Ca^{2+} Oscillations Regulate Contraction Of Intrapulmonary Smooth Muscle Cells

Michael J. Sanderson, Yan Bai, and Jose Perez-Zoghbi

Abstract Pulmonary blood pressure is a function of the resistance of the intrapulmonary blood vessels. Consequently, the mechanisms controlling blood vessel smooth muscle cell (SMC) contraction serve as potential sites for hypertension therapy. To explore these mechanisms, access to the intrapulmonary vessels is required and this is provided by the observation of a unique lung slice preparation with microscopy. There are 2 major processes that determine SMC tone; the intracellular Ca^{2+} concentration and the sensitivity of the SMCs to Ca^{2+}. Agonist-induced increases in Ca^{2+} occur in the form of propagating Ca^{2+} oscillations that predominately utilize internal Ca^{2+} stores and inositol trisphosphate receptors. The frequency of these Ca^{2+} oscillations correlates with contraction. Agonists also increase Ca^{2+} sensitivity of SMCs to enhance contraction. Changes in membrane potential mediated by KCl also stimulate contraction via slow Ca^{2+} oscillations and increased sensitivity. However, these slow Ca^{2+} oscillations rely on Ca^{2+} influx to drive the cyclic release of over-filled Ca^{2+} stores via the ryanodine receptor. The relaxation of SMC tone can be induced by the reduction of the frequency of the Ca^{2+} oscillations and the Ca^{2+} sensitivity by b_2-adrenergic agonists or nitric oxide.

Keywords Pulmonary hypertension • Confocal microscopy • Lung slices • Mouse • Arterioles • Airways

1 Introduction

Increased pulmonary vasculature resistance is a key parameter associated with the various forms of pulmonary hypertension.[1] In this chapter, we address the normal Ca^{2+}-based mechanisms that lead to acute vasoconstriction and increased vasculature resistance.

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A major concern associated with the investigation of the mechanisms of pulmonary vasoconstriction is that the small intrapulmonary arterioles, which are thought to respond to hypoxia and other molecular stimuli (agonists) associated with pulmonary hypertension,[2] are the focus of attention. This issue is not easily addressed because these vessels are widely distributed and form an integral part of the lung tissue. A common technique used to investigate the responses of these blood vessels within the lung has been the measurement of pulmonary blood pressure of whole lungs (either in vivo or in vitro) in response to blood-borne agonists. This approach has a number of disadvantages when attempting to understand smooth muscle cell (SMC) physiology. For example, the responses to the blood-borne agonist are likely to be filtered by endothelial cell responses. More important, the measured parameter of pressure provides little information regarding the mechanisms that regulate contraction. The usual alternative to overcome this problem is the study of isolated or cultured individual SMCs. But, this approach also has its limitations. While changes in intracellular signals are accessible in single cells, the correlation of these responses with the contraction of the blood vessel is lost. In addition, the isolation of SMCs from blood vessels leads to the loss of their extracellular matrix and interactions with neighboring cells and the lung parenchyma. In addition, even short periods of cell culturing can alter SMC function, and the adherence of cells to the culture surface limits their contractility. Moreover, the isolation of pure vascular SMCs from small intrapulmonary blood vessels is extremely difficult because, within the lung, the arteries are very close to the airways; therefore, the isolation of SMCs does not render a purified population of vascular cells. The use of isolated large vessels, such as the extrapulmonary arteries, can facilitate the isolation of specific SMCs, but these SMCs would not seem to be the most relevant cells with which to study physiological responses related to pulmonary hypertension. Of course, without cell culture techniques, many important advances in SMC biology would not have been possible, but in this case, where the cumulative output of the cell response is a change in blood vessel diameter, the disparate scales of cell physiology and blood vessel dynamics need to be bridged.

2 Lung Slices

One experimental approach to study intracellular signals in vascular SMCs while retaining the morphology and the dynamic state of the blood vessel is the use of thin, live lung slices.[3,4] Lung slices are prepared by filling the alveolar spaces with warm (~37°C) fluid agarose via the trachea.[5] The gelling of the agarose by cooling transforms the spongy soft lung parenchyma into a more solid gel that can be sectioned with a vibratome. Lung slices ranging from 75 to 250 μm thick can be cut and are suitable to investigate SMC physiology. However, slices cut too thin can result in damage to long cells, such as the SMCs, that may spiral around the lumen of the vessels. Each lung slice predominantly consists of alveoli tissue; within this, the cross sections of arteries, veins, and airways can be found (Fig. 5.1). The airways are easily identified by their epithelium with active cilia. The arteries or

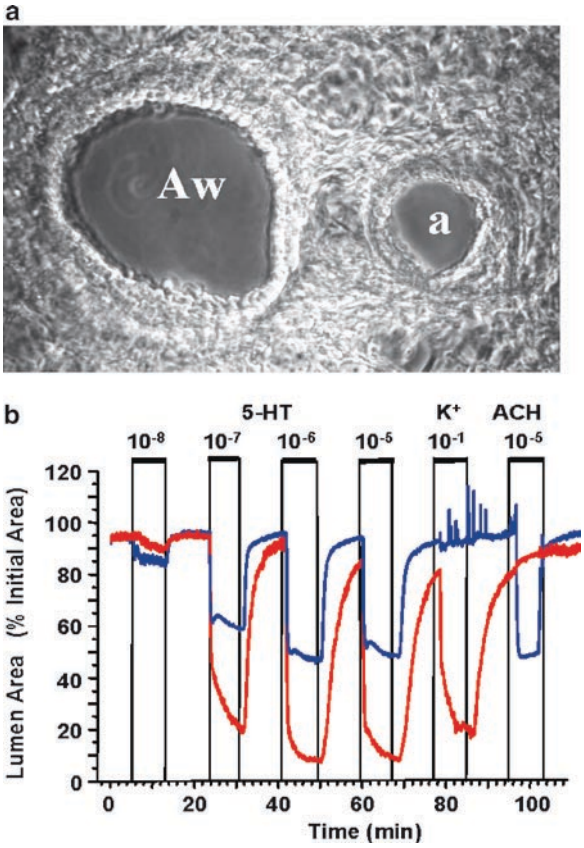

Fig. 5.1 (**a**) The appearance of an arteriole (*a*) and airway (*Aw*) in a mouse lung slice as observed with phase-contrast microscopy. (**b**) The contractile response of the arteriole and airway to a range of increasing concentrations of 5-hydroxytryptamine (5-HT), high K^+, and acetylcholine (ACh)(molar). The extent of contraction is expressed as a percentage of the initial lumen size. The arteriole (*red trace*) displays a slow contraction and relaxation rate in response to 5-HT in comparison to the airway (*blue trace*). Although K^+ induces twitching in both the airway and arteriole SMCs, a greater cumulative contraction is observed in the arteriole. The arteriole does not contract in response to ACh. Hanks' balanced salt solution (HBSS) is used to wash the lung slice between agonist applications

arterioles are also easily found because these vessels partner the airways through the lung (Fig. 5.1). Veins are generally found as independent structures. Using this technique, live lung slices have been prepared from a variety of animals, including mice,[6] rats,[7] guinea pigs,[8] and humans.[8,33]

