness [126]. Focal adhesion kinase (FAK), a 125 kDa non-receptor tyrosine kinase forming part of the adhesion complex [127], has also been implicated in regulating cell migration through the regulation of the Rho family proteins [128]. FAK phosphorylates one or more Rho GEFs and may therefore increase sensitivity to Ca^{2+} by activating the RhoA/Rho kinase pathway [129]. Recently, it has been shown that FAK may also facilitate cycles of RhoA inactivation and activation through its association with p190RhoGEF and p190RhoGAP [130]. The paper demonstrated that p190RhoGEF over-expression enhances RhoA activation and focal adhesion formation. Further to this, $FAK^{-/-}$ fibroblasts demonstrate reduced p190RhoGEF and cessation of dysregulated RhoA activity [130]. Conversely, over-expression of p190RhoGAP is associated with decreased RhoA

activity and cell motility demonstrating that FAK expression and activity are required for the recruitment of p190RhoGAP to focal adhesions to promote the RhoA inhibition that is required for directional migration [131].

A new paradigm to treatment of obstructive airway diseases such as asthma has been proposed based on the findings that at short ASM lengths there is an increase in cytoskeletal recruitment dissociated from cross-bridge cycling leading to bronchodilator insensitive airway distensibility [132]. Therefore, targeting the signalling molecules that are involved in cytoskeletal recruitment, such as PIP2, Rho, Ca^{2+} and adhesion junction proteins may provide a novel approach to the treatment of obstructive airway diseases.

Calcium sensitisation and RhoA/Rho kinase

As previously described, $[Ca^{2+}]_i$ is the key driving force behind smooth muscle contraction. The degree of force and threshold for contraction can however be altered in a Ca^{2+} independent manner termed "Ca^{2+} sensitisation". RhoA, a monomeric GTPase and its downstream effector Rho-Kinase increase myosin light chain (MLC) phosphorylation through inhibition of myosin light chain phosphatase (MLCP) leading to enhanced contraction for a given $[Ca^{2+}]_i$ [133–136]. MLCP activity is regulated by the phosphorylation status of the myosin binding subunit MYPT-1. Two key sites, thr 696 and 853 are phosphorylated by Rho-kinase leading to inhibition of activity [137,138]. In addition to mediating Ca^{2+} sensitisation, RhoA catalyses the assembly of adhesome complexes, inducing actin polymerisation in an essential step during airway smooth muscle contraction [139]. Indeed, it has been reported that inhibition of RhoA activation robustly suppresses tension development with little effect on MLC phosphorylation [139].

Tyrosine phosphorylation

The involvement of protein tyrosine phosphorylation in smooth muscle contraction and Ca^{2+} sensitisation has been recognised for a while, though not the specific members involved [140–142]. With the development of specific tyrosine kinases inhibitors, it has now emerged that non-receptor tyrosine kinases (NRTKs) are important regulators of RhoA and Ca^{2+} sensitisation. NRTKs include the Src family kinases (Src-FK), which are the largest subfamily of NRTKs, and FAK. Several groups have implicated a role for specific NRTKs in Ca^{2+} sensitisation in

