A Ca\(^{2+}\)- and PKC-driven regulatory network in airway smooth muscle

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In the lungs, contraction of the smooth muscle cells (SMCs) lining the walls of the airways decreases the luminal diameter of these hollow structures, increasing resistance to airflow. Chronic pathological increases in airway smooth muscle contraction have been implicated in the chain of events leading to asthma and chronic obstructive pulmonary disease (COPD). In this issue of The Journal of General Physiology, Mukherjee et al. describe an elegant series of experiments designed to investigate the role of protein kinase C (PKC) in small airway contraction. Here, we discuss the implications of their findings both for our understanding of the mechanisms regulating contraction of airway smooth muscle and for raising the intriguing possibility that PKC could provide a target for therapeutic strategies for treating pathological conditions such as asthma and COPD.

A summary of the pathways regulating airway smooth muscle contraction is depicted in Fig. 1. The contractile state of airway smooth muscle is ultimately determined by the relative activities of myosin light chain (MLC) kinase (MLCK) versus those of the opposing phosphatase (MLCP). This ratio is influenced by the frequency of Ca\(^{2+}\) oscillations and the degree of Ca\(^{2+}\) sensitivity. Contractile agonists that stimulate airway smooth muscle and produce Ca\(^{2+}\) oscillations include substances that promote membrane depolarization (e.g., KCl) and ligands of G protein–coupled receptors that initiate signaling via either the G\(_{q/11}\) or the G\(_{12/13}\) signaling pathways (Mukherjee et al., 2013). Membrane depolarization (with KCl) has been reported to stimulate Ca\(^{2+}\) entry into airway SMCs via L-type voltage-gated calcium channels; this elevates free intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)\(_i\)]) and leads to loading of the SR Ca\(^{2+}\) stores and consequently to release of Ca\(^{2+}\) from the SR into the cytosol. Further amplification of Ca\(^{2+}\) signaling occurs via the mechanism of calcium-induced calcium release via RyRs. This manifests as low frequency Ca\(^{2+}\) oscillations associated with SMC twitching and small unsynchronized reductions in airway luminal diameter (Perez and Sanderson, 2005).

In contrast, contractile agonists such as acetylcholine or 5-hydroxytryptamine produce high frequency Ca\(^{2+}\) oscillations that are correlated with more sustained contractions of SMCs and an associated reduction in airway luminal diameter (Perez and Sanderson, 2005). These agonists are G protein–coupled receptor ligands that activate specific signaling pathways to induce cyclic calcium release via the PLC/iPL3 pathway and Ca\(^{2+}\) sensitization via PKC/CP17 or RhoA/ROCK pathways (Wright et al., 2012). During these more sustained stimulations, SR store depletion leads to oligomerization of STIM and translocation to store-operated Ca\(^{2+}\) channels, which open to replenish the SR stores.

Increased [Ca\(^{2+}\)]\(_i\) promotes Ca\(^{2+}\) binding to the protein calmodulin. Ca\(^{2+}\)-bound calmodulin activates MLCK. Phosphorylation by MLCK of a serine at position 19 on the regulatory MLC initiates cycling of the cross-bridges between myosin and actin, leading to cell shortening and force development. MLCP dephosphorylates this serine, thereby opposing these actions of MLCK. Thus, relaxation can take place if [Ca\(^{2+}\)]\(_i\) decreases, MLCK activity decreases, or MLCP activity dominates over MLCK activity. Ca\(^{2+}\) sensitivity is altered when MLCP activity is inhibited via the RhoA pathway or via phosphorylation of CPI-17 by PKC or ROCK. MLC phosphorylation and shortening velocity of smooth muscle increase rapidly and then decay, even during sustained stimulation. Indeed, MLC phosphorylation decays before maximal force develops. To explain these results, a “latch-bridge” model has been proposed (Hai and Murphy, 1988).

The latch-bridge model suggests that phosphorylation of MLC is essential for actin–myosin cross-bridge formation. Activation of MLCK phosphorylates MLC, allowing cross-bridge cycling and thus development of force. However, once phosphorylated, MLC allows a cross-bridge to form; it may be dephosphorylated by MLCP without detachment of the bridge (creating a noncycling latch-bridge). These latch-bridges have unaltered force-generating capacity but presumably detach at a much slower rate than phosphorylated cross-bridges and so are thought to contribute to sustained tension (Murphy and Rembold, 2005). This model implies that inhibition of MLCP could increase the magnitude of smooth muscle contraction at any Ca\(^{2+}\) concentration.
Lung slices, which preserve most of the macroscopic and microscopic features of the lung within a single slice of tissue (Cooper et al., 2009), have been used for many studies of respiratory physiology and pathology (Liberati et al., 2010; Sanderson, 2011). In a lung slice, one can directly visualize intrapulmonary bronchioles with actively beating ciliated epithelial cells alongside intrapulmonary arterioles, all embedded within the normal lung parenchyma of alveolar tissue. Optical sectioning of lung slices with a confocal or two-photon microscope permits direct visualization of individual cells within the intrapulmonary airways. Changes in airway contractility can then be correlated with simultaneous changes in \([\text{Ca}^{2+}]_i\) when slices are loaded with \([\text{Ca}^{2+}]_i\) indicator dyes (Bergner and Sanderson, 2002; Perez and Sanderson, 2005; Bai and Sanderson, 2006; Sanderson et al., 2008).