The ability to retain blood vessel morphology in mouse lung slices has been more challenging than for the airways. For the most part, the airways are found, after slicing, to be fully relaxed and open (mouse and rat). However, the mouse blood vessels have a tendency to collapse and appear to have undergone an irreversible contraction. The underlying cause of this response is not fully understood, but it does underscore that there is a significant difference in the way airway and blood vessel SMCs respond to the same stimulation (cutting). To obtain noncollapsed blood vessels, we

modified the preparation of the lungs for sectioning by the additional instillation of warm liquid gelatin into the blood vessels via the pulmonary artery.[4] This has the effect of resisting the contraction that appears to occur in response to sectioning. Gelatin was used instead of agarose because gelatin dissolves at 37°C and, as a result, can be easily removed after slicing, leaving the blood vessel lumen empty and without lumen resistance to contraction for future experiments. However, we observed that some blood vessels also collapsed after the gelatin was dissolved at 37°C. From a practical point of view, the retention of the agarose in the alveoli serves as a replacement for pleural pressure and cannot be removed, otherwise the whole slice would collapse. The collapse of the blood vessels appears to result from a change in its interaction with the lung parenchyma, as indicated by an open or expanded appearance of the vessel adventitia. By contrast, the airways do not show this morphology and may have a stronger connection with the lung parenchyma and the associated tethering forces that maintains their relaxed or open state. In view of weak tethering of blood vessels, a positive pulmonary blood pressure, although normally low, may be more important for the dilation of the blood vessels. As a result, the use and retention of agarose in the blood vessels is being evaluated.

Lung slices provide many experimental advantages. These include the ability to examine the responses of different size vessels[9] and the applicability of the technique to a variety of animals and humans. Lung slices are viable for several days and appear to retain most of their physiological responses to agonists. The adjacent location of the blood vessel to the airway also provides a unique opportunity for comparative studies of vascular and airway SMC activity.[4,10,11] This is particularly useful because each SMC type serves as a control for the other in experiments in which activities of blood vessels and airways are recorded simultaneously. Perhaps the most important aspect of the lung slice is that individual cells are readily observed by microscopy techniques. In combination with fluorescence Ca^{2+} reporter dyes and confocal or two-photon microscopy, the changes in $[Ca^{2+}]_i$ (intracellular calcium concentration) have been imaged in individual SMCs, and this activity has been correlated with blood vessel and airway contraction.[4,12]

3 Response of Arterioles to Agonists

Perfusion of mouse lung slices with a variety of agonists, such as 5-hydroxytryptamine (5-HT) (100–1,000 n*M*), induces contraction of the blood vessels (Fig. 5.1). Although this is expected, the advantage of the lung slices is that they provide the ability to assess the dynamics and magnitude of this contraction, especially because the activity can be compared with the airway SMCs. In comparison to the airway, 5-HT induces a large but slower contraction of the blood vessel. Interestingly, on the removal of 5-HT, the relaxation rate of the blood vessel is also slower than the airway (Figs. 5.1 and 5.2). Blood vessels also strongly, but slowly, contract in response to low concentrations (10 n*M*) of endothelin (ET)[12] (Fig. 5.3). The response to ET is only slowly reversed by extensive washing. However, mouse intrapulmonary blood vessels do not respond to acetylcholine (Fig. 5.1) or phenylephrine.[4]

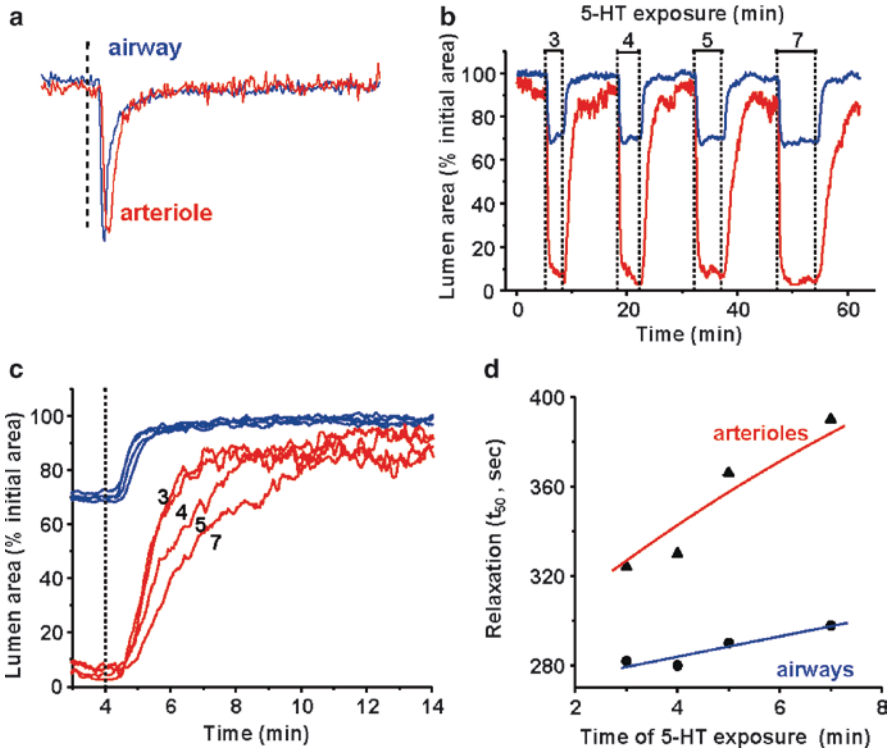

Fig. 5.2 The relaxation rate of arteriole and airway SMCs as a function of agonist exposure. (**a**) The dynamics of a similar size transient contraction induced by 20 m*M* caffeine in an arteriole (*red*) and airway (*blue*) appear similar. (**b**) The sequential contraction of an arteriole (*red*) and airway (*blue*) in response to increasing exposure times (minutes) of 1 μ*M* 5-HT. (**c**) The alignment (at 5-HT washout time) of the four maximal contractions induced by 5-HT shown in **b** to demonstrate the effect of contraction time on the relaxation time of both the airway and arteriole. (**d**) The time to attain 50% of relaxation (t_{50}) as a function of the exposure time to 5-HT from the data shown in **c**. In the airway, the duration of relaxation is slightly increased, whereas in the arteriole the relaxation time substantially increases with exposure time to 5-HT

A simple explanation for a slower relaxation rate of the arteries with respect to the airways is the idea that the blood vessels are weakly tethered to the lung parenchyma compared to the airways. This, in addition to the lack of vascular lumen pressure in the lung slices, would be consistent with the constriction of the blood vessels during sectioning. However, the rates of relaxation of an arteriole and an airway were comparable when transiently contracted to a similar extent using caffeine to induce a transient release of internal Ca^{2+} in both SMC types (Fig. 5.2a) This response suggests that during Ca^{2+}-induced contraction, similar tethering forces act on the airway and arteriole.

