Disrupting Actin-Myosin-Actin Connectivity in Airway Smooth Muscle as a Treatment for Asthma?

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Breathing is known to functionally antagonize bronchoconstriction caused by airway muscle contraction. During breathing, tidal lung inflation generates force fluctuations that are transmitted to the contracted airway muscle. *In vitro*, experimental application of force fluctuations to contracted airway smooth muscle strips causes them to relengthen. Such force fluctuation-induced relengthening (FFIR) likely represents the mechanism by which breathing antagonizes bronchoconstriction. Thus, understanding the mechanisms that regulate FFIR of contracted airway muscle could suggest novel therapeutic interventions to increase FFIR, and so to enhance the beneficial effects of breathing in suppressing bronchoconstriction. Here we propose that the connectivity between actin filaments in contracting airway myocytes is a key determinant of FFIR, and suggest that disrupting actin-myosin-actin connectivity by interfering with actin polymerization or with myosin polymerization merits further evaluation as a potential novel approach for preventing prolonged bronchoconstriction in asthma.

Keywords: asthma; bronchoconstriction; myosin; actin; contractile

Excessive contraction of airway smooth muscle (ASM) leads to bronchoconstriction and airflow obstruction during an asthma attack. In nonasthmatic airways, the ASM is found in thin bundles arranged essentially circularly around the airway, likely providing structural support. In normal lungs, ASM also likely assists in regulation of gas exchange, mucus clearance, defense mechanisms, and coughing, though it is uncertain whether any of these roles is required for normal lung function (1). Indeed, it may be that airway smooth muscle is a vestigial remnant of lung development (2), during which its periodic contractions might raise luminal pressure and thereby help to advance the lung buds (3). In the airways of individuals with asthma, the smooth muscle layer becomes thicker by increasing cell size and/or cell number. Such ASM over-accumulation is thought to facilitate the excessive bronchoconstriction that occurs characteristically in asthma. Furthermore, evidence connects smooth muscle in the pathogenesis of airway inflammation and remodeling (4, 5).

SMOOTH MUSCLE CONTRACTION AND AIRWAY CONSTRICTION

Smooth muscle contracts when filaments (thick) of myosin motors hold and ratchet actin filaments (thin) in opposite

directions within the cell cytoplasm (Figure 1). These contractile actin filaments are oriented roughly along the long axis of each myocyte, and are mechanically interconnected by co-anchorage within dense bodies, or by inter-filament linkage by thick filaments; some thin filaments are also anchored to dense plaques at the cell membrane and (through integrin complexes) to surrounding tissue matrix. Thus, myosin-mediated translation of thin filaments in opposite directions shortens individual myocytes along their long axis, and in concert they shorten the muscle bundles in which they share long axis orientation.

Because the airways are embedded within the lung parenchyma, the load against which muscle bundles contract is determined in part by lung inflation; thus, breathing imposes a fluctuating load against which shortening must occur or must be maintained. While the steady mean load against which airway smooth muscle contracts partially determines its extent of shortening during maximal contractile stimulation, the influence of superimposed load fluctuations (such as those imposed by breathing) appears to be of even greater importance. For example, Shen and coworkers demonstrated that repeated single boluses of intravenous methacholine caused bronchoconstriction that varied considerably according to the depth of tidal breathing (at fixed frequency) in mechanically ventilated rabbits (6). Airway resistance rose considerably in response to methacholine administration when tidal volume was zero, whereas the bronchoconstrictor response was progressively attenuated when the same methacholine dose was given during mechanical ventilation with higher and higher tidal volumes; indeed, bronchoconstriction was prevented essentially completely by mechanical ventilation with large (20 ml/kg) tidal volumes. Hyperpnea similarly suppresses bronchoconstriction in other animals and in humans (7–10), and accounts for the delayed onset of bronchoconstriction induced in subjects with asthma by exercise (which provokes hyperpnea) or by eucapnic voluntary hyperventilation of dry air, until after cessation of exercise or hyperpnea. Thus, even in asthma, deep breathing functionally antagonizes bronchoconstriction, though studies (11–15) suggest that the degree to which breathing antagonizes bronchoconstriction may be abnormally reduced in subjects with asthma.