Mukherjee et al. (2013) used low magnification (10×) phase-contrast microscopy to obtain time-lapse (0.5-Hz) images of lung slices and monitored changes in the

Mukherjee et al. (2013) addressed an elusive issue: a possible role for PKC, which is activated by phorbol esters, and \([\text{Ca}^{2+}]_i\) oscillations in tuning excitation–contraction coupling in airway smooth muscle. To do this, they used the mouse lung slice preparation. This preparation, briefly described, involves inflation of the lungs with liquid agarose, which is then cleared from the airways and forced into the alveolar spaces by subsequent inflation of the lungs with a small volume of air. The agarose is gelled by cooling the lungs, permitting slicing of the stiffened tissue on a tissue slicer or microtome. The agarose-filled alveolar tissue mimics positive alveolar pressure of the lung, exerting tensile forces on the airway walls and preventing the slice from collapsing.
cross-sectional area of airway lumens in response to perfusion with various contractile agonists, PKC activators, and PKC antagonists. Additionally, they used confocal microscopy to examine lung slices loaded with the calcium indicator dye Oregon green, permitting visualization of Ca^{2+} signaling within individual airway SMCs. They made four key discoveries (Mukherjee et al., 2013). First, activation of PKC caused repetitive, unsynchronized, and transient contractions in the SMCs lining the airway lumen that resulted in small reductions in airway luminal area. Second, this contractile activity correlated with low frequency Ca^{2+} oscillations in airway SMCs. Third, PKC activation with phorbol esters and thrombin produced a strong Ca^{2+} sensitization of SMC contraction; that is to say, it increased the contractile response of the airways to Ca^{2+}-elevating stimuli. Finally, PKC activation induced reversible phosphorylation of CPI-17 and the regulatory MLC. Because phosphorylation of CPI-17 inhibits MLCP, phosphorylation of the regulatory MLC and CPI-17 together accelerate cross-bridge cycling and thus increase contraction. This mechanism is independent of the Rho/Rho kinase pathway and could lead to G protein–coupled induced Ca^{2+} sensitization of smooth muscle.

Previous lung slice studies have reported that the frequency of Ca^{2+} oscillations in airway smooth muscle correlates with the magnitude of contraction such that high frequency oscillations lead to larger, more sustained decreases in airway luminal diameter than do low frequency oscillations (in other words, airways seem to function as biological integrators) (Perez and Sanderson, 2005; Sanderson, 2011). Mathematical modeling has suggested that the sustained contractions achieved with high frequency oscillations occur because the time required for MLCP activation and cross-bridge detachment exceeds that of the period of oscillations so that only a small fraction of bound myosins has time to detach from actin during the interspike interval, leading to a sustained contraction (Wang et al., 2008). Mukherjee et al. (2013) found that the low frequency Ca^{2+} oscillations initiated when PKC phosphorylates CPI-17, leading to inactivation of MLCP, produce only small transient contractions of the SMCs and small reductions in airway luminal area. Because [Ca^{2+}], initially rises during the upstroke of the oscillation, MLCK activity increases while MLCP is inhibited by phosphorylation, these Ca^{2+} channels increase their opening probability, producing localized areas of sustained, persistent Ca^{2+} influx (Navedo et al., 2008, 2010; Cheng et al., 2011; Dixon et al., 2012). Accordingly, local L-type Ca^{2+} channel activity depends on the relative activities of AKAP150–associated kinases (PKA or PKC) and the phosphatase (calcineurin) on Ca^{2+} channels.

In this context, the work of Mukherjee et al. (2013) provokes several interesting questions. For example, which specific PKC isoform is involved in the development of slow Ca^{2+} oscillations and associated contractions? This is important because conventional calcium-dependent isoforms of PKC have a distinct pharmacology from novel and atypical isoforms such as PKCe (Steinberg, 2008), which would be critical for any future pursuit of therapeutic strategies involving PKC inhibition. Is local targeting of PKC by an AKAP critical for these events to take place? Does loss of PKC anchoring abolish the actions of PKC agonists on Ca^{2+} and contractility of airway smooth muscle? Finally, does inhibition of PKC in airway smooth muscle increase airflow during asthma and COPD?

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