On the other hand, the rate of blood vessel relaxation appears to be influenced by the duration of agonist stimulation (Fig. 5.2). The relaxation time of the arterioles, after reaching a similar contractile state, was increased by extending the duration of sustained contraction induced by 5-HT (Fig. 5.2d). This behavior would not be

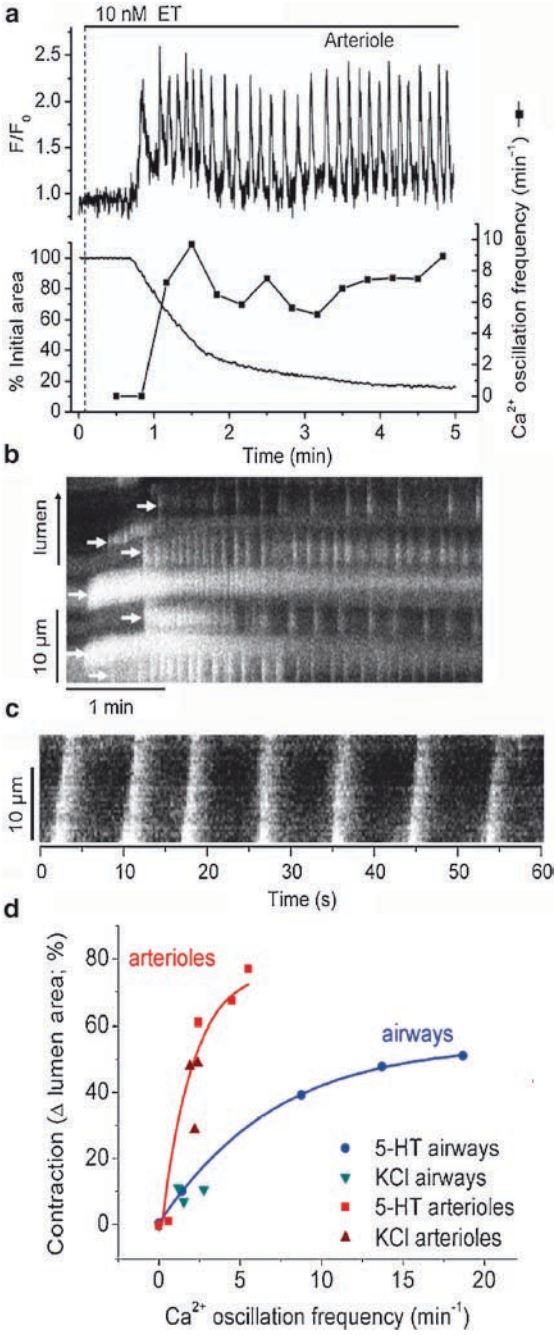

Fig. 5.3 (**a**) Stimulation of Ca^{2+} oscillations in a single SMC (*top*) and contraction (*bottom*, *continuous line*) in a mouse arteriole in a lung slice in response to endothelin (ET). The increase in the frequency of the Ca^{2+} oscillations in response to ET is shown (*bottom*, *squares* and *line*).

expected to result from loose or weak tethering or a lack of blood pressure. Because the relaxation rate after a brief contraction was initially fast (Fig. 5.2a), it would appear that the blood vessel SMCs have stiffened to a greater extent than airways in proportion to the duration of agonist exposure. This observation suggests a difference in the intrinsic mechanism for maintaining a contractile force between arteriole and airway SMCs. The development in the arterioles of a more extensive latch state than in the airways could explain this difference, and this property may be a useful mechanism to help arterioles develop a sustained contraction against blood pressure.

4 Ca^{2+} Signaling of Intrapulmonary SMCs

It has been well established that increases in intracellular calcium concentration ($[Ca^{2+}]_i$) stimulate the contraction of SMCs. However, due to the lack of imaging technologies with adequate spatial and temporal resolution and the use of cultured cells to study Ca^{2+} signaling, the dynamics of SMC Ca^{2+} signaling in situ appears to have been greatly underestimated. The classic "textbook" description of agonist-induced increases in $[Ca^{2+}]_i$, in both vascular and airway SMCs, consists of an initial spike followed by a decreased and sustained plateau and force production by the SMCs correlated with the sustained phase of Ca^{2+} increase. This activity is in stark contrast to the Ca^{2+} signals we have recorded from blood vessel and airway SMCs in lung slices with confocal or two-photon laser scanning microscopy and recording rates of 15–30 images per second. We found that the agonists 5-HT and ET induced Ca^{2+} signaling in the SMCs consisting of a series of Ca^{2+} oscillations[4,12] (Fig. 5.3). The frequency of these Ca^{2+} oscillations increases with agonist concentration; importantly, the magnitude of the blood vessel and airway contraction increases with the increasing frequency of the Ca^{2+} oscillations (Fig. 5.3). The frequency of the Ca^{2+} oscillations in arteriole SMCs reaches a maximum of about 10 per minute (10 n*M* ET after 5 min at room temperature).

Each Ca^{2+} oscillation also has a spatial organization; in general, the Ca^{2+} oscillation begins as an increase in Ca^{2+} at one end of the cell, and this propagates as a wave of Ca^{2+} to the other end of the cell (Fig. 5.3). It is not uncommon for the direction

Fig. 5.3 (continued) (**b**) The asynchronous nature of Ca^{2+} oscillations occurring in multiple adjacent SMCs represented by a line-scan analysis. The line of interest (distance, *vertical axis*) is oriented parallel to the vessel and across the circumferential SMCs. Each individual cell is represented by a horizontal trace (time) indicated by a *white arrow*. Each Ca^{2+} oscillation appears as a *white vertical line* and occurs asynchronously in each cell. The initiation of the Ca^{2+} oscillations by ET is also asynchronous. (**c**) A line-scan analysis of a single SMC demonstrating that with each Ca^{2+} oscillation a Ca^{2+} wave is propagated along the length of the cell. In this case, the line of interest is aligned along the cell (vertical distance). Because the Ca^{2+} wave propagates along the cell, the trace has a *slope* to the *right* (with respect to time) that indicates wave propagation velocity. (**d**) The relationship between the frequency of the Ca^{2+} oscillations and the contraction of arterioles (*red*) and airways (*blue*) in response to 5-HT and KCl. Slow Ca^{2+} oscillations induced in arterioles by 5-HT and KCl induce a larger contraction compared to the 5-HT-induced contraction of airways that is mediated by faster Ca^{2+} oscillations. Slow Ca^{2+} oscillations induced by KCl in airways induce little contraction

of the wave propagation to reverse. This usually occurred when a Ca^{2+} wave prematurely dissipated and failed to propagate along the full length of the cell. Under these circumstances, a second initiation site emerged near the opposite end of the cell and propagated a Ca^{2+} wave in the opposite direction. This spatial behavior of Ca^{2+} waves has interesting implications; the initiation of Ca^{2+} waves at the end of the cells suggests that these regions have the highest pacemaker activity, and this may be the result of a narrowing of the SMC toward its ends. It is possible that the concentrations of the internal messengers (in this case, inositol 1,4,5-trisphosphate, IP_3) are higher in the smaller cell volumes (assuming equal production per unit membrane) near the end of the cell. A higher concentration of IP_3 would be predicted to lead to the earlier activation of the IP_3 receptors (IP_3Rs) and the origination of the Ca^{2+} waves (Fig. 5.4). An alternative possibility is that the density of the IP_3Rs is higher toward the ends of the cell.

