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Ion channel regulation of intracellular calcium and airway smooth muscle function

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Asthma-associated airway hyper-responsiveness (AHR) is primarily mediated by excessive airway smooth muscle cell (SMC) contraction, yet the mechanisms responsible for this behavior remain unknown. A hypothesis commonly favored to explain excessive force production by individual SMCs is that the intracellular calcium concentration ($[Ca^{2+}]_i$) that regulates SMC contraction is abnormally elevated in asthmatic airway SMCs. However, in view of the importance of Ca^{2+} as a second messenger, an elevated $[Ca^{2+}]_i$ would have other wide-ranging ramifications for SMC physiology and this may be expressed in terms of altered cell proliferation or phenotype. SMC contraction is also determined by the sensitivity of the SMC contractile apparatus to the $[Ca^{2+}]_i$ and AHR may be explained by an abnormal increase

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in Ca^{2+} sensitivity. Similarly, airway constriction also depends on the number and size of the SMCs and the resistive load experienced by these SMCs. As a result, increased SMC proliferation and airway wall remodeling, processes commonly associated with airway inflammation, are believed to significantly contribute to AHR.

A common theme linking these varied processes is that each one is regulated or influenced by $[\text{Ca}^{2+}]_i$ which, in turn, is determined by the activity of a wide variety of ion channels, in balance with membrane Ca^{2+} pumps. This increase in $[\text{Ca}^{2+}]_i$ can be achieved in two fundamental ways; by the release of Ca^{2+} from internal stores via specialized receptor/channels on the sarcoplasmic reticulum or by Ca^{2+} influx from the extracellular space via a variety of plasma membrane ion channels. The objective of this review is to focus on the nature of this channel activity and how it relates to changes in $[\text{Ca}^{2+}]_i$ and the regulation of SMC contraction, proliferation and secretion, with the idea that ion channels may serve as a therapeutic targets for AHR.

Mechanisms of internal Ca^{2+} release

Agonist-induced SMC contraction is predominately dependent on increases in $[\text{Ca}^{2+}]_i$ resulting from the release of Ca^{2+} from internal stores, namely the sarcoplasmic reticulum. Importantly, this increase in $[\text{Ca}^{2+}]_i$ is not static, but rhythmically increases and decreases in the form of Ca^{2+} oscillations. This reliance on internal stores is emphasized by the observations that both agonist-induced airway SMC contraction and Ca^{2+} oscillations can occur in the absence of extracellular Ca^{2+} [1,2] and that, inhibitors (cyclopiazonic acid and thapsigargin) of the sarcoplasmic/endoplasmic reticulum Ca^{2+} ATPase (SERCA) that lead to the run-down or emptying of the internal Ca^{2+} store, abolish Ca^{2+} oscillations [2,3].

Agonist-induced Ca^{2+} oscillations (using ACh or 5HT) occur in tracheal, bronchial and small airways of mice [1,4–6], pigs [2,7,8], and humans [9]. Importantly, the frequency of these Ca^{2+} oscillations generally increases with the concentration of the agonist [1,5,10]. However, the Ca^{2+} oscillations are usually initiated at a frequency slightly higher than the steady frequency that is subsequently expressed in the continual presence of agonist. Ca^{2+} oscillations commonly occur as Ca^{2+} waves in SMCs. Increases in $[\text{Ca}^{2+}]_i$ are often initiated at one end of the SMC and propagate, as a Ca^{2+} wave, along the entire length of the cell. However, the position of the initiation site can fluctuate and relocate to the opposite end of the cell, with the consequence that the direction of the Ca^{2+} wave propagation reverses [1,3,11]. The velocity of the propagating Ca^{2+} wave is relatively constant but may increase with agonist concentration in range from 8 to 24 $\mu\text{m/s}$ in tracheal SMCs [11] and from 27 to 35 $\mu\text{m/s}$ in mouse small airways [1,3].

Mechanisms of Ca^{2+} oscillations

The model for the generation and maintenance of Ca^{2+} oscillations in airway SMCs appears to be similar to many other cell types [12,13]. The activation of G-protein coupled receptors (GPCR) by agonists, such as ACh or 5HT, in airway SMCs leads to $\text{G}\alpha_q$ -mediated activation of phospholipase C (PLC) to synthesize the second messengers inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol from plasma membrane phosphatidylinositol 4,5-bisphosphate [14–16]. Subsequently, IP_3 diffuses through the cytosol and binds to IP_3 receptors (IP_3R) in the sarcoplasmic reticulum. The IP_3R is a specialized Ca^{2+} channel with a gating mechanism regulated by IP_3 and Ca^{2+} (Figure 1).