FORCE FLUCTUATION-INDUCED RELENGTHENING OF CONTRACTED AIRWAY SMOOTH MUSCLE

Superimposition of force fluctuations (that mimic the load fluctuations created by breathing; frequency 12/min) upon a sub-maximal steady load against which ASM strips that had been isotonically contracted results in their partial relengthening *in vitro* (Figure 2) (16–20). Such “force fluctuation-induced relengthening” (FFIR) of shortened ASM strips can occur even during continued maximal contractile stimulation (e.g., continued

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Figure 1. Model of smooth muscle contraction. In smooth muscle cells, actin filaments (orange lines) are anchored to dense bodies (magenta ovals); for clarity, only one actin filament is shown emanating in each direction from each dense body, though it is thought that many such filaments actually emanate in each direction. Side polar myosin filaments are arranged between actin filaments anchored to two different dense bodies. Activated myosin filaments pull on the actin filaments, translating the entire actin filament–dense body–actin filament structure in the direction shown by the black arrow, resulting in muscle shortening.

Figure 2. Force fluctuation-induced relengthening (FFIR) of acetylcholine-contracted bovine tracheal muscle strips contracted against load equivalent to 32% of maximal force generated by each strip (F_o), plus superimposed force oscillations as indicated on the x-axis. Acetylcholine

exposure caused shortening of untreated (control, red symbols and line) control trachealis strips against steady load, and there was progressive relengthening as the magnitude of superimposed force fluctuations was increased. This FFIR was physiologically regulated by p38 MAPK signaling, because p38 MAPK inhibition with SB203580 enhanced FFIR (blue symbols and line). Note that the “control” condition is a state of active p38 MAPK signaling. Adapted by permission from Reference 18.

high concentration acetylcholine exposure) and continued maximal contractile activation (reflected in high level phosphorylation of the regulatory 20-kD myosin light chain). Clearly, FFIR is a different phenomenon from muscle relaxation, even though both result in relengthening of the shortened muscle. Furthermore, FFIR of maximally contracting ASM strips is a physiologically regulated phenomenon, for the extent of relengthening, and the time course over which it occurs varies considerably with the mode of contractile activation (e.g., acetylcholine versus KCl exposure; see below) and with manipulation of intracellular signaling pathways (18).

ACTIN-MYOSIN-ACTIN CONNECTIVITY AS A DETERMINANT OF FFIR

We propose that the connectivity between actin filaments constitutes a major determinant of the magnitude of FFIR

Figure 3. Proposed mechanism by which actin filament length influences FFIR. (A) In contracted smooth muscle cells in which contractile actin filaments are short in length, once force fluctuations begin, the stretch imposed upon the cells pulls the actin filaments (orange) sufficiently far that overlap and connection by myosin filaments (blue), which had been developing force in parallel, is now lost. For contraction to occur once again there must be a rearrangement of the contractile apparatus such that myosin filaments now act in series. The change from parallel (high force) to a series (low force) configuration diminishes the ability of the muscle to exert sufficient force to resist relengthening during force oscillation, and the muscle lengthens. (B) In contracted smooth muscle cells with longer actin filaments, the stretch imposed on the cell during force oscillation does not break the actin-myosin-actin connection, so that rearrangement of myosin filaments is not required, and force-generating capacity is not diminished. Presumably, the force generating capacity is maintained at a level sufficient to resist relengthening. Reprinted by permission from Reference 42.

exhibited by contracted ASM strips *in vitro*. As noted above, actin filaments may be physically connected within ASM cells by virtue of their simultaneous attachment to opposite faces of a common myosin filament (Figure 1). We and others have gathered evidence that conditions which modulate the strength or nature of such actin filament–myosin filament–actin filament connectivity dramatically influences the magnitude and time course of FFIR of contracted airway smooth muscle.

Modulation of Actin Filament Length

It seems likely that forcibly extending airway myocytes with short actin filaments could cause sufficient sliding of the filaments relative to each other as to lose a zone of “overlap” in which both filaments could be connected together through their common attachment to a single myosin filament. Were such loss of connectivity to happen as a result of stretching during a force fluctuation, then internal rearrangement and reconnection of the actin and myosin filaments might occur in such a way as to reduce the force-generating capacity of the contractile apparatus, as illustrated in Figure 3A (21). Essentially, the rearrangement of actin and myosin filaments in a forcibly lengthened muscle cell with short actin filaments might require that previously parallel force-generating myosin motors now assume a series distribution, which reduces their concerted force-generating capacity, thus reducing the myocyte’s ability to resist lengthening in response to force fluctuations and increasing FFIR. In contrast, myocytes with long actin filaments might not undergo parallel-to-series rearrangement of their myosin filaments, and so might not lose force-generating capacity with which to restore myocyte length after stretching (Figure 3B).