The spatial behavior also emphasizes that the mechanism propagating the Ca^{2+} wave is regenerative, and this is believed to be mediated by Ca^{2+}-induced Ca^{2+} release (CICR) from the sarcoplasmic reticulum (SR) via IP_3-sensitized IP_3Rs (Fig. 5.4). Although the Ca^{2+} waves spread throughout the cell, they do not appear to be propagated to adjacent cells. As a result, agonist-induced Ca^{2+} oscillations appear asynchronously in multiple SMCs (Fig. 5.3). It is important to point out that each agonist-induced Ca^{2+} oscillation does not normally initiate a wave of contraction or "twitch" response in each SMC. This implies that the regulation of the cellular contractile apparatus by the phosphorylation state of the myosin light chain (MLC) has a time constant significantly slower than the frequency of the Ca^{2+} oscillations and therefore integrates the Ca^{2+} oscillations into an average response (Fig. 5.4). This has the advantage that sustained contraction can be maintained without a sustained increase in $[Ca^{2+}]_i$, which can be deleterious to the cell. In a similar way, the asynchronous Ca^{2+} oscillations may help the muscle tissue, as a whole, to further integrate the individual Ca^{2+} pulses into a steady, sustained contraction.

Fig. 5.4 (continued) phospholipase C (PLC) to synthesize IP_3 from membrane lipids phosphatidylinositol 4,5-bisphosphate (PIP_2). IP_3 stimulates the release of Ca^{2+} from the sarcoplasmic reticulum (SR) via the IP_3 receptor (IP_3R). Ca^{2+} is returned to the SR via the sarcoplasmic/endoplasmic reticulum Ca^{2+} ATPase (SERCA). The cyclic release and reuptake of Ca^{2+} leads to Ca^{2+} oscillations. Ryanodine receptors (RyRs) do not seem to be activated by Ca^{2+} released from the IP_3R during agonist stimulation. Ca^{2+} oscillations are integrated via calmodulin to stimulate myosin light chain kinase (MLCK) which in turn phosphorylates the myosin light chain (MLC) and initiate contraction ("on rate"). Agonists may also inactivate MLC phosphatase (MLCP) via receptors, protein kinase C (PKC) or Rho kinase (ROK) to decrease the rate of MLC dephosphorylation ("off rate") and enhance contraction. SMC relaxation can be induced by reducing the Ca^{2+} oscillation frequency via the action of cGMP/cAMP on the IP_3R. In addition, cAMP can induce relaxation by stimulating MLCP activity. (**b**) Hypothetical relative speeds of MLCK-mediated contraction and MLCP-mediated relaxation; mouse arterioles have a slower relaxation rate than airways but similar contraction rates. (**c** and **d**) Contraction in arteries (*red line*) and airways (*blue line*) induced by slow Ca^{2+} oscillations (*gray lines*) is cumulative in arterioles but transient (twitching) in airways. The fast relaxation rate of airway SMCs allows them to fully relax in the time between each Ca^{2+} oscillation. (**e**) Sustained airway contraction (*blue*) is achieved in response to fast agonist-induced Ca^{2+} oscillations because the time between each oscillation is insufficient to allow relaxation

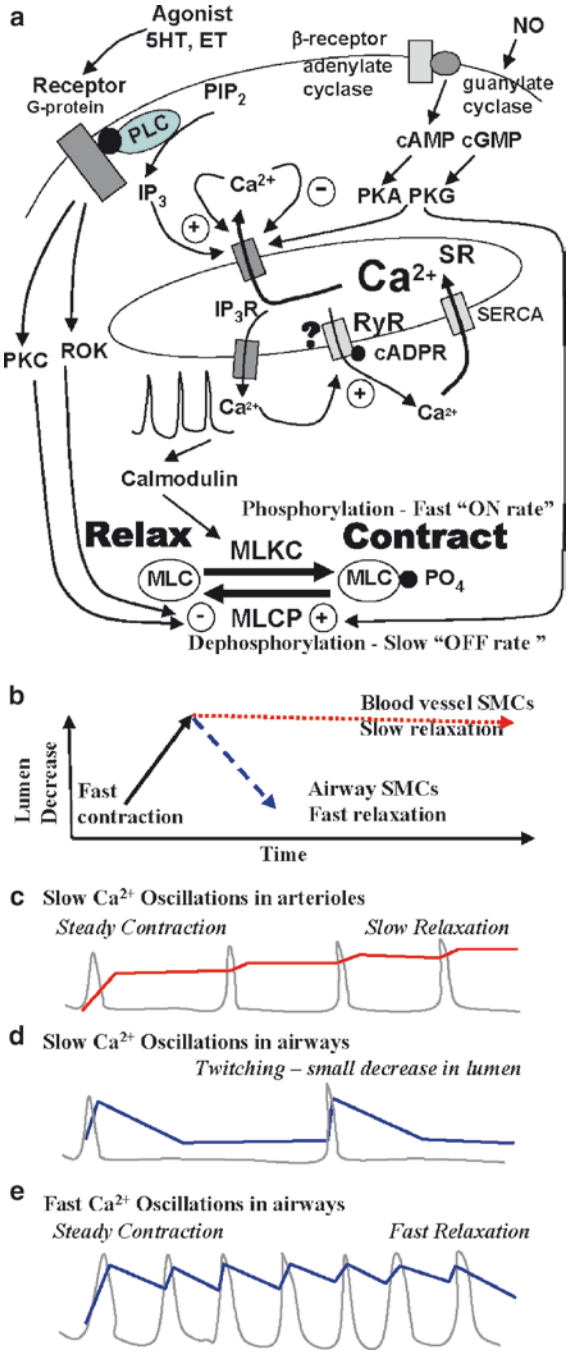

Fig. 5.4 (**a**) A general scheme of the molecular mechanisms contributing to contraction of arteriole SMCs. Agonists (5-HT, ET) stimulate their specific G protein-coupled receptors to stimulate

4.1 Roles of Ca^{2+} Influx and Internal Ca^{2+} Release

A second classical idea that features in many studies aimed at understanding the regulatory mechanisms of pulmonary SMC contraction is that the steady plateau phase of elevated $[Ca^{2+}]_i$, responsible for maintaining force, is primarily determined by Ca^{2+} influx via membrane channel activity. The loss of force after the removal of extracellular Ca^{2+} is consistent with this view.