In response to the initial rise of $[\text{IP}_3]_i$, cytoplasmic Ca^{2+} opens the IP_3R at a local initiation site (generally situated close to one end of the SMC) to release Ca^{2+} from the sarcoplasmic reticulum. The released Ca^{2+} diffuses away and activates neighboring IP_3 -sensitized IP_3Rs to initiate the further release of Ca^{2+} . In this manner, a wave of increased $[\text{Ca}^{2+}]_i$ propagates

through the cell. The resulting local increase in $[Ca^{2+}]_i$ promotes the binding of Ca^{2+} to a second site on the IP_3R which inactivates and closes the receptor to terminate Ca^{2+} release. In addition, the reduction in the $[Ca^{2+}]$ within the sarcoplasmic reticulum also contributes to the termination of the Ca^{2+} release process [13]. The subsequent decrease in $[Ca^{2+}]_i$ results from the combined effects of SERCA activity in the sarcoplasmic reticulum to replenish the Ca^{2+} stores, the extrusion of Ca^{2+} by plasma membrane Ca^{2+} -ATPases and Na^+/Ca^{2+} exchangers and the binding of Ca^{2+} to protein buffers in the cytosol. In addition, the reduction of $[Ca^{2+}]_i$ leads to the release of Ca^{2+} from the IP_3R to reset the receptor. In this manner, repetitive Ca^{2+} oscillations result from the cyclic Ca^{2+} -induced activation and deactivation of IP_3 -sensitized IP_3Rs (Figure 1).

It seems clear that IP_3Rs participate in agonist-induced generation of Ca^{2+} oscillations in airway SMCs: The inhibition of IP_3R with heparin (an IP_3R antagonist) or with an IP_3R monoclonal antibody blocks Ca^{2+} oscillations (recorded as Ca^{2+} -activated Cl^- currents) [10]. However, the additional contribution of a second sarcoplasmic reticulum Ca^{2+} release channel, the ryanodine receptor (RyR), to Ca^{2+} oscillations and the propagation of Ca^{2+} waves appears to vary between SMCs at different locations in the airway or with the species. In this alternative model of Ca^{2+} oscillations, the participation of both the IP_3R and the RyR is suggested because ryanodine or ruthenium red (RyR antagonists) blocked Ca^{2+} oscillations whereas heparin, only partially inhibited Ca^{2+} oscillations induced by ACh [17] in pig airway SMCs. By contrast, ryanodine was unable to inhibit or alter the properties of the Ca^{2+} oscillations of SMCs from mice or rats suggesting that Ca^{2+} oscillations and waves are dependent only on IP_3Rs in bronchioles of these animals [18].

SMC contraction and Ca^{2+} oscillation frequency

By measuring airway SMC contraction and Ca^{2+} signaling, it has been found that increased contraction of small airways and tracheal SMC strips in response to increasing concentrations of agonists correlated with an increase in the frequency, but not the amplitude, of Ca^{2+} oscillations [1,5,19]. This observation supports the hypothesis that airway SMC contraction is regulated by the frequency of the Ca^{2+} oscillations. In addition, mathematical modeling of the contractile responses of airway SMCs upholds the idea that Ca^{2+} oscillations are more effective than steady-state Ca^{2+} for the generation of contraction [20]. However, further analysis suggested that the frequency component of the Ca^{2+} oscillations alone may not be responsible for this effect. These mathematical models showed that it is more likely that the change in the average $[Ca^{2+}]_i$ resulting from the integration of the Ca^{2+} oscillations has more influence in determining the extent of contraction, a conclusion supporting previous suggestions [11]. In addition, there are significant differences in the contraction-frequency relationship induced by different agonists and between SMCs from different species. For example, 5HT induced Ca^{2+} oscillations with slower frequencies in bronchiole airway SMCs than those induced by an equal concentration of ACh, but the associated contraction in both cases was similar [1]. Likewise, the frequency of Ca^{2+} oscillations induced by 5HT was approximately three times slower in the SMCs of intrapulmonary arterioles than it was in airway SMCs, but the arterioles contracted more than the airways [3]. These differences in the contraction-frequency relationships suggested differences in the Ca^{2+} sensitivity of the SMCs. This idea was confirmed with Ca^{2+} permeabilized cells in which the $[Ca^{2+}]_i$ was set at a high steady level; under these conditions of constant $[Ca^{2+}]_i$, exposure to equal concentrations of different agonists (e.g. methacholine or 5HT) resulted in different amounts of contraction [20,21]. These observations indicate that, in addition to the frequency of Ca^{2+} oscillations, the Ca^{2+} sensitivity of SMCs is a major factor contributing to the regulation of SMC contraction [22].

With this insight into airway SMC contraction, we can turn our attention to the important mechanism of broncho-dilation that must be acutely induced to counter airway hyper-

responsiveness occurring during an asthma attack. Over the longer term, the therapeutic aim is the prevention of airway hyper-responsiveness. These goals require the relaxation and desensitization of the airway SMCs. A current primary approach used to relax airway SMCs is the inhalation of β_2 -agonists that mediate their effects via increases in cAMP and the activation on protein kinase A [18,23]. However the mechanism of SMC relaxation is not well understood. Activation of β_2 -receptors with agonists, such as, salbutamol [24], isoproterenol [18] and albuterol [23], reduces the frequency of agonist-induced Ca^{2+} oscillations in SMCs and this response correlates with airway relaxation. Airway relaxation and a similar reduction of the Ca^{2+} oscillation frequency is induced by nitric oxide acting via cGMP [25,26]. As outlined above, Ca^{2+} oscillations primarily rely on internal Ca^{2+} release. Consistent with this idea, is the observation that the slowing of the frequency of the Ca^{2+} oscillations appears to mainly result from the inhibition of Ca^{2+} release from internal stores by blocking the IP_3R [18,26]. The inhibition of Ca^{2+} release by NO/cGMP has been shown to involve protein kinase G-mediated phosphorylation of an IP_3R associated protein, but it is not clear if cAMP in airway SMCs also acts via protein kinase A-mediated phosphorylation of a similar associated protein [27–30]. Ca^{2+} oscillations could also be slowed by a reduction in the $[\text{IP}_3]_i$, but β_2 -agonists, forskolin or 8-Br-cAMP have been reported to not interfere with the synthesis of IP_3 [31,32]. In addition, β_2 -agonists have been reported to activate K_{Ca} channels which, as discussed later, would hyperpolarize the cell membrane and reduce Ca^{2+} entry through voltage activated Ca^{2+} channels [33]. A limited Ca^{2+} entry would be expected to lead to a decrease in Ca^{2+} oscillation frequency by depleting and delaying the re-loading of the internal Ca^{2+} stores [34].