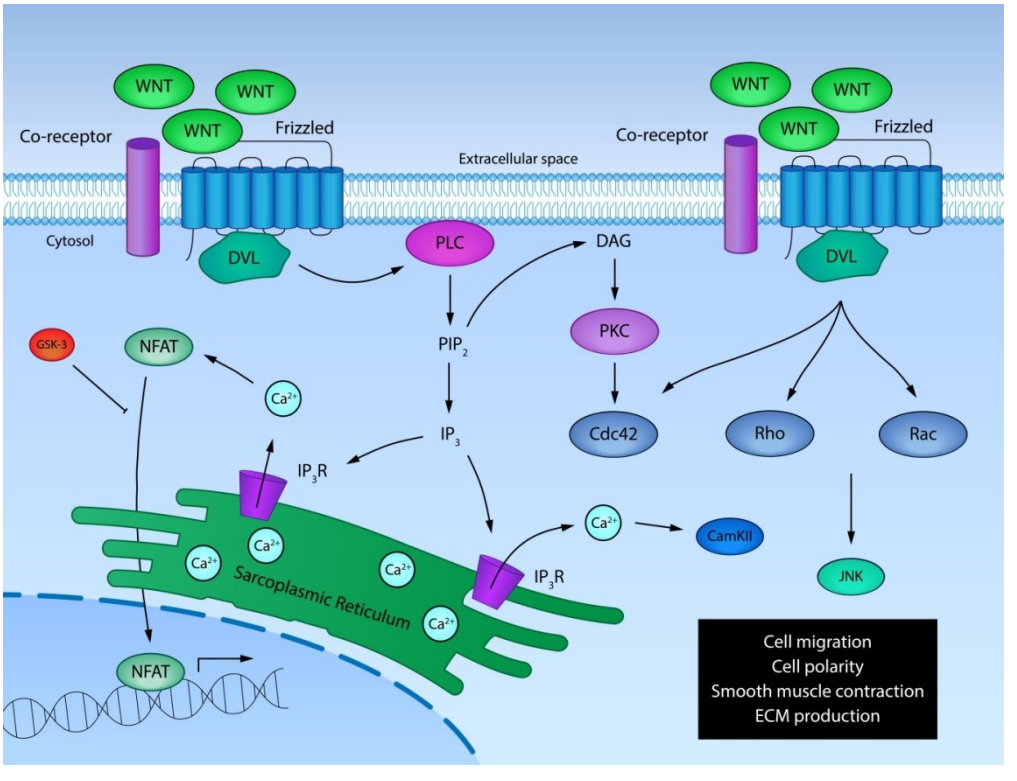

Figure 3. A simplified scheme of the β-catenin independent (non-canonical) signalling pathway, as currently evidenced in ASM. The WNT-Ca^{2+} pathway on the left-hand side acts through Ca^{2+} released from the SR via the IP3R which activates calmodulin-dependent kinase II (CaMKII) and protein kinase C (PKC), finally resulting in activation of nuclear factor of activated T cells (NFAT). The Planar cell polarity (PCP) pathway on the right-hand side triggers activation of small GTPases, leading to actin polymerisation and microtubule stabilisation. Although limited at this point, there is evidence of an overlap between both systems, suggesting a role for Ca^{2+} in the PCP pathway as well.

vascular smooth muscle, in particular Src Family Kinases (Src-FK) [143,144]. FAK has also been implicated in having an upstream role in the activation of RhoA. FAK tyrosine phosphorylates one or more Rho GEFs and may contribute to the increased Ca^{2+} sensitivity of myosin, by the activation of RhoA and its downstream effector, Rho-kinase [145].

Src-FK

Src-family kinases (Src-FK) form a group of closely related NRTKs including Src, yes and fyn [146], that are expressed in rat pulmonary arterial smooth muscle cells (PASMC) [143] and human ASM [147]. Inhibition of Src-FK has been shown to abolish sphingosylphosphorylcholine (SPC) and PGF2α induced Ca^{2+} sensitisation in vascular smooth muscle by abolishing Rho-kinase translocation from the cytoplasm [143,144]. Knock et al., further demonstrated that MYPT-1 is phosphorylated at the aforementioned key sites and MLC20 at ser-19 in a Src-FK dependent manner following PGF2α stimulation, without affecting basal phosphorylation [143]. Basal phosphorylation of MYPT-1 and MLC20 were however reduced by Rho-kinase inhibition, suggesting Src-FK involvement only during GPCR stimulation. Furthermore, when Rho-kinase and Src-FK were both inhibited, there was no additive effect on either PGF2α-induced MYPT-1 and MLC20 phosphorylation, or PGF2α-induced contraction suggesting that Rho-kinase and Src-FK may act on the same pathway.

Both Src-FK and FAK play a role in signal transduction pathways downstream of GPCR activation and it is possible that Src-FK and FAK provide the signalling link between G-protein receptor activation and RhoA activation. Therefore, targeting NRTKs such as Src-FK and FAK which have been shown to activate RhoA and Ca^{2+} sensitisation pathways, could present a new and promising therapeutic intervention for the treatment of diseases characterised by abnormal smooth muscle contraction.

The role of PKC in the regulation of airway smooth muscle contraction

An important upstream signal transduction molecule that regulates smooth muscle contraction is the protein kinase C (PKC) family of isoenzymes. These serine–threonine kinases can be activated by DAG, increases in $[Ca^{2+}]_i$ (conventional and novel PKCs) or neither (atypical PKCs). Since most agonists that stimulate airway contraction activate PLC-β to synthesise DAG

and IP3 to increase $[Ca^{2+}]_i$ (see Fig. 1) the activation of PKC is thought to play an important role in the regulation of airway tone, as demonstrated in murine model of asthma [148].