In our studies with lung slices, it is clear that the steady-state contraction is related to the frequency of the Ca^{2+} oscillations rather than a steady level of $[Ca^{2+}]_i$. Consequently, the mechanism generating these Ca^{2+} oscillations is vital to understanding SMC physiology. The first issue to address is the source of the Ca^{2+} used in the Ca^{2+} oscillations. In the absence of extracellular Ca^{2+}, Ca^{2+} oscillations can both be initiated by agonist and can persist for some time. As a result, a substantial contraction is observed under Ca^{2+}-free conditions. However, in the prolonged absence of Ca^{2+}, the Ca^{2+} oscillations run down and terminate. The frequency of the Ca^{2+} oscillations was not altered by nifedipine, a voltage-dependent Ca^{2+} channel blocker, but the Ca^{2+} oscillations were abolished by agents such as thapsigargin or cyclopiazonic acid (CPA) that inhibit sarcoplasmic/endoplasmic reticulum Ca^{2+} ATPase (adenosine triphosphatase) (SERCA) pumps and lead to an emptying of internal Ca^{2+} stores (Fig. 5.4). The conclusion that can be drawn from these experiments is that Ca^{2+} oscillations primarily utilize internal Ca^{2+} stores. Each Ca^{2+} oscillation requires a release of Ca^{2+} from the SR, and although most of this Ca^{2+} is returned to the SR after the Ca^{2+} release terminates (Fig. 5.4), invariably, some Ca^{2+} is lost to the extracellular environment. As a result, Ca^{2+} oscillations are dependent on external Ca^{2+} to refill the SR store to prevent its depletion. Thus, the classical view would benefit from a redefinition of the roles of Ca^{2+} release and Ca^{2+} influx related to the cellular mechanism of maintaining SMC contraction, and the role of Ca^{2+} influx needs to be investigated in terms of its effect on Ca^{2+} oscillation frequency rather than the steady state $[Ca^{2+}]_i$.

The oscillatory nature of the Ca^{2+} signals in blood vessel SMCs is consistent with the release of Ca^{2+} from the SR via the IP_3R (Fig. 5.4). This process is found in vascular SMCs from other types of blood vessels.[13] With lung slices, we have also found that airway SMCs display Ca^{2+} oscillations in response to agonist (5-HT and acetylcholine [ACh]), and that the frequency of these Ca^{2+} oscillations also correlates with contraction[10] (Fig. 5.3). The Ca^{2+} oscillations in airway SMCs are similar in many ways to those of the pulmonary blood vessels and require external Ca^{2+} for prolonged activity. Importantly, these oscillations can be initiated or increased in frequency by the photolytic release of IP_3 in the SMCs.[14,15] Also consistent with the idea that Ca^{2+} oscillations are mediated by increases in IP_3 is the finding that 5-HT appears to act via $5\text{-}HT_2$ receptors that are linked via G proteins to the activation of phospholipase-β (PLC-β) to produce IP_3. The $5\text{-}HT_2$-receptor-specific antagonist ketanserin blocked the action of 5-HT, whereas 2, 5-dimethoxy-4-iodoamphetamine (DOI), an agonist of this receptor, induced 5-HT-like changes.[4] By contrast, an agonist of the $5\text{-}HT_3$ receptor that mediates Ca^{2+} influx had little effect.

Likewise, the use of specific agonists and antagonists indicates that Ca^{2+} oscillations induced by ET are mediated by an equal contribution by ET_A and ET_B receptors[12] that act via activation of PLC-β to produce IP_3.

4.2 Effect of KCl on Arteriole SMC Physiology

From these data, it appears that pulmonary arteriole SMCs utilize SR Ca^{2+}. However, another idea, which is frequently incorporated into experimental regimes, is that SMC depolarization is a major control signal for the contraction of pulmonary SMCs. Because a common way to experimentally induce membrane depolarization in SMCs is their exposure to high extracellular concentrations of KCl (~100 m*M*), we examined the responses of SMCs in lung slices to this treatment.[4,10]

Although exposure to KCl induces pulmonary arterioles to contract, this contraction is usually slower and smaller in magnitude than that induced by agonists and often displays some regional twitching of the blood vessel. The contractile response to KCl was abolished in the absence of extracellular Ca^{2+} or presence of nifedipine and Ni^{2+}, indicating Ca^{2+} influx is a primary mediator of the response and consistent with the idea that membrane depolarization leads to the opening of voltage-dependent Ca^{2+} channels.

To confirm these ideas, we examined the Ca^{2+} responses of the SMCs to KCl and were surprised to observe that, in place of a steady-state elevation of $[Ca^{2+}]_i$, which might be expected with sustained depolarization, there occurred a series of Ca^{2+} oscillations. However, these Ca^{2+} oscillations were significantly different from those induced by agonists; their frequency was very slow (1–3 per minute), and the duration of the Ca^{2+} increase associated with each Ca^{2+} oscillation was greatly extended.[4,10] Extremely similar Ca^{2+} oscillations were observed in airway SMCs in response to KCl, and in contrast to agonist-induced Ca^{2+} oscillations, a clear correlation between each Ca^{2+} oscillation and an airway SMC twitch could be made. While a similar effect can be observed in vascular SMCs, this effect is reduced. Such twitching behavior associated with KCl-induced Ca^{2+} oscillations suggests that their slow frequency is at the limits of the integration time of the enzymes of the contractile process; in other words, at slow frequencies, especially in the absence of agonist, contraction begins to follow the changes in $[Ca^{2+}]_i$.

A closer examination of the KCl-induced Ca^{2+} oscillations with higher-speed recordings revealed that a series of elemental and localized Ca^{2+} signals with increasing frequency occurred prior to the development of a propagating Ca^{2+} wave or Ca^{2+} oscillation.[4,10] Immediately after a Ca^{2+} oscillation had subsided, these elemental Ca^{2+} signals could not be detected, but with time, they began to reappear with increasing frequency until the next Ca^{2+} oscillation was triggered. Similar activity was observed in airway SMCs. Elemental Ca^{2+} signaling, in the form of Ca^{2+} sparks, has been observed in other SMCs and cardiomyocytes, and these signals have been determined to be the result of localized Ca^{2+} release from the SR via a small grouping of ryanodine receptors (RyRs), specialized SR Ca^{2+} channels.[16,17] Although the

Ca^{2+} elemental events we observed are somewhat bigger than those associated with traditional Ca^{2+} sparks, we found that ryanodine (a RyR antagonist) abolished KCl-induced Ca^{2+} oscillations. Again, similar results were observed in airway SMCs.

From these data, we propose the following mechanism to explain KCl-induced Ca^{2+} oscillations: KCl depolarizes the cell membrane and initiates an initial influx of Ca^{2+} into the cell. To compensate and restore the $[Ca^{2+}]_i$, the cell transports the excess Ca^{2+} from the cytosol not only to the extracellular medium but also to the SR. This redistribution of Ca^{2+} will continue while there is a Ca^{2+} influx, with the result that the SR Ca^{2+} content increases. RyRs appear to be sensitive to this SR Ca^{2+} content and become more likely to open.[16] The opening of a RyR releases Ca^{2+}, which itself can stimulate adjacent sensitized RyRs, with the result that a localized Ca^{2+} event occurs. Because the Ca^{2+} discharge associated with these local events does not counter the accumulation of Ca^{2+} in the SR, the SR Ca^{2+} content continues to increase with time. This in turn further sensitizes the RyRs to elicit more Ca^{2+} elemental events. However, when the Ca^{2+} content of the SR reaches a threshold level and the RyRs are very sensitive to Ca^{2+}, an elemental Ca^{2+} release event quickly turns into a propagating Ca^{2+} wave by the process of CICR. This global increase in $[Ca^{2+}]_i$ has the effect of lowering the Ca^{2+} content of the SR, which desensitizes the RyRs, and the process can begin again to generate the slow Ca^{2+} oscillations.