Airway SMC relaxation mediated by Ca^{2+} de-sensitization, commonly involves an increase in the activity of myosin light chain phosphatase to antagonize the activating phosphorylation of the myosin light chain by myosin light chain kinase. In acute responses, β_2 -agonists and cAMP analogs have been shown to reduce the Ca^{2+} sensitivity of airway SMC [18,23,35]. However, NO does not seem to have a large effect on the Ca^{2+} sensitivity of mouse airway SMCs [26].

Over the long-term, it is not clear how the SMCs become hyper-responsive, but stimulation of the mechanisms of Ca^{2+} release and re-uptake could result in an increase the frequency of agonist-induced Ca^{2+} oscillations which, in turn, would increase the myosin light chain kinase activity, myosin phosphorylation and force production. In addition, hyper-responsiveness could also result from an increase in Ca^{2+} sensitivity which may be mediated by alterations in myosin phosphatase activity (inhibition) or one of its regulators such as Rho-kinase [22,36]. There are a number of additional factors that could also be part of the problem of airway hyper-responsiveness, for example the accumulation of more SMCs in the airway wall. This form of sensitization is often countered by steroid therapy but, as mentioned later, the inhibition of K_{Ca} channels to reduce cell proliferation may be a viable alternative.

Routes of Ca^{2+} influx

In view of the excess of extracellular Ca^{2+} , especially in comparison to the limited Ca^{2+} of the internal stores of SMCs, an obvious alternative way to increase $[\text{Ca}^{2+}]_i$ in SMCs is by the influx of Ca^{2+} . Despite the wide variety of different channel types in the plasma membrane through which this can occur, a clear correlation of a specific Ca^{2+} influx pathway with a physiological function of SMC, other than refilling of Ca^{2+} stores, is, at present, lacking. A common idea has been that Ca^{2+} influx directly contributes to airway SMC contraction, but, as described above, Ca^{2+} release from internal stores in the form of Ca^{2+} oscillations appears to be more important in agonist-induced SMC contraction. However, even though Ca^{2+} oscillations repetitively utilize much of the Ca^{2+} released from the sarcoplasmic reticulum, it is inevitable that some Ca^{2+} is lost to the extracellular environment. Consequently, for sustained Ca^{2+}

oscillations and contraction, it is essential to replenish the $[Ca^{2+}]$ of the sarcoplasmic reticulum and this requires Ca^{2+} influx.

Ca^{2+} influx through plasma membrane ion channels may also have other important physiological functions in SMCs. For example, increases in $[Ca^{2+}]_i$ caused by Ca^{2+} influx can lead to alterations in proliferation and secretion (see later). Furthermore, an absence of Ca^{2+} influx will eventually result in the depletion of the Ca^{2+} of the sarcoplasmic reticulum. This will affect many of the fundamental functions of the sarcoplasmic reticulum, related to protein folding and maturation, stress responses and apoptosis [37]. It is therefore essential to characterize and determine the molecular identity of the various ion channels involved in Ca^{2+} influx to understand their role in the physiological and pathophysiological processes of airway SMCs.

Store-operated Ca^{2+} entry

The process of replenishing the $[Ca^{2+}]$ of the sarcoplasmic reticulum by agonist-induced Ca^{2+} influx is termed capacitative calcium entry or store-operated calcium entry and, as the name implies, Ca^{2+} influx occurs upon the emptying of the internal Ca^{2+} stores via the activation of store-operated channels in the plasma membrane [38,39]. However, agonists acting on membrane receptors can also initiate Ca^{2+} entry by stimulating the production of diacylglycerol or its derivatives to directly activate membrane ion channels called receptor-operated channels (Figure 1). Ca^{2+} entry via these receptor-operated channels may accompany or precede Ca^{2+} influx via store-operated channels [40–43]. Even though opinions differ with respect to the contribution of receptor-operated and store-operated Ca^{2+} channels to store refilling, both processes have been shown to be necessary to sustain Ca^{2+} oscillations in various cell types [39,44]. Other routes for Ca^{2+} entry are voltage-gated Ca^{2+} channels and the sodium-calcium exchanger operating in the reverse-mode [45]. However, the activation of voltage-operated Ca^{2+} channels for long time periods requires successive cycles of membrane depolarization and hyperpolarization in order to avoid channel inactivation. Although oscillations in membrane voltage during agonist stimulation have been observed in airway SMCs, the changes in membrane potential are usually inadequate to stimulate the opening of L-type Ca^{2+} channels [46]. In addition, the contribution of voltage-gated Ca^{2+} channels appears to be minimal because inhibitors of these channels had little effect on agonist-induced SMC contraction or Ca^{2+} oscillations [1] or as therapies for AHR [46].