Ca^{2+} signalling activated by PKC in ASM

The role of PKC activation on Ca^{2+} signalling in small ASM cells was studied in precision cut lung slices (PCLS) using confocal microscopy [149]. PKC activation with phorbol esters induced repetitive Ca^{2+} oscillations that persisted in the continuous presence of the stimuli. The frequency of the oscillations was 0.5–2.5 cycles/min, significantly less than the frequency induced by most contractile agonists (15–30 cycles/min). These Ca^{2+} oscillations, as with those induced by isotonic KCl (50 mM) were blocked by inhibition of SERCA or by the inhibition of RyR, indicating that they were mediated by Ca^{2+} release from intracellular stores through RyRs [4,150]. In contrast, the high-frequency Ca^{2+} oscillations induced by contractile agonists are IP3 dependent as previously described [150]. Thus, all PKC, KCl, and agonist-stimulated Ca^{2+} oscillations are mediated, in part, by Ca^{2+} release from the SR. PKC-activated Ca^{2+} oscillations, like those induced by KCl, were inhibited by removal of extracellular Ca^{2+} or by L-type voltage-gated Ca^{2+} channel (LVGC) blockers such as nifedipine suggesting that activation of PKC results in an initial increase in Ca^{2+} influx through LVGCs.

Relationship between PKC-activated Ca^{2+} oscillations and ASM cell contraction

Simultaneous measurements of Ca^{2+} signalling and ASM cell contraction in PCLS showed that the low-frequency Ca^{2+} oscillations stimulated by PKC were accompanied by concomitant ASM cell twitching in small airways [149]. This temporal association between low-frequency Ca^{2+} oscillations and ASM twitching is similarly observed during the stimulation of PCLS with KCl [4]. In both cases, ASM twitching resulted in a very low reduction in airway lumen area. In contrast, high frequency Ca^{2+} oscillations induced by contractile agonists including ACh (or MCh), 5-HT, histamine, cysteinyl leukotrienes and endothelin-1 (ET-1) are accompanied by concentration-dependent sustained ASM cell contraction that results in a strong reduction in airway lumen [4,13,28]. This close association between Ca^{2+} oscillations and contraction support the hypothesis that small-airway contraction is regulated, at least in part, by the frequency of Ca^{2+} oscillations in ASM cells. Similarly, the concomitant

reduction in Ca^{2+} oscillation frequency and increase in airway lumen (relaxation) during exposure of PCLS to bronchodilators including β_2AR agonists, NO, bitter-taste receptor agonists, and H2S (see below) further support the regulation of ASM cell contraction by Ca^{2+} oscillation frequency.

PKC activation induces strong Ca^{2+} sensitisation

Experiments with Ca^{2+}-permeabilised PCLS from mice suggest that small airways have a low intrinsic (basal) Ca^{2+} sensitivity since they remain relaxed under conditions in which there is a sustained increase in ASM cell $[Ca^{2+}]_i$ [28,30,151]. The addition of agonists to these Ca^{2+}-permeabilised lung slices elicits a strong airway contraction, indicating that they induce Ca^{2+} sensitisation. Similarly, activation of PKC with phorbol esters induces a strong airway contraction in Ca^{2+}-permeabilised lung slices [149], in contrast to the small decrease in lumen elicited in non-permeabilised lung slices. Thus, to induce a strong sustained contraction, a stimulus must substantially increase both the $[Ca^{2+}]_i$ and the Ca^{2+} sensitivity [68] which isn't achieved by the low frequency oscillations induced by phorbol esters. Similarly, KCl induces low-frequency Ca^{2+} oscillations, weak Ca^{2+} sensitisation and, as a result, ASM twitching and weak sustained contraction in mouse small airways. Evidence for Ca^{2+} sensitisation by PKC activation with phorbol esters was also obtained in non-permeabilised (normal) airways. Thus, pre-exposure of mouse bronchial rings or lung slices to phorbol esters increases force development in response to KCl [149,152]. All the experimental evidence indicates that PKC activation in ASM cells induces Ca^{2+} sensitisation.

