Latrunculin B increases force fluctuation-induced relengthening of ACh-contracted, isotonically shortened canine tracheal smooth muscle

M. L. Dowell,1 O. J. Lakser,2 W. T. Gerthoffer,3 J. J. Fredberg,4 G. L. Stelmack,5 A. J. Halayko,5 J. Solway,1,* and R. W. Mitchell1,*

1Section of Pulmonary and Critical Care Medicine, University of Chicago, and 2Children’s Memorial Hospital and Northwestern University, Chicago, Illinois; 3Department of Pharmacology, University of Nevada School of Medicine, Reno, Nevada; 4Physiology Program, Harvard School of Public Health, Boston, Massachusetts; and 5Section of Respiratory Diseases, University of Manitoba, Winnipeg, Manitoba, Canada

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Dowell, M. L., O. J. Lakser, W. T. Gerthoffer, J. J. Fredberg, G. L. Stelmack, A. J. Halayko, J. Solway, and R. W. Mitchell. Latrunculin B increases force fluctuation-induced relengthening of ACh-contracted, isotonically shortened canine tracheal smooth muscle. J Appl Physiol 98: 489–497, 2005. First published October 1, 2004; doi:10.1152/japplphysiol.01378.2003.—We hypothesized that differences in actin filament length could influence force fluctuation-induced relengthening (FFIR) in short, isometrically contracted airway smooth muscle and tested this hypothesis as follows. One-hundred micromolar ACh-stimulated canine tracheal smooth muscle (TSM) strips set at optimal reference length (L_{ref}) were allowed to shorten against 32% maximal isometric force (F_{max}) steady preload, after which force oscillations of ±16% F_{max} were superimposed. Strips relengthened during force oscillations. We measured hysteresivity and calculated FFIR as the difference between muscle length before and after 20-min imposed force oscillations. Strips were relaxed by ACh removal and treated for 1 h with 30 nM latrunculin B (sequesters G-actin and promotes depolymerization) or 500 nM jasplakinolide (stabilizes actin filaments and opposes depolymerization). A second isotonic contraction protocol was then performed; FFIR and hysteresivity were again measured. Latrunculin B increased FFIR by 92.2 ± 27.6% L_{ref} and hysteresivity by 31.8 ± 13.5% vs. pretreatment values. In contrast, jasplakinolide had little influence on relengthening by itself; neither FFIR nor hysteresivity was significantly affected. However, when jasplakinolide-treated tissues were then incubated with latrunculin B in the continued presence of jasplakinolide for 1 more h and a third contraction protocol performed, latrunculin B no longer substantially enhanced TSM relengthening. In TSM treated with latrunculin B + jasplakinolide, FFIR increased by only 3.03 ± 5.2% L_{ref} and hysteresivity by 4.14 ± 4.9% compared with its first (pre-jasplakinolide or latrunculin B) value. These results suggest that actin filament length, in part, determines the relengthening of contracted airway smooth muscle.

actin filament dynamics; force oscillations; isotonic contractions; hysteresivity

A NUMBER OF PREVIOUS STUDIES in animals (22, 24, 25, 27) and humans (7, 29) have demonstrated that tidal breathing per se reduces airway constriction during contractile stimulation, and deep breathing does so more effectively. Even a single deep inspiration can substantially reverse experimentally induced bronchoconstriction in normal individuals (1, 4, 11). However, the ability of deep breathing to dilate the airways is absent in asthmatic subjects (4, 11). Understanding why this protective mechanism fails in asthma is the focus of ongoing investigation in several laboratories and is the ultimate goal of the present study.

Fredberg and coworkers (13, 14) studied the influence on airway smooth muscle shortening of the load fluctuations imposed by breathing. They stimulated bovine trachealis strips with ACh and allowed them to shorten isotonically against a constant load to a steady-state length and then superimposed sinusoidal force oscillations of increasing amplitudes (to simulate tidal breathing) on the constant mean load (5). These increasing force oscillations caused substantial smooth muscle relengthening, despite continued contractile stimulation. They showed that relengthening could be explained in large part by perturbed equilibria of myosin binding (5), but, when oscillations were stopped, the muscle failed to reshorten. This result could not be explained by myosin binding dynamics (which predict complete reshortening) and so indicated that another nonbridge mechanism of muscle plasticity (beyond perturbed myosin equilibrium) must be operative. Subsequent studies showed that oscillation-induced relengthening of contracted airway smooth muscle is a physiologically regulated phenomenon. For example, treatment with the p38 MAPK inhibitor SB-203580 enhanced force fluctuation-induced relengthening (13). One potential consequence of p38 MAPK inhibition is shortening of contractile actin filaments, because p38 MAPK activation leads to the phosphorylation of heat shock protein 27 (HSP27), an actin “capping protein.” When unphosphorylated, HSP27 limits addition of globular (G)-actin monomers to the actin filament barbed end, and actin filament shortening occurs over time. When phosphorylated, HSP27 loses its capping function, and filament lengthening can ensue.

Based on these observations, we hypothesized that actin filament length plays an important role in determining force fluctuation-induced relengthening of contracted airway muscle (2, 28). To test this hypothesis, we assessed the effects of latrunculin B (which sequesters G-actin, thus preventing filament polymerization and lengthening) and jasplakinolide (which stabilizes actin filaments by blocking their degradation), on isotonic, auxotonic, and isotonic contraction of canine tracheal smooth muscle (TSM). We reasoned that sequestration of actin monomers with latrunculin B should decrease contractile filament length and so should result in increased force fluctuation-induced relengthening, as our lab-

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Exploratory has proposed previously (2, 28). Furthermore, stabilization of polymerized actin with jasplakinolide should block the latrunculin effect.

METHODS

Tissue preparation. In accordance with Institutional Animal Care and Use Committee approved protocols, random source dogs were anesthetized and killed by overdose with pentobarbital sodium (30 mg/kg iv); tracheas were excised and placed in physiological saline solution for transport to the laboratory. On arrival, tissues were rinsed several times and maintained in Krebs-Henseleit (K-H) solution of the following composition (in mM): 115 NaCl, 25 NaCO₃, 1.38 KH₂PO₄, 2.51 KCl, 2.46 MgCl₂, 2.5 CaCl₂, and 11.2 dextrose. This solution was gassed with 95% O₂-5% CO₂ to maintain a pH between 7.3 and 7.5. All studies were conducted at 37°C in K-H solution.

Parallel fibered bundles of epithelium-