The identities of the channels involved and the mechanism of store-operated channel activation have been long-standing questions since the discovery of store-operated Ca^{2+} entry [47]. Multiple mechanisms of channel activation via direct contact with store-associated Ca^{2+} sensing molecules or by soluble factors have been proposed but without much consensus. Therefore, it was an important advance when the molecular identities of store-operated channels and their regulatory proteins were associated with Orai family proteins [48–50] and stromal interacting molecule 1 (STIM1), respectively [51–53]. Receptor-operated channels may also involve these proteins [54–56]. Orai1 proteins form a highly Ca^{2+} selective store-operated ion channel in the plasma membrane that is responsible for the well characterized Ca^{2+} release-activated Ca^{2+} current, I_{CRAC} . STIM1 is Ca^{2+} -binding membrane protein present in the sarcoplasmic reticulum where it appears to function as a Ca^{2+} sensor and transmits information regarding the Ca^{2+} content of the sarcoplasmic reticulum to plasma membrane store-operated channels (Figure 1). The fall in Ca^{2+} in the internal store leads to the aggregation of STIM1 into punctate structures within the sarcoplasmic reticulum, but close to the plasma membrane, where it can interact with the Orai1 or other store-operated channels [57,58]. siRNA mediated knock-down of STIM1 significantly reduced store-operated Ca^{2+} entry following Ca^{2+} store depletion by histamine but not bradykinin in human airway SMCs [59]. In addition, it has been shown that Orai1 and potentially Orai3 contribute to store-operated channels in

these cells [60]. Importantly, Orai1, -2, -3 as well as STIM1 have been found to be expressed in human airway SMCs.

Ca²⁺ influx via TRP Ca²⁺ channels

The molecular identities of all store-operated and receptor-operated channels in SMCs are not yet fully characterized and the non-selective characteristics of the Ca²⁺ influx mechanism suggests the involvement, in addition to Orai1, of other protein channels. In this respect, it appears that the transient receptor potential (TRP) proteins constitute some or parts of these channels [61,62]. The mammalian TRP protein super-family is comprised of multiple subfamily members [63]. In airway SMCs, several members of the three biggest and most characterized subfamilies (TRPC, TRPM, TRPV) have been proposed to form agonist-activated, cation permeable channels that contribute to Ca²⁺ influx to regulate contraction and proliferation [64]. Furthermore, members of all three families are reported to be expressed in human airway SMCs including TRPC1, -3, -4, -5, -6, TRPM4, -7 and TRPV1, -2, -4 [65–69].

TRP proteins assemble into homo- and/or heteromeric tetramers, generating cation permeable channels [70] which are permeable to a range of mono- and divalent cations. However, permeability differs greatly between the various family members. The TRPCs form largely non-selective cation channels with the selectivity for Na⁺ and Ca²⁺ being determined by the specific components of the hetero-multimers [63]. TRP channels are activated by a wide variety of stimuli including mechanical, osmotic and thermal stress and chemical compounds and the expression of some TRP channels may be regulated by growth factors and cytokines [68,71, 72].

The TRPC family is the subfamily classically associated with store-operated and receptor-operated Ca²⁺ entry. They all appear to be activated by agonist stimulation via the PLC pathway [61], but there is considerable controversy regarding the relationship of TRPC channels to store-operated and receptor-operated channels. On the basis of over-expression systems, it has been suggested that the activating mechanism may depend on the expression level [73,74]. The TRPC1 channel is important for agonist-induced Ca²⁺ entry in SMCs. Generally, TRPC1 has been proposed to form a store-operated channel [66,75], except in vascular SMCs, where store-operated channels appear to be independent of TRPC1 even though the channel is present and functional [76]. In airway SMCs TRPC1 has been reported to be involved in proliferation and therefore may play a role in airway remodeling [65].

By over-expression of STIM1 in HEK293 cells, STIM1 has been shown to interact with all TRPC proteins except TRPC7, binding directly to TRPC1, 4 and 5, and promoting hetero-multimerisation of TRPC3 and 1, and TRPC4 and 6. STIM1 seems necessary for these channel to act as store-operated channels, although it does not appear to be required for channel function per se [58]. This may be consistent with the findings in vascular SMCs where STIM1 is required for store-operated channel, but TRPC1 is not [76].

TRPC3 is also expressed in vascular SMCs where its activation results in Ca²⁺ influx and vasoconstriction, whereas its inhibition attenuates depolarization and contraction [77]. The channel is thought to be primarily receptor-operated [78] although in human airway SMCs the expression of TRPC3 channels can be increased by treatment with TNF- α which leads to increased store-operated Ca²⁺ entry in response to GPCR agonists such as ACh and bradykinin [68]. Dietrich et al., suggested that TRPC3 channel homo-multimers may be constitutive active in vascular and airway SMCs, whereas channels formed of TRPC3 and TRPC6 hetero-multimers require activation via the PLC pathway [79].

There has been considerable focus on TRPC6 channels in pulmonary vascular SMCs where they are believed to be involved in cell proliferation and may contribute to pulmonary arterial

hypertension [80] and hypoxic pulmonary vasoconstriction [81]. Interestingly, TRPC6-deficient mice showed enhanced agonist-induced contraction of aortic rings, a response perhaps explained by a compensating up-regulation of TRPC3. These results highlight some of the complexity in studying the role of these channels [79]. From these findings, it has been proposed in human airway SMCs that TRPC6 serve as a receptor-operated channel, activated by diacylglycerol and its analogue 1-oleoyl-2-acetyl-sn-glycerol (OAG) [66]. However, in guinea pig airway SMCs, TRPC6 knock-down did not affect OAG-induced Ca^{2+} influx [82].