Whether contractile agonists, or rather, other stimuli such as inflammatory molecules activate the PKC pathway in ASM cells to increase contraction is still uncertain. An interesting molecule that may activate PKC in airway ASM cells is thrombin. This molecule is a well-known central protease in the coagulation cascade, however thrombin levels have been found increased in the small airways and the alveolar space of asthma patients compared to those of controls [153,154]. In addition, thrombin can cause airway hyperresponsiveness in experimental asthma [155] and is suggested to be involved in its pathogenesis [156]. Interestingly, thrombin induces Ca^{2+} sensitisation of the contraction mediated by stress fibres in retinal pigment epithelial cells by

activating PKC and thereby promoting phosphorylation of the PKC-potentiated PP1 inhibitory protein of 17 kDa (CPI-17) and MLC20 [157]. In small airways thrombin was found to induce a weak airway contraction in non-permeabilised lung slices [149] and a strong, PKC dependent contraction in Ca^{2+} permeabilised slices, suggesting induction of sensitisation. Furthermore, thrombin was found to markedly potentiate the KCl-induced airway contraction [149]. Thus, activation of PKC in ASM cells in airways exposed to increase levels of thrombin could sensitise them to contractile agonists such that these agonists cause stronger contraction.

The mechanism of Ca^{2+} sensitisation

In many experimental models, Ca^{2+} sensitisation in smooth muscle is initiated by activation of PKC and Rho-kinase [129]. The phosphoprotein CPI-17, expressed in smooth muscle, is a target for PKC that, when phosphorylated, binds to the PP1 subunit of MLCP to cause its inhibition, thereby causing Ca^{2+} sensitisation, previously reviewed [158]. In the small airways, PKC activation induces ~60% phosphorylation of CPI-17 in tissues with "normal" or "Ca^{2+}-permeabilised" ASM cells [149] suggesting it is a mediator of PMA-induced Ca^{2+} sensitisation in small airways [149]. Finally, PKC activation was demonstrated to cause a reversible increase in MLC20 phosphorylation in small airways [149], as expected if phosphorylated CPI-17 inhibits MLCP. Thus, the data in small airways support a mechanism in which CPI-17 relays the signal from PKC in the intracellular cascade that increases MLC20 phosphorylation and Ca^{2+} sensitisation in ASM cells.

Pharmacological targeting and modulators of Ca^{2+} oscillations and sensitisation

Most, if not all, bronchodilators studied reduce $[Ca^{2+}]_i$ in the ASM cells of small as well as large airways exposed to contractile agonists [13,159–166]. Furthermore, simultaneous measurements of airway contraction and Ca^{2+} signalling in ASM cells in PCLS has shown that inhibition of agonist-induced intracellular Ca^{2+} oscillations is quickly followed by airway relaxation, during exposure to bronchodilators [161–167]. Because agonist-induced Ca^{2+} oscillations are initiated and maintained by cyclic Ca^{2+} release through IP3R (and perhaps RyR), it is not surprising that many bronchodilators inhibit Ca^{2+} oscillations by inhibiting IP3R activity (Fig. 1). However, not all bronchodilators inhibit the IP3R activity by the same

mechanisms. Below we describe the experimental evidence for each bronchodilator studied that suggest that inhibition of Ca^{2+} release through IP3R and Ca^{2+} oscillations is a common cellular mechanism for bronchodilation and that the IP3R in the airway smooth muscle is a common target for most bronchodilators.

Beta-2 adrenergic receptor (β_2AR)

Beta-2 adrenergic receptor (β_2AR) agonists exert their effects via dissociation of the Gαs subunit from the receptor which activates adenylyl cyclase (AC) to generate cAMP. The increase in cAMP can activate the canonical effector molecule cAMP-dependent protein kinase A (PKA) and/or the exchange protein directly activated by cAMP (Epac) [168]. The downstream effects are regulation of Ca^{2+} signalling, Ca^{2+} sensitivity, and rMLC phosphorylation in airway ASM cells to induce bronchodilation (Fig. 1). There have been many emerging concepts regarding β_2AR pharmacology which is of great interest to the respiratory field, such as using full agonists comprised of a single enantiomer to off-set loss of desensitisation through an increased spare receptor reserve [169]. Or using an inverse agonist to suppress inflammation and persistent AHR while maintaining the use of agonists as rescue therapy taking advantage of the "sensitising" effect of the inverse agonist [170]. Such revelations are still clinically unproven but could improve the clinical outcome for many patients.