4.3 Ca^{2+} Sparks: A Relaxation Mechanism in Pulmonary SMCs?

In almost all respects, the contractility and Ca^{2+} signaling of the SMCs in the lung slices is highly reproducible and consistent. The tissue morphology appears normal, and as mentioned, the SMCs retain their cell contacts and extracellular matrix. In our opinion, lung slices appear to represent a healthy preparation of lung tissue. Yet, we have failed to observe Ca^{2+} sparks in the multitude of SMCs we have examined in relaxed blood vessels, even though Ca^{2+} sparks have been suggested to serve as a fundamental relaxation mechanism for vascular SMCs. One explanation could be that the sensitivity of our confocal or two-photon microscope systems is inadequate. However, we clearly observed elemental signals associated with KCl-induced Ca^{2+} oscillations and Ca^{2+} sparks within cardiomyocytes in the myocardial sheath surrounding the pulmonary vein in mouse lung slices.[18]

The Ca^{2+} sparks are believed to induce SMC relaxation by hyperpolarizing the cell membrane via the localized activation of spontaneous transient outward currents (STOCs) carried by K^+ though Ca^{2+}-activated K^+ channels[19]; hyperpolarization would counter Ca^{2+} influx via voltage-dependent Ca^{2+} channels. However, contraction of intrapulmonary vascular SMCs appears to be regulated by internal Ca^{2+} release in the form of agonist-induced Ca^{2+} oscillations rather than by membrane depolarization and activation of voltage-dependent Ca^{2+} channels.[20] Thus, membrane hyperpolarization would appear to have a minimal contribution to relaxation in intrapulmonary blood vessel SMCs.

4.4 The Role of Ryanodine Receptors

The inhibition of elemental Ca^{2+} signaling in cells exposed to KCl by ryanodine supports the idea that RyRs are present in intrapulmonary SMCs. However, if Ca^{2+} sparks do not strongly feature in intrapulmonary SMC physiology, what role might the RyR have? Because RyRs can be gated open by increases in $[Ca^{2+}]_i$, RyRs may contribute to the propagation of Ca^{2+} waves associated with Ca^{2+} oscillations by amplifying or replacing the Ca^{2+} release initiated via IP_3Rs (Fig. 5.4). Surprisingly, we have found that ryanodine has no influence on the frequency or form of the agonist-induced Ca^{2+} oscillations of both blood vessel and airway SMCs.[34] Similar findings apply to agonist-induced Ca^{2+} oscillations in other SMCs.[21,22] This suggests that the RyR does not respond to the local changes in $[Ca^{2+}]_i$ associated with agonist-induced Ca^{2+} oscillations. Part of the reason for this might be that agonist-induced Ca^{2+} oscillations lower the SR Ca^{2+} content, and as a result, the RyRs become desensitized.[16] In airway SMCs, it has been suggested that increased cyclic adenosine diphosphate (cADP)-ribose production associated with inflammation can sensitize the RyR for it to take part in cell signaling.[23] It is therefore possible that the RyR contributes more to the pathologic changes associated with the SMCs in pulmonary hypertension, such as those of cell growth and proliferation, when the activity of CD38 may be increased.

5 Frequency-Modulation as Method for Contraction Regulation

In blood vessels, as well as in airways, an increase in the frequency of the Ca^{2+} oscillations correlated with an increase in contraction (Fig. 5.3). This has led to the suggestion that extent of contraction is regulated by a frequency-modulated (FM) mechanism. This approach has the potential advantage of generating sustained contraction without sustained increases in $[Ca^{2+}]_i$. In addition, a reliance on distinct Ca^{2+} oscillations rather than steady-state $[Ca^{2+}]_i$ may be a more accurate way to detect regulatory signals because Ca^{2+} oscillations will be less susceptible to small variations in cytoplasmic $[Ca^{2+}]_i$.

Multiple steps of phosphorylation of Ca^{2+}/calmodulin-dependent protein kinase II (CaM-kinase II) in response to sequential Ca^{2+} oscillations have been proposed as a specialized mechanism mediating FM regulation.[24] However, the concept of FM regulation only requires that the forward activation of the system ("on rate") has faster kinetics than the reverse inactivation ("off rate"). Rather than requiring a specialized single molecule such as CaM-Kinase II, this arrangement can be achieved by a pair of antagonistic enzymes that have different kinetics. In this respect, SMCs appear to have the ideal molecular control to take advantage of FM regulation. The forward step, stimulated by Ca^{2+}, is mediated by calmodulin and

MLCK (myosin light chain kinase) and results in phosphorylation of MLC. The reverse step, dephosphorylation of MLC, is mediated by MLCP (MLC phosphatase); slower kinetics of this enzyme will result in sustained contraction during the presence of Ca^{2+} oscillations (Fig. 5.4).

Although it appears, at first sight, that the frequency of Ca^{2+} oscillations dominates the control of contraction (perhaps because of their visual impact and reliable observation), comparative studies with different agonists, KCl, or airway SMCs revealed that their influence on the extent of contraction is variable. In mouse blood vessels, the Ca^{2+} oscillations occurred with frequencies up to a maximum of 10 per minute and induced a large contraction (about 80% with ET). Yet, in airway SMCs, the Ca^{2+} oscillations occurred at higher frequencies (25 per minute) but were associated with smaller contractions (Fig. 5.3d). The Ca^{2+} oscillations induced by KCl were very slow and induced a small contraction with pronounced twitching in airway SMCs but greater contraction in blood vessels with less twitching (Fig. 5.3d).

To explain these variable responses, we have proposed a hypothesis based on the duration of the off rate of the control of contraction (Fig. 5.4). If we assume that the on rate of contraction is similar between SMCs, an idea that mathematical modeling of the Ca^{2+}-dependent kinetics of calmodulin and MLCK activation upholds,[25] then the off rate (MLC dephosphorylation by MLCP) will determine the duration and extent of contraction. With a slow off rate, slow Ca^{2+} oscillations can stimulate a sustained-steady contraction; this is the case of agonist-induced contraction in blood vessels. With a fast off-rate, slow Ca^{2+} oscillations can only stimulate a weak, twitch contraction; this is the case with KCl-induced contraction in airways. Fast Ca^{2+} oscillations are required for a sustained contraction with a fast off rate; this is the case with agonist-induced contraction of airways (Fig. 5.4).