In summary, human airway SMCs express several members of the TRPC family, which have been shown to be associated with Ca^{2+} influx, contraction and proliferation in other SMC types. However the functional importance of TRPC as well as Orai proteins in human airway SMCs still needs to be confirmed, although progress towards this goal is hampered by the lack of specific channel agonists and antagonists. In addition, the findings that TRPC proteins can form hetero-multimers and the suggestions that Orai1 proteins can interact with TRPCs [83] raises the possibility that several different store- and receptor-operated channels exist in airway SMCs. These could also be dynamically regulated by factors that change their expression (e.g. cytokines and growth factors) during disease. In view of this association of TRP channels with Ca^{2+} influx, contraction and proliferation, TRP channels may be potentially valuable pharmaceutical targets for the treatment of asthma.

Ca^{2+} influx via stretch-activated channels

Airway SMCs are exposed to various physical forces such as gravity, compression and rhythmic mechanical strain. Because these forces act on the plasma membrane, Ca^{2+} -permeable cation channels, activated by mechanical stretch or stretch-activated channels provide the SMC with the ability to respond to mechanical stimuli [84–87] (Figure 1). Importantly, these Ca^{2+} permeable stretch-activated channels occur in human airway SMCs [88].

The mechanisms by which stretch activates these channels are not fully understood, but a direct and indirect mechanism has been proposed [87]. The finding that Gd^{3+} blocks stretch-induced Ca^{2+} influx in various cells, including airway SMCs, supports the idea of direct activation [88–90]. Currently, there are two models to explain direct gating of stretch-activated channels; a “bilayer model” in which plasma membrane tension itself is sufficient to gate the mechanosensitive channel and a “tethered model” in which mechanical force is transmitted to the ion channel through a linkage formed by the extracellular matrix, integrin proteins and cytoskeleton [86,87]. For the indirect mechanism, it has been proposed that the activation of PLC or the release of another signaling molecule (e.g. ATP) following application of mechanical stress elicits intracellular Ca^{2+} mobilization and subsequent activation of the channels at the plasma membrane [87,91]. It is also possible that Ca^{2+} influx via stretch-activated channels secondly activates voltage-dependent channels by causing membrane depolarization [85,87]. In human ASM cells, stretch-induced Ca^{2+} influx is not blocked by the PLC inhibitor, U-73122, the IP_3R blocker, xestospongin C, or the voltage-gated channel blocker, nifedipine [88]; these results suggest that Ca^{2+} -permeable stretch-activated channels in human airway SMCs are activated directly by stretch rather than indirectly via intracellular messenger production or voltage-dependent channel gating.

TRPV channels: a candidate for stretch-activated channels?

The TRPV subfamily members are activated by a wide variety of stimuli including agonists, heat and changes in osmolarity [61,92] and there is increasing evidence that a putative mechano-sensitive channel is one of the members of the TRPV subfamily [93–95]. Human airway SMCs express TRPV1, -2 and -4 genes [67,88] and it appears that TRPV2 and -4 mediate Ca^{2+} influx in response to membrane stretch or hypo-osmotic induced cell swelling

[67,95,96]. Stretch-induced Ca^{2+} influx is inhibited by ruthenium red, a TRPV inhibitor, but not by capsazepine, a TRPV-1 inhibitor [88]. Therefore, TRPV2 and -4 are candidate genes for stretch-activated channels in airway SMCs. However, it should be noted that TRPC6 [97] and TRPM7 [98] channels have also been linked to the stretch-induced Ca^{2+} influx in other cell types. TRPV4 can also be activated by moderate heat ($>24^{\circ}\text{C}$), 4 α -phorbol 12,13-didecanoate (4 α -PDD), and endogenous substances like endocannabinoid anandamide, arachidonic acid and 5',6'-EET [67] and this suggests a role in thermosensing and Ca^{2+} homeostasis. TRPV2 is also activated by insulin growth factor (IGF-1) by the insertion of the channel into the cell membrane. Intracellular vesicles, that arise from the Golgi and incorporate the channel, fuse with the plasma membrane upon stimulation with IGF-1 and this process may underlie IGF-1-induced contraction and proliferation of airway SMCs [64]. TRPV2 might also be upregulated in capsaicin-induced asthma and it can be upregulated by growth factors [72].

Stretch-activated Ca^{2+} influx in disease

The lungs are continuously exposed to a mechanically dynamic environment during breathing [99] and excessive strain and pressure occurs in the airway walls during bronchoconstriction in asthmatic patients [100]. Therefore, mechanical stress is likely to have an impact on cellular function in normal and diseased lungs [101,102]. Indeed, the development [103], contraction [104,105], stiffness [106–108], proliferation [109], migration [110], organization of the actin cytoskeleton [106,108,111,112] and protein synthesis [113,114] of airway SMCs are influenced by the mechanical stress. These cellular responses of airway SMCs to mechanical strain are mediated via multiple intracellular pathways including RhoA, myosin light chain kinase, cyclooxygenase (COX), MAP-kinases (ERK1/2 and p38) and various transcription factors [104,113,115–118]. Although the involvement of stretch-activated channels is currently ill-defined, airway SMC contraction or IL-8 production induced by cyclic mechanical strain are significantly inhibited by the stretch-activated channel inhibitor Gd^{3+} [104,113]. Therefore it is important to further characterize stretch-activated channels and their role in airway SMCs as they too may serve as drug targets for the treatment of airway disease [94, 119].