The β_2AR agonists formoterol and salbutamol decrease the frequency of contractile agonist induced Ca^{2+} oscillations in a concentration-dependent fashion [13,160,163]. cAMP partially inhibits Ca^{2+} entry through plasma membrane Ca^{2+} channels but not Ca^{2+} release stimulated by ryanodine receptor activation [160]. The effect of cAMP was further explored in the small airways of mice using the PCLS technique with a variety of agonists [161,163]. Relaxation of pre-contracted airways and a simultaneous reduction in oscillation frequency was demonstrated. The authors proposed that β_2AR agonists and cAMP elevating agents inhibit Ca^{2+} oscillations by reducing the IP3R sensitivity to IP_3, as release of caged IP_3 partially reversed the effect (Fig. 1). There is still a lack of consensus on the role of the primary downstream effectors of cAMP signalling, PKA and Epac (Fig. 1) on cAMP-induced inhibition of Ca^{2+} signalling and ASM cell relaxation. The role of these signalling molecules have been reviewed in detail [171,172].

β_2AR agonists induce Ca^{2+} desensitisation in airway smooth muscle

The first suggestion of β_2AR agonists affecting Ca^{2+} sensitisation were shown by measuring higher levels of relaxation than the simultaneously measured decrease in $[Ca^{2+}]_i$ [173]. In subsequent studies in lung slices with a clamped $[Ca^{2+}]_i$, salbutamol and formoterol induced relaxation of MCh-contracted airways in the absence of $[Ca^{2+}]_i$ changes, indicating that these β_2AR agonists caused Ca^{2+} desensitisation [162,163]. Importantly, in human lung slices with Ca^{2+} permeabilised airway ASM cells, formoterol relaxed histamine-contracted airways, indicating that formoterol induces Ca^{2+} desensitisation in the ASM cells of human small airways [13]. In conclusion β_2AR agonists, by elevating the intracellular cAMP concentration, cause Ca^{2+} desensitisation and this mechanism contributes to the bronchodilation effect.

Nitric oxide (NO)

Nitric oxide (NO) is produced in the small airways by epithelial cells, resident immune cells, and endothelial cells of neighbouring intrapulmonary arteries, and relaxes ASM (Fig. 1). NO enters the cytosol of ASM cells where it stimulates the soluble guanylyl cyclase (sGC) to produce cGMP, leading to relaxation of the airways in a protein kinase G (PKG) dependent manner [164]. Exposure of agonist-contracted airways to NO rapidly and strongly inhibited agonist-induced Ca^{2+} oscillations and induced airway relaxation. In the first minute following NO addition, NO caused an immediate and complete suppression of Ca^{2+} oscillations; these subsequently resumed but with a lower frequency than prior to NO addition. Concomitant with the changes in Ca^{2+} oscillations, NO induced a biphasic airway relaxation with a rapid and strong relaxation that peaked in the first minute followed by partial contraction [164]. These results suggest that NO-induced airway relaxation is at least in part mediated by a reduction in the frequency of Ca^{2+} oscillations. A decrease in the $[Ca^{2+}]_i$ in smooth muscle is expected to result in a reduction in activated Ca^{2+}-calmodulin that regulates MLCK and therefore in a decrease in MLC20 phosphorylation that regulates contraction. However, whether inhibition of Ca^{2+} oscillations by NO is accompanied by a reduction in MLC20 phosphorylation has not been investigated in the small airways.

In the presence of NO both IP_3-induced Ca^{2+} release and airway contraction were fully inhibited and these effects were reversed by NO washout [164], suggesting that NO inhibits Ca^{2+} release

via IP3R to reduce the frequency of Ca^{2+} oscillations. The effect was PKG dependent, however the precise interplay between NO/cGMP/PKG to inhibit the IP3R has not been investigated. A putative candidate is the PKG substrate protein IRAG, present in the SR membrane of ASM. When IRAG is phosphorylated by PKG it associates with the IP3R and blocks IP3R activation by IP_3 and Ca^{2+} [174,175]. Contrary to β2AR agonists, NO failed to induce relaxation after clamping of $[Ca^{2+}]_i$, suggesting it plays no role in sensitisation [164].