Because the activity of MLCP is the major determinate of the off rate, MLCP activity should be considered to be equally important as the Ca^{2+}-based mechanisms that regulate SMC contraction. It should also be noted that although most agonists that regulate contraction commonly stimulate Ca^{2+} oscillations and increases in $[Ca^{2+}]_i$, they also simultaneously regulate MLCP activity.

6 The Influence of Ca^{2+} Sensitivity on Contraction

The variability in the extent of contraction that occurs in the presence of a constant level of $[Ca^{2+}]_i$ (i.e., a constant on rate) is referred to as the Ca^{2+} sensitivity of the SMCs (i.e., the off rate). A high Ca^{2+} sensitivity implies a strong contraction and hence low MLCP activity, whereas low Ca^{2+} sensitivity indicates a tendency to relax or give weak contraction and is mediated by high MLCP activity. In keeping with its definition, the method of measuring Ca^{2+} sensitivity requires the stimulation of SMCs with a constant level of $[Ca^{2+}]_i$ and the measurement of the extent of contraction. This can only be achieved if the $[Ca^{2+}]_i$ of the SMCs can be experimentally controlled.

6.1 A Unique Nondestructive Technique for Ca^{2+} Permeabilization

Control of $[Ca^{2+}]_i$ requires the circumvention of the ability of the cells to regulate a Ca^{2+} gradient across its membranes. This can be achieved by "permeabilizing" the cell membrane with bacterial toxins or mild detergents. However, such treatments have the disadvantage that they also increase the permeability of the membrane to a number of other ions and cytoplasmic constituents, including important messenger molecules, by forming large pores in the plasma membrane. In addition, it is unclear if these treatments alter the membrane permeability of intracellular organelles, including the SR.

We have adapted an alternative approach in our laboratory to specifically elevate membrane Ca^{2+} permeability that exploits the ion channels of the cells and induces little membrane damage. This technique involves the treatment of lung slices simultaneously with caffeine and ryanodine.[11] Caffeine serves as an agonist of the RyR and stimulates its activation. By contrast, ryanodine serves as an antagonist of the RyR, and when RyR is activated by caffeine, ryanodine irreversibly locks the RyR in an open state. As a result, the SR is depleted of Ca^{2+}. Normally, the emptying of Ca^{2+} from the SR leads to the opening of store-operated channels (SOCs) in the plasma membrane, allowing a Ca^{2+} influx that can be used to replace the Ca^{2+} lost from the SR.[26] However, after caffeine and ryanodine treatment, any Ca^{2+} returned to the SR will not accumulate because of the open RyRs. Thus, the cell has a persistent influx of Ca^{2+} via SOCs. The $[Ca^{2+}]_i$ can now be manipulated by varying external $[Ca^{2+}]$. The advantage of this technique is that the cell and tissue structure are unaffected, and agonist receptor function remains viable. In addition, Ca^{2+} mobilization by agonists is prevented because the SR remains empty. There appears to be no loss of cell constituents with this technique; as a result, the Ca^{2+}-permeabilized lung slices can be experimentally used for many hours to investigate the influence of Ca^{2+} sensitivity.[11]

6.2 Agonist-Induced Increases in Ca^{2+} Sensitivity

After Ca^{2+} permeablization with caffeine and ryanodine and the exposure of the lung slices to Ca^{2+}-free saline, the $[Ca^{2+}]_i$ falls to zero, and the blood vessels, as well as the airways in both mouse and rat lung slices, are relaxed (Fig. 5.5). On exposure to high extracellular $[Ca^{2+}]$, the $[Ca^{2+}]_i$ is increased in the SMCs, and the mouse blood vessel partially contracts. This indicates that resting arteriole SMCs have a basal level of sensitivity to Ca^{2+}, implying that the activity of the MLCP is not high. Interestingly, under the same conditions, the mouse airway initially contracts but subsequently relaxes even though the $[Ca^{2+}]_i$ remained high (Fig. 5.5). This result implies that in mouse airway SMCs the increase in $[Ca^{2+}]_i$, in addition to initially activating MLCK, also slowly activated MLCP activity, to a level that eventually fully countered the activity of MLCK.[11,25] Because rat airways, rat blood vessels,

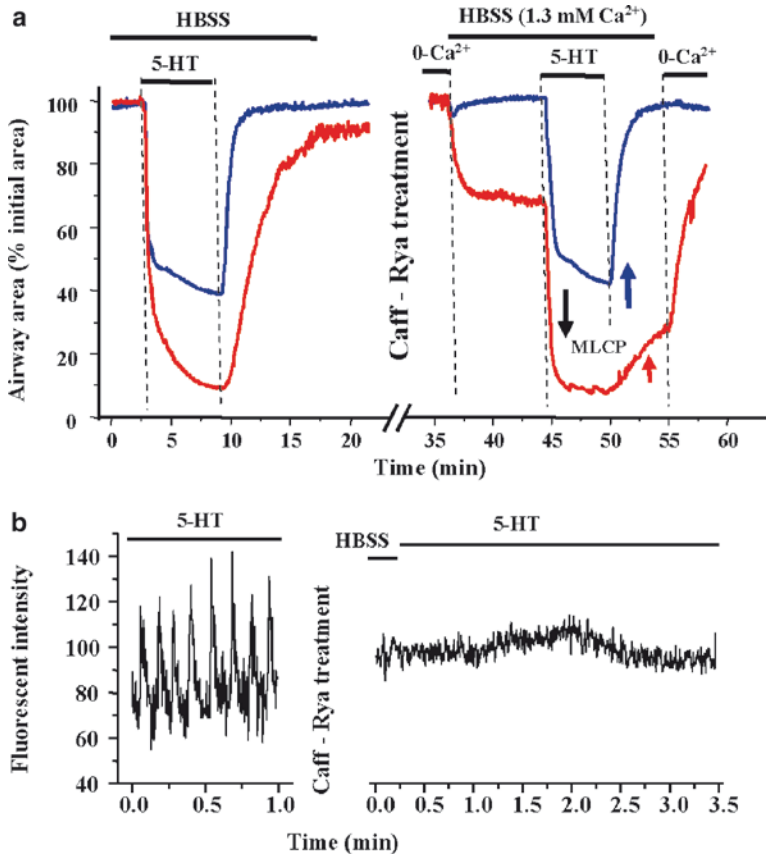

Fig. 5.5 Changes in Ca^{2+} sensitivity induced by increases in $[Ca^{2+}]_i$ and agonists in mouse arterioles (*red*) and airways (*blue*). (**a**) The contraction induced by 5-HT in normal arterioles and airways (*left*) and in Ca^{2+}-permeabilized lung slices (*right*) following treatment with caffeine and ryanodine. In zero extracellular Ca^{2+}, the arteriole and airway are relaxed. An increase in $[Ca^{2+}]_i$ induced by the addition of HBSS containing 1.3 m*M* $CaCl_2$ induces a partial contraction of the arteriole. By contrast, the airway transiently contracts and relaxes. On the addition of 5-HT, both the arteriole and airway display contraction to a level equivalent to that observed in normal lung slices prior to caffeine/ryanodine treatment. Both the airway and arteriole relax on the removal of 5-HT. Because there are no changes in $[Ca^{2+}]_i$ during the addition and removal of 5-HT, the rates of change of the airway and arteriole lumen size indicate the rates of inactivation (*black arrow*, contraction) and reactivation (relaxation; *blue arrow*, airway; *red arrow*, arteriole) of MLCP activity. The relaxation rate of the arteriole is slower than that of the airway. (**b**) Representative changes in $[Ca^{2+}]_i$ in the SMCs (both airway and artery) associated with the treatments shown in **a**. In normal lung slices (*left*), 5-HT induced Ca^{2+} oscillations to mediate contraction. In Ca^{2+}-permeablized slices (*right*), the $[Ca^{2+}]_i$ was sustained at an elevated level after HBSS addition but did not change on the addition or removal of agonist (5-HT)

and human airways all respond to a sustained increase in $[Ca^{2+}]_i$ in a manner similar to mouse blood vessels, it appears that a Ca^{2+}-induced increase in MLCP activity is a species-specific (mouse) response.