Calcium-dependent K^{+} channels

Although acute changes in membrane potential do not seem to be required to stimulate airway SMC contraction, it appears that $[\text{Ca}^{2+}]_i$ and membrane potential are interconnected by Ca^{2+} -dependent K^{+} channels and that this regulation is designed to operate over a longer time course to mediate changes in SMC phenotype.

Characteristics of K_{Ca} channel family

The three major families of human Ca^{2+} -activated K^{+} channels (K_{Ca}); the large conductance ($\text{K}_{\text{Ca}}1.1$), intermediate conductance ($\text{K}_{\text{Ca}}3.1$) and small conductance channels (3 varieties, $\text{K}_{\text{Ca}}2.1$, 2.2 and 2.3) [120], share the property of forming an open conformation in response to increased $[\text{Ca}^{2+}]_i$. Each family differs in their Ca^{2+} -sensing mechanism and voltage sensitivity. While $\text{K}_{\text{Ca}}1.1$ channels bind Ca^{2+} directly at the C-terminus, $\text{K}_{\text{Ca}}3.1$ channels form a constitutive molecular partnerships with the Ca^{2+} -binding protein calmodulin which results in a greater sensitivity to $[\text{Ca}^{2+}]_i$ [120,121]. In addition, the $\text{K}_{\text{Ca}}1.1$ channel, but not the $\text{K}_{\text{Ca}}3.1$ channel, is voltage-gated. These properties of high Ca^{2+} -sensitivity and voltage-insensitivity allow the $\text{K}_{\text{Ca}}3.1$ channel to open during membrane hyper-polarization when it is likely that $\text{K}_{\text{Ca}}1.1$ is inactive. Such distinct functional properties suggest that these channels may operate during different conditions in the same cell, such as occur following a phenotypic alteration in airway SMC.

K_{Ca} channels also differ in their sensitivity to inhibitors allowing their different actions to be assessed pharmacologically; K_{Ca}1.1 channels are inhibited by TEA, iberiotoxin and charybdotoxin whereas K_{Ca}3.1 channels are inhibited by TRAM-34, clotrimazole, ICA17043 and charybdotoxin [120,122].

The role of K_{Ca} channels in SMC Contraction

A Ca²⁺-dependent and voltage-gated outward K⁺ current that is sensitive to charybdotoxin and iberiotoxin is widely found in isolated tracheal SMCs [123–125]. The properties of this current are consistent with the large conductance K_{Ca} channel (BK or K_{Ca}1.1); a channel commonly responsible for spontaneous transient outward currents (STOCs) that are associated with 'Ca²⁺ sparks' [126]. In vascular SMCs, the Ca²⁺-activation of K_{Ca}1.1 channels leads to membrane hyper-polarization and the inhibition of voltage-gated L-type Ca²⁺ channels. This in turn, reduces the amount of Ca²⁺ influx, and thereby decreases SMC tone [127]. The activation of the K_{Ca}1.1 channel by physiological (nitric oxide) or therapeutic bronchodilators (isoproterenol) is consistent with this paradigm, are activated by these agonists that appear to reduce Ca²⁺ by other although it is not clear how K_{Ca} mechanisms [18]. However, the observations that voltage-gated Ca²⁺-channel inhibitors have no effect on agonist-induced Ca²⁺ signaling in airway SMCs [1], are ineffective bronchodilators [128] and that the deletion of K_{Ca}1.1 channels in transgenic mice reduced airway contractility [22,46,128] all suggest that the contribution of membrane potential to regulation of [Ca²⁺]_i in airway SMCs is minimal. Consequently, the role of K_{Ca}1.1 in airway SMC contraction remains controversial. Airway SMCs also contain Ca²⁺-activated Cl[−] channels [7] and these have been found to be responsible for spontaneous transient inward currents (STICS) [126]. The exact role for Ca²⁺ activated Cl[−] currents in airway SMCs is also unclear, but these currents may enhance Ca²⁺ loading of the sarcoplasmic reticulum [129] and influence cell proliferation.

The role of K_{Ca} channels in SMC proliferation

Airway SMCs are adaptable and may adopt a 'synthetic' or 'proliferative' phenotype in chronic asthma [130]. SMC proliferation leads to hyperplasia in asthma and is a feature of isolated human airway SMCs *in vitro* [131]. This entry into and progression through the cell-cycle requires a careful orchestration of the membrane potential with the [Ca²⁺]_i [132] and this appears to be associated with distinct patterns of K_{Ca} channel expression.