Hydrogen sulphide

Hydrogen sulphide (H_2S) is a gaseous molecule that is produced in the cytosol. It appears to regulate diverse cell physiological processes including smooth muscle contractility [176,177] and has been linked with asthma in both adult and paediatric patients [178,179]. In ASM cystathionine γ-lyase (CSE) produces H_2S [178] and its expression as well as the levels of endogenous H_2S in lung tissue are reduced in rodent models of allergic asthma [180,181]. As in vascular ASM cells, H_2S relaxed ASM cells in mice [182], and exogenous treatment with H_2S improved lung function and decreased airway inflammation in an allergic murine model of asthma [180]. These findings suggest that the CSE/H_2S system plays a critical protective role in the development of asthma.

H_2S is believed to mediate ASM relaxation independently of KATP channel activation [182–184] via inhibition of IP_3-induced Ca^{2+} release and oscillations akin to β_2AR agonists [165]. The IP3Rs have cysteine residues in their luminal side that are redox sensitive [185]. Significant conformational changes in this part of the protein induced by a reducing agent such as H2S could alter its affinity for regulatory proteins [186,187]. Relaxations induced by H_2S were reversed by the addition of the oxidising agent diamide and mimicked by the thiol-reducing agent dithiothreitol (DTT) [165].

The effect of H_2S on Ca^{2+} sensitisation was investigated in PCLS with Ca^{2+} permeabilised ASM cells. However, H_2S induced only a small and slow airway relaxation in these slices suggesting that Ca^{2+} desensitisation is not the major mechanism that mediates the airway relaxation induced by H_2S [165]. In conclusion, endogenous H_2S is a potent bronchodilator that blocks Ca^{2+} release through the IP3R and thus inhibits agonist-induced Ca^{2+} oscillations to relax airway ASM cells.

Bitter taste receptors

Recently, several members of the TAS2R bitter-taste receptors were found expressed on human [188], mouse [166,189], and guinea pig [190] ASM cells and its agonists reversed bronchoconstriction. In taste-bud cells, TAS2R activation is linked to the activation of PLCβ2 leading to IP3R-mediated increases in $[Ca^{2+}]_i$ [191,192], which would be expected to induce ASM cell contraction. While TAS2R agonist-induced Ca^{2+} signalling has been observed in ASM cells [166,188,189], it has only occurred in the absence of any contractile agonist and usually when high millimolar concentrations of TAS2R agonist are used. Under these conditions, it is difficult to ascertain whether TAS2R agonist-mediated Ca^{2+} signalling underlies the ability of TAS2R agonists to reverse contractile agonist-induced ASM contraction.

Hence, it is possible that TAS2R agonist-mediated Ca^{2+} signals only occur with high agonist concentration and when the SR Ca^{2+} store is full. However, when the SR Ca^{2+} store is partially empty or when the IP3Rs are in an open state, such as the case during Ca^{2+} oscillations, TAS2R agonists do not alter IP3R open probability or Ca^{2+} efflux rate [166,189]. This has been demonstrated where in the presence of methacholine, TAS2R agonist application did not augment methacholine-induced Ca^{2+} signals, but instead inhibited Ca^{2+} signals [166,189]. Importantly, this inhibitory effect on methacholine-induced Ca^{2+} signalling is consistent with the mechanism of other known bronchodilators discussed above, and indicates that TAS2R also mediate bronchodilation via common pathways. Similar to β_2AR agonists, TAS2R agonists also decreases Ca^{2+} sensitisation in small ASM cells [166]. This dual inhibitory action makes TAS2R agonists effective bronchodilators. However, further studies are required to determine the exact consequence of TAS2R agonist-induced Ca^{2+} signals in the absence of contractile agonists since clearly they do not induce ASM cell contraction.

SOCE

Targeting a process involved in both hyper-contractility and aspects of ASM cell remodelling occurring in an inflammatory environment could have great therapeutic potential. However the difficulty in designing high affinity and selective SOCE blockers on top of the ubiquitous nature of the process poses drug development problems. Recent work has identified novel pyrazole compounds that selectively target Orai1-mediated Ca^{2+} entry [193],

which might facilitate further rational drug design to develop therapeutically viable SOCE blockers. To address selectivity to the lung, local administration of drugs (e.g. in aerosols) or an 'antedrug' (a ligand that is rapidly metabolised in plasma) could be exploited [194]. Alternatively, further clarification of the potential contribution of additional subunits to SOCE in an asthmatic setting in ASM cells such as TRPC3 [53] or other Orai members might provide more ASM-selective molecular targets.