If these same Ca^{2+}-permeabilized lung slices are subsequently exposed to a contractile agonist, the blood vessels show a further substantial increase in contraction, albeit without any change in $[Ca^{2+}]_i$. Perhaps more impressively, the mouse airway also displays an increase in contraction that is similar in magnitude to that displayed in a normal slice (Fig. 5.5). The implication is that agonist, acting via the same G protein-coupled receptors that mediate increases in $[Ca^{2+}]_i$ via Ca^{2+} oscillations (now disabled by caffeine/ryanodine treatment), decreases the MLCP activity (increases Ca^{2+} sensitivity) of both arteriole and airway SMCs. Similar findings apply to rat and human airway SMCs. The exact degree of this enhancement of the Ca^{2+} sensitivity by agonist, like the exact stimulation of Ca^{2+} oscillations by agonist, is both agonist and species specific. Thus, the final contraction is a function of the precise changes in Ca^{2+} signaling and Ca^{2+} sensitivity (Fig. 5.3d).

As might be expected, the removal of agonist results in the relaxation of arterioles and airways and implies a reactivation of the MLCP. We had proposed that the relaxation rates differ between arterioles and airways, and that this accounted for differences in the Ca^{2+} oscillation frequency-contraction relationships. With the Ca^{2+}-permeablized lung slices, this difference in relaxation rate can be observed (Fig. 5.5). With a constant MLCK activity, induced by the sustained elevated $[Ca^{2+}]_i$, the removal of 5-HT reveals that the airways quickly and fully relax, indicating a fast and strong reactivation of the MLCP activity (Fig. 5.5, blue arrow). By contrast, the arteriole displays a slow and partial relaxation in the same time (red arrow). Thus, with a similar Ca^{2+} stimulation, the arterioles display greater contraction than airways as a result of their MLCP activity.

7 Relaxation Mechanisms of SMCs

A major goal of therapy for pulmonary hypertension is a reduction in arteriole resistance. In conditions for which increased vessel resistance is primarily due to increased muscle tone rather than changes in arteriole structure, a suppression of Ca^{2+} signaling and Ca^{2+} sensitivity may be an effective therapy. A similar goal is addressed in asthma; acute airway SMC relaxation and relief of dyspnea is achieved by the inhalation of β_2-agonists (steroids are required to counter inflammation for the long-term control of asthma). In lung slices, we have shown that β_2-agonists such as isoproterenol,[14] albuterol,[27] and formoterol[33,35] all induced airway SMC relaxation by reducing the frequency of the Ca^{2+} oscillations. Although β_2-agonists have little effect on blood vessels, we have found similar results with nitric oxide (NO) that reduces Ca^{2+} oscillations in both airways[28] and arterioles of mouse lung slices. The implication of these findings is that the mechanisms of the Ca^{2+} oscillations serve as key contractile signals in arterioles and therefore represent a therapeutic target. The investigation of Ca^{2+} influx, without reference to its role in Ca^{2+} oscillations, is less likely to identify a key regulator.

The mechanism of Ca^{2+} oscillations in pulmonary arteriole SMCs is not well understood, but it is likely that it has many features in common with Ca^{2+} oscillations

in airway SMCs or systemic arterioles.[16] Our experiments with β_2-agonists and NO indicate that a reduction in the frequency of the Ca^{2+} oscillations is achieved by a reduction in the sensitivity of the IP_3R to IP_3.[14,28] This mechanism appears to involve phosphorylation by either cyclic adenosine monophosphate (cAMP)/protein kinase A or cyclic guanosine monophosphate (cGMP)/protein kinase G (PKG) (Fig. 5.4 a) A similar conclusion has been reached with bovine tracheal SMCs, in which IP_3R-associated cGMP kinase substrate (IRAG) protein phosphorylated by PKG associates with and downregulates the IP_3R.[29] However, the frequency of the Ca^{2+} oscillation can be influenced by Ca^{2+} influx as this is required to fully replenish the SR Ca^{2+} content; the period of the Ca^{2+} oscillation is determined, in part, by the time taken to refill the SR.[30] The channels directly contributing to the process of Ca^{2+} influx appear to be associated with the recently identified Orai proteins which are, in turn, regulated by a Ca^{2+} sensor, STIM1 (stromal interacting molecule 1), within the SR.[31] In addition, transient receptor potential (TRP) channels may contribute to this process.[32] While it is reasonable that Ca^{2+} influx via a variety of channels may contribute to the frequency of the Ca^{2+} oscillations, a direct demonstration of this connection would be a major step toward identifying specific channels playing a significant role in contraction and relaxation of pulmonary blood vessels.

In addition to the Ca^{2+} oscillations, as we have emphasized, Ca^{2+} sensitivity plays a major role in contraction. Therefore, it might be no surprise to point out that β_2-agonists exert a substantial part of their relaxing effect on airway SMCs by simultaneously decreasing Ca^{2+} sensitivity by presumably increasing MLCP activity.[27] However, NO appears to much have less effect on Ca^{2+} sensitivity in airway SMCs, and this correlates with its weak action on airway relaxation.[28] The role of MLCP in SMC contractility makes it an attractive therapeutic target that has perhaps been overlooked by our focus on Ca^{2+}.

8 Conclusions

Contraction of intrapulmonary blood vessel SMCs is regulated by dynamic Ca^{2+} signaling in the form of agonist-induced Ca^{2+} oscillations and waves rather than by steady-state $[Ca^{2+}]_i$. The extent of this contraction is also strongly regulated by agonist-induced changes in Ca^{2+} sensitivity. While Ca^{2+} influx via a variety of ion channels can occur, it is unclear how this contributes to contraction. An understanding of the relationship of this Ca^{2+} influx with Ca^{2+} oscillations and contraction would be valuable as this would help identify pivotal therapeutic sites to induce SMC relaxation. It must also be emphasized that SMC contraction and, importantly, relaxation belong to an active process driven by MLCP activity. Because this mechanism can have an equal contribution to SMC contraction as Ca^{2+}-based mechanisms, it also has important therapeutic potential.

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