Snetkov et al [133] demonstrated that K_{Ca}1.1 currents, found in isolated human bronchial SMCs, diminished with *in vitro* culture and suggested that a reduction in the expression of a major K⁺ channel correlated with a switch to a proliferative phenotype [133,134]. Similarly, outwardly rectifying currents associated with K_{Ca} channels in proliferating human airway SMCs were relatively insensitive to iberiotoxin, implying a relatively small contribution by K_{Ca}1.1 [135]. Under these conditions, few cells expressed currents typical of K_{Ca}3.1 channels, but in response to TGF-β, the majority of cells increased the expression and activity of K_{Ca}3.1 channels. A loss of K_{Ca}1.1 channels and gain in K_{Ca}3.1 channels also occurs when vascular SMCs switch from a contractile to a proliferative phenotype [136,137]. Thus, the switch from a contractile to a proliferative phenotype by SMCs in response to injury is associated with the replacement of relatively Ca²⁺-insensitive, voltage-gated K_{Ca} channels with voltage-insensitive, but more Ca²⁺-sensitive K_{Ca} channels that may facilitate the regulation of the cell-cycle.

The role of K_{Ca} channels in cell-cycle regulation

The idea that the cell cycle is regulated in SMCs by K_{Ca} channels is attractive but, as yet, not well documented. On the other hand there is considerable evidence supporting the idea that a change in membrane potential serves as a critical determinant of cell cycle progression in other cells. A well-studied example is found with MCF-7 breast cancer cells in which membrane

hyper-polarization, mediated by the activation of K^+ channels, correlates with the progression of cells from the G_0 phase into, and to the end of, the G_1 phase [138,139]. We review the data from MCF-7 breast cancer cells with the objective of gaining an insight to the mechanisms that may apply to SMCs expressing similar K_{Ca} channels. An arrest of the cell cycle can be induced by the inhibition of K^+ channels but the particular K^+ channel involved varies in different cell types. Ouadid et al., [140] also investigating MCF-7 cells found that although the early phases of hyper-polarization (up to mid G_1) were accompanied by increases in K^+ channels related to the human *ether-a-go-go related gene* (K_v10), this channel density and mRNA expression fell later in G_1 despite persistent membrane hyper-polarization. This sustained hyper-polarization was explained by the identification of a clotrimazole and TRAM-34 sensitive, Ca^{2+} activated-channel with the characteristics of $K_{Ca3.1}$ [140]. The mRNA and currents associated with $K_{Ca3.1}$ channel increased throughout G_1 and were maximally elevated in early S-phase. $K_{Ca3.1}$ channels have also been implicated in proliferation of mesenchymal cells, vascular SMCs and transformed fibroblasts when stimulated by growth factors [141,142]. Similarly, inactivation of $K_{Ca3.1}$ channels, with inhibitors such as TRAM-34 and clotrimazole, prevented human airway SMC proliferation [135], SMC re-growth in a model of post-angioplasty restenosis [137,143] and cell proliferation induced by mitogens including serum, basic FGF and thrombin. By contrast, $K_{Ca1.1}$ and K_{Ca2} inhibitors, such as iberiotoxin, TEA and apamin do not inhibit proliferation [144]. These results indicate that a differential expression of K^+ channels regulates membrane potential throughout the cell cycle and that expression of $K_{Ca3.1}$ channels is necessary to progress into the S-phase. Given the inhibitory effect of membrane hyper-polarization on voltage-gated Ca^{2+} channels, it is possible that the additional $K_{Ca3.1}$ channel activity provides the necessary electrochemical gradient to enhance Ca^{2+} entry, perhaps via another voltage-independent Ca^{2+} channel [145,146].

K^+ channels as a therapeutic target

$K_{Ca3.1}$ is expressed by proliferating airway SMCs rather than 'normal' contractile cells [135] and its expression is driven by the pathological mediator TGF β that is increased in asthmatic airways. Its presence thus characterizes SMCs involved in disease progression making it an attractive therapeutic target in chronic asthma. In addition $K_{Ca3.1}$ channels are found in mesenchymal stem cells, epithelial cells and mast cells, all of which contribute to the pathology of chronic asthma [147,148]. Inhibitors of $K_{Ca3.1}$ channels have proven to be safe during clinical trials for sickle cell disease and clinical trials with these inhibitors for the treatment of asthma are being developed [122].

The role of $[Ca^{2+}]_i$ in SMC-mediated inflammation

In addition to their contractile and proliferative activities, airway SMCs have also been implicated as a target, as well as a source, of a variety of pro-inflammatory factors and extracellular matrix proteins. Indeed there is evidence that their proliferative and synthetic functions can overlap [149] and that growth factors such as TGF- β , not only induce proliferation, but also induce extracellular matrix deposition [150]. Airway SMCs also respond to cytokines and produce the chemokines CXCL10 (IP-10), in response to interferon (IFN)- γ [151,152], and IL-8 and eotaxin, in response to IL-4 and IL-13 [153]. Moreover, pro-inflammatory cytokines such as TNF- α , IL-1 β and IFN- γ induce the expression of multiple chemokines and adhesion molecules in airway SMCs [154–156]. Thus, airway SMCs can perpetuate the inflammation of asthmatic airways [157–159].

There is growing evidence that Ca^{2+} signaling is important in the regulation of the production of cytokines and chemokines in other cell types such as T cells [160]. In human adipocytes, secretion of macrophage inhibitory factor (MIF), CD14, macrophage colony-stimulating factor, IL-6 and expression of monocyte chemoattractant protein-1 were all Ca^{2+} -dependent [161]. IL-1 β production by murine peritoneal macrophages [162] and exercise-induced IL-6

expression by skeletal muscle cells [163] were also regulated by $[Ca^{2+}]_i$. In human bronchial epithelial cells, particulate matter induced IL-1 β and IL-8 mRNA expression with an elevation of $[Ca^{2+}]_i$ [164]. Additionally, perturbing Ca^{2+} homeostasis affects mRNA expression of heme oxygenase-1 in human endothelial cells [165] and nuclear factor κ B (NF κ B) activation and reactive oxygen species production in monocytes [166].

In airway SMCs, thapsigargin, an inhibitor of SERCA [167–169], modulated the differential effect of TNF- α on the production of IL-6 and RANTES (regulated on activation, normal T cell expressed and secreted) [155]. Thapsigargin also reduced TNF- α -induced expression of intercellular adhesion molecule 1 (ICAM-1) by suppressing the activation of NF κ B [154]. Moreover, nickel ions (Ni^{2+}) which act as a broad Ca^{2+} channel blocker to reduce Ca^{2+} influx, abrogated CD40-induced NF κ B activation in human airway SMCs [156]. These findings suggest that the $[Ca^{2+}]_i$ homeostasis is also involved in cytokine/chemokine production in airway SMCs. Furthermore, cytokine exposure affects airway SMC Ca^{2+} signaling by modulating of the sarcoplasmic reticulum Ca^{2+} stores [154].

One of the key proteins used to transduce increases in $[Ca^{2+}]_i$ is calmodulin (CaM), a 148-amino acid protein that binds up to four Ca^{2+} ions. An important action of CaM is the activation of a family of Ca^{2+} /CaM-dependant protein kinases including CaM kinases I, II, III, and IV, myosin light chain kinase, and phosphorylase kinase [170]. In NIH 3T3 cells, an increase in $[Ca^{2+}]_i$ induced by IFN- γ activates CaM kinase II, which in turn, phosphorylates signal transducers and activators of transcription-1 (STAT-1) on serine 727 for maximum transcription activation [171]. In contrast, the inhibition of CaM kinases suppresses IFN- α induced STAT-1 tyrosine phosphorylation and the activation of macrophages [172]. It would be of interest to determine if Ca^{2+} regulates airway SMC STAT-1 activation and cytokine production stimulated by the IFN family.

Summary

Airway SMCs possess a wide array of ion channels which reside in plasma membrane, including receptor- or store-operated channels that may be comprised of a variety of TRP channels, stretch-activated channels, voltage-gated channels and Ca^{2+} -dependent K^+ channels. In addition, SMCs have specialized receptor/channels that reside in the sarcoplasmic reticulum. The activity of these channels significantly contributes to changes in the $[Ca^{2+}]_i$, which, in turn, determines, in the short term, the magnitude of SMC contraction and, in the longer term, SMC proliferation and the production and secretion of pro-inflammatory factors. In an exaggerated state, these cell activities are believed to be collectively expressed as airway hyper-responsiveness. While changes in $[Ca^{2+}]_i$ are common to each processes, the question is raised of how this single ion messenger mediates this diversity of responses. Although the nature of the Ca^{2+} signals associated with proliferation or secretion are uncharacterized, the Ca^{2+} waves and oscillations associated with SMC contraction clearly indicate that airway SMC Ca^{2+} signaling is spatially and temporally dynamic. The key implication of this is that pattern of the changes of the $[Ca^{2+}]_i$ are also important. In this respect, the distribution and temporal expression of a variety of ion channels that alter $[Ca^{2+}]_i$ provides the necessary plasticity to regulate different SMC functions. As a result, these ion channels form an attractive target for therapeutic approaches for asthma.

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Figure 1. Ion channels and their contribution to Ca^{2+} changes in airway smooth muscle cells
 The binding of agonist to its receptor activates phospholipase C (PLC) via membrane G-proteins to synthesize inositol trisphosphate (IP_3) and diacylglycerol (DAG) from membrane lipids. IP_3 (blue triangles) diffuses throughout the cell and binds to the IP_3R , sensitizing it to Ca^{2+} , which stimulates the opening of the receptor to allow the release of Ca^{2+} from the sarcoplasmic reticulum (SR). The released Ca^{2+} in turn stimulates (+ black arrow) Ca^{2+} -induced Ca^{2+} release from adjacent, sensitized IP_3Rs which results in the propagation of a Ca^{2+} wave. The released Ca^{2+} subsequently inactivates the IP_3R (– red arrow) and the Ca^{2+} is re-accumulated in the SR via Ca^{2+} pumps (SERCA). The repetitive release and uptake of Ca^{2+} results in Ca^{2+} oscillations. DAG and its derivatives (e.g. arachidonic acid, AA) move in the membrane to activate receptor-operated Ca^{2+} influx or channels (ROC) to replenish internal Ca^{2+} stores. During the Ca^{2+} oscillations, the Ca^{2+} store is partially depleted and this is sensed by STIM1 in the SR. STIM1 translocates through the SR membrane to aggregate with and activate store-operated Ca^{2+} entry or channels (SOC) (consisting of Orai1 or TRP proteins) to replenish the internal Ca^{2+} store. Ryanodine receptors (RyR) may release internal Ca^{2+} in the form of Ca^{2+} sparks to activate near by K_{Ca} channels to induce membrane hyperpolarization. $\text{K}_{\text{Ca}1.1}$ channels may be replaced with $\text{K}_{\text{Ca}3.1}$ channels during cell proliferation to alter membrane voltage which may influence voltage-dependent Ca^{2+} channels (VDC). Stretch-activated channels (SA) are directly gated by physical stimuli.