ROCE

ROCCs hold tremendous promise as new drug targets in airway diseases. However, much is still to be learnt, such as the complex multimerisation of the TRP family proteins leading to distinct biophysical characteristics. Presence of multiple ROCC combinations reflect (in part) the heterogeneity of $[Ca^{2+}]_i$ patterns that arise upon stimulation with agonists. The emergence of several novel non-TRP ROCE mechanisms in ASM such as the ARC, P2X and NMDA channels may provide additional targets for modulating Ca^{2+} dynamics. How they correlate with the altered ASM phenotype observed in respiratory diseases and the potential for therapeutic intervention is still to be elucidated.

Rho kinase

Inhibitors of Rho kinase have been instrumental in furthering the understanding of the Rho kinase signalling pathway and Ca^{2+} sensitisation. Inhibitors of Rho kinase relax elevated airway smooth muscle contraction and may prevent cellular migration thus providing attractive therapeutic targets for both respiratory and vascular diseases [195,196]. Their effectiveness to reduce AHR and inflammation in in vivo animal models of asthma have been well described in the literature [197–199]. Current research is focussing on the upstream regulation of Rho kinase and its activation with the aim to identify a new class of bronchodilators with potentially anti-inflammatory effects.

Future directions

There is still a lot to be learnt with regard to this rapidly advancing field, which due to its universal nature has the potential to translate into numerous other tissues and diseases. Very little is still known about the extent many proteins play in dictating Ca^{2+} dynamics within the ASM such as the PMCA pump. While the Ca^{2+} buffering capacity of SERCA2 is well described and its expression has been reported to

be altered in asthma [200], the field is in need of a thorough characterisation of the role of PMCA. Furthermore, the emerging roles of mechanisms outlined in this review which have been more extensively described in other systems, such as the non-canonical WNT pathway and various ROCE pathways, need to be better defined in ASM. Structural components such as the mitochondria [201,202], lysosomal stores [203] and caveolae [39,204] all further add to the complexity of calcium signalling, and their influence must be considered in future research. An area of potentially great therapeutic promise are those mechanisms that lead to changes in ASM Ca^{2+} sensitivity in asthma, which is likely to play a significant role in AHR.

There are practical limitations involved in measuring Ca^{2+} oscillations in tandem with other functional endpoints such as Ca^{2+} sensitivity, therefore results often have to be extrapolated from single transients, to oscillations and finally to cellular function. These assumptions have largely been validated in in vitro experiments by correlating various Ca^{2+} parameters such as decreased peaks, prolonged sequestration and enhanced basal concentration to functional endpoints including enhanced proliferation, secretion of inflammatory mediators [200,205]. The development and wider spread usage of PCLS has produced interesting revelations in the last few years and further technical advances to come will highlight the true complexity of this system.

Conclusions

Many physiologically relevant contractile agonists including acetylcholine, 5-hydroxytriptamine, endothelin-1, histamine, and cysteinyl leukotrienes elevate $[Ca^{2+}]_i$ in the form of intracellular Ca^{2+} oscillations, and concomitantly increase Ca^{2+} sensitivity in small ASM cells [3,4,13,28,154]. In contrast, bronchodilators such as β2-agonists, nitric oxide (NO), TAS2R agonists, and endogenous hydrogen sulfide (H_2S) induce airway relaxation by inhibiting one or both of these processes [161,163–165]. Subsequent changes in $[Ca^{2+}]_i$ can affect other signalling pathways such as WNT leading to profound physiological changes. The regulation of ASM $[Ca^{2+}]_i$ and Ca^{2+} sensitivity is highly complex. However, recent work has sought to fully describe the cellular mechanisms that mediate changes in Ca^{2+} handling, sensitivity and the formation of the contractile apparatus which forms an important part of ASM migration and airway remodelling. Full characterisation of the signalling pathways de-

scribed here will undoubtedly provide new, interesting avenues for therapeutic intervention.

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