In support of this notion are results from two studies in which different pharmacologic interventions that might have shortened actin filaments also increased FFIR. Lakser and colleagues (18) showed that inhibition of p38 mitogen-activated protein kinase (MAPK) with SB203580 increased FFIR exhibited by maximally activated bovine tracheal smooth muscle strips shortened against 32% (mean) of their maximal force (Figure 2). p38 MAPK proteins are expressed in ASM and become activated during acetylcholine (ACh)-induced contrac-

tion (and in response to diverse stress stimuli, including hypersomolarity, cooling/rewarming, and IL-1 β or TNF- α exposure). Acting through its downstream targets and effectors, MAPKAP kinase 2/3, activated p38 MAPK stimulates phosphorylation of HSP27 on three serine residues (22). Nonphosphorylated HSP27 is thought to bind to and cap the barbed ends of actin filaments, thereby preventing the addition of monomers and lengthening of actin filaments (23). Phosphorylated HSP27 can no longer bind to actin filaments, and their barbed ends are thus available for actin monomer addition and consequent filament lengthening (24). In the study of Lakser and coworkers, pharmacologic p38 MAPK inhibition likely also blunted HSP27 phosphorylation, thereby promoting actin filament capping and shortening actin filaments. More direct evidence that actin polymerization influences FFIR was gathered when Dowell and colleagues (25) demonstrated that sequestration of actin monomers with latrunculin B augmented FFIR in contracted canine tracheal muscle strips. Actin filament length is ordinarily determined by the balance of addition and loss of actin monomers from actin filaments. By sequestering actin monomers, latrunculin treatment should have prevented the addition of G-actin to actin filaments, thereby shortening these filaments (though actin filament length was not directly measured in that study). Thus, two different interventions that both should have resulted in shorter actin filaments, and so should have changed the susceptibility of actin filament–actin filament connectivity to force fluctuations, each enhanced the magnitude of FFIR.

Modulation of Myosin Filament Length

It is the side-polar myosin filaments that actually connect actin filaments to one another in contracting ASM, through myosin-actin crossbridge interactions along both sides of the myosin filament. The number of crossbridge attachments in the cross-section of the tissue determines the force supported by a muscle. For a given contractile activation state (i.e., a given likelihood that each myosin head is attached to actin), a longer myosin filament with more myosin heads should more tightly interconnect its two flanking actin filaments, by virtue of a greater number of crossbridge attachments to each actin filament. Thus, longer myosin filaments should produce stronger actin filament–

Figure 4. (A) Typical time courses of FFIR in ACh- and KCl-contracted ASM shortened initially for 20 minutes against steady load of 32% F_{max} (induced by ACh or KCl exposure, respectively) after which load fluctuations of $\pm 16\%$ F_{max} are superimposed for another 20 minutes. In ACh-stimulated ASM strips (top panel), the immediate relengthening that attends application of force oscillations (osc) is quite small. In contrast, tissues contracted with KCl (middle panel) exhibit very considerable immediate relengthening upon application of force fluctuations. Force tracing (bottom panel) depicts the timing of the force fluctuations applied to the KCl-contracted strip; force fluctuations applied to the ACh-contracted strip reflect its own F_{max}, but were otherwise analogous. (B) Phosphorylation of the regulatory 20-kD myosin light chain in ACh- and KCl-contracted ASM. Total protein was extracted from tissues contracted isotonicly against 32% F_{max} for 20 minutes with either ACh or KCl

(left) or from tissues relaxed post contraction with either ACh or KCl (right), then size fractionated by SDS-PAGE, and phosphorylated (top) or total (bottom) MLC₂₀ bands revealed by immunoblotting. Considerably greater MLC₂₀ phosphorylation is evident in ACh-stimulated than in KCl-stimulated tissues.

Figure 5. Mechanical system that could simulate the relengthening response of KCl-contracted ASM to onset of force fluctuations. Two pathways connect anchors (yellow) in muscle isotonic shortened by KCl exposure; the shorter pathway has low tensile strength ("wet spaghetti"), whereas the longer pathway has high tensile strength ("steel chain"). The "tug" that attends the onset of force fluctuations breaks the wet spaghetti, and the system snaps to a longer length until relengthening is stopped abruptly when the steel chain is fully extended and now supports the entire load.

actin filament connectivity than would shorter myosin filaments. Importantly, myosin filament length is not fixed (26), but rather is dynamically determined, changing even during the course of a single contraction (27, 28). In addition, myosin filament length appears to be influenced by recent muscle length history (e.g., length oscillation of relaxed ASM shortens myosin filaments) (29), by the length of actin filaments which seem to provide

a template for myosin polymerization (30–32), and by the phosphorylation state of the 20-kD regulatory myosin light chain (MLC₂₀) (33–38). We exploited the latter to obtain indirect evidence suggesting that myosin filament length modulates FFIR. We chose to compare FFIR on ACh- and KCl-elicited contractions because we wanted to be able to assess differences in contraction, FFIR, and selected protein activation in canine tracheal smooth muscle (TSM) strips that had been activated by two diverse mechanisms: G protein-coupled receptors and voltage-dependent calcium channels, respectively. While either ACh or KCl can stimulate substantial force generation and ASM shortening, each agent does so employing signaling pathways that only partially overlap with those activated by the other. Consequent to these different signals, greater MLC₂₀ phosphorylation is seen in maximally ACh-stimulated than in maximally KCl-stimulated tissues (Figure 4B). We did not use lesser concentrations of ACh to elicit after-loaded isotonic contractions in the TSM strips because of the difficulty in "titrating" equivalent levels of isometric, isotonic, or after-loaded isotonic activation among several strips from the population of canine tracheas. Lesser concentrations of ACh would be on the linear portion of the sigmoidal concentration-response relationship and subject to greater variability. Also, G protein-coupled receptor activation with ACh at a lower concentration would still activate MEK and p38 MAPK pathways that promote contractile filament assembly.

The time course of FFIR is dramatically different between ASM strips that have been contracted with ACh versus strips contracted with KCl. Figure 4A shows the length changes typically observed when canine trachealis strips stimulated with 10^{-4} M ACh or 43 mM KCl-substituted Krebs-Henseleit solution are shortened isotonic against 32% F_{max} steady load (as determined during isometric contraction activated by each agonist, respectively), then subjected to superimposed load fluctuations ($\pm 16\%$ F_{max}) during continued contractile activation. Each muscle strip shortens by about half of its reference

Figure 6. Hypothetical connections among actin and myosin filaments in KCl-contracted ASM before and after length oscillation ("tug"). Before the onset of force oscillations (*upper panel*), the shortest axial path through the muscle contractile apparatus contains weak myosin connections between actin filaments (red, illustrated with only one crossbridge per side), analogous to the wet spaghetti path of Figure 5. Some stronger myosin connections between actin filaments (blue, illustrated with two crossbridges per side) are scattered throughout the muscle, but this stronger pathway, analogous to the steel chain path of Figure 5, is slack. Once the tug of force oscillations is imposed (*lower panel*), the weak connections are broken (note loss of red crossbridges) and muscle snaps to a longer length determined by the path composed of only stronger connection, which now supports the increased load of the force oscillation/tug.

Figure 7. Hypothetical connections among actin and myosin filaments in ACh-contracted ASM immediately before or after initiating force oscillations (these are unchanged by force oscillations). Pre-tug, the shortest axial path through the muscle contractile apparatus contains very strong connections, with many myosin heads interacting with actin filaments (shown in green). A tug from force oscillations does not break these connections and the muscle does not have a large immediate relengthening response.

length against the steady load. Application of load fluctuations to the ACh-contracted muscle strip induces a small immediate increase in length, followed by progressive relengthening toward an asymptote thereafter. In marked contrast, the KCl-contracted strip exhibits a dramatic immediate increase in length upon application of load fluctuations, and settles at a final length soon after the abrupt relengthening induced by force fluctuations.

A mechanical system that could mimic the immediate relengthening response to force fluctuations in KCl-contracted ASM strips is depicted in Figure 5. In this schema, the cell at the top of Figure 5 presents intramyocyte conditions after isotonic shortening and just before superimposition of force fluctuations. A pair of nodes mechanically anchoring the contractile apparatus (akin to dense bodies or dense plaques) have been pulled toward each other during contraction, and end up connected by two types of mechanical pathways. One is shorter and taut, and actually supports the load against which contraction has occurred; the other is longer and slack, and at this point in time is not actually supporting much load. Imagine further that the tensile strength of the shorter connector is relatively low (think “wet spaghetti”), so that the load it now bears is near its breaking point. In contrast, the tensile strength of the longer connector is great (think “steel chain”)—more even than the greatest load that it could ever experience within the myocyte. Now let us superimpose force fluctuations upon the steady load that the cell at the top of Figure 5 had shortened against. As the load fluctuates above the previously steady level, it exceeds the tensile strength of the wet spaghetti path (which had borne the entire load by virtue of its shorter length), and the wet spaghetti path breaks. No longer held in its shortened configuration by the wet spaghetti, the myocyte quickly relengthens until the steel chain path is fully extended, whereupon it supports the entire external load and prevents further relengthening. The integrated system thus snaps from a steady shorter length during isotonic contraction to a new steady but longer length during load fluctuation, in a pattern that mimics the relengthening of KCl-contracted canine trachealis muscle. Our model depicts contractile units within an isolated cell. It is conceivable that through adherens junctions (force transmission sites formed between dense plaques of adjacent cells), contractile units in different cells may similarly interact.

We speculate that because there is relatively low MLC_{20} phosphorylation in KCl-contracted ASM, the distribution of lengths of myosin filaments interconnecting actin filaments is skewed toward predominance of shorter myosin filaments (Figure 6, *top*), which as discussed above might result in weaker actin filament–myosin filament–actin filament connectivity. A contractile pathway containing such weak actin-myosin-actin connections would break when the tensile load imposed (e.g., during force fluctuations) exceeded the connection strength (39). Contractile paths with low tensile strength (due to their containing actin-myosin-actin connections through short myosin filaments, as shown in *red* in Figure 6) would behave like the wet spaghetti paths in Figure 5. Upon their rupture during force fluctuations, the muscle would lengthen quickly until pathways composed of only stronger actin-myosin-actin connections (i.e., through longer myosin filaments, as shown in *blue* in Figure 6) support the entire load and prevent further relengthening. We imagine that the very minor immediate relengthening that occurs in ACh-contracted ASM strips subjected to force fluctuations (Figure 4A) reflects a predominance of longer myosin filaments (whose polymerization had been promoted by increased MLC_{20} phosphorylation; Figure 4B), as shown in Figure 7. In such muscle, virtually all the paths are “kryptonite chains,” due to the very strong actin filament–actin filament

interconnections by long myosin filaments. Perhaps the prolonged FFIR that occurs in ACh-contracted muscle strips reflects the sequential breakages of the weakest of the strong pathways, each fracture allowing for tiny length increments that accumulate slowly (39). Alternatively, perhaps other mechanisms, such as those influenced primarily by actin filament length, account for the slow relengthening phase.

SPECULATION ON THERAPEUTIC IMPLICATIONS

If it occurs *in vivo*, which seems extremely likely, then force fluctuation–induced relengthening of contracted airway smooth muscle would have the effect of reversing bronchoconstriction. Thus, intervention to enhance FFIR could represent a novel therapeutic approach for airflow obstruction in asthma, one that does not rely on relaxing airway muscle (the principal goal of acute reliever medications) or subduing airway inflammation (the principal goal of long-term controller medications).

As discussed above, it seems likely that strong connectivity between actin filaments reduces force fluctuation–induced relengthening of contracted ASM. Thus, disrupting actin-myosin-actin connectivity seems worthy of exploration as a potential treatment for asthma. We suggest two approaches for disrupting actin-myosin-actin connectivity: (1) shorten actin filaments; and (2) shorten myosin filaments. Actin filament length is regulated physiologically through the balance of addition and loss of actin monomers and severing or stabilization of actin filaments, as we reviewed previously (21). These processes are not unique to airway smooth muscle. Given the ubiquitous importance of actin filaments in a wide range of cell types, it might be challenging to design an intervention that shortens actin filament length selectively within airway smooth muscle without disrupting other important actin functions in the lung, such as maintenance of airway epithelial or vascular endothelial barrier integrity. In contrast, smooth muscle myosin differs structurally from skeletal or cardiac myosin, and it may be easier to inhibit smooth muscle myosin polymerization selectively. Although we are not aware of drugs developed specifically for this purpose, monoclonal antibodies directed toward specific parts of the myosin II tail prevented or even reversed myosin polymerization *in vitro* (40, 41). Perhaps small-molecule drugs could be designed to achieve this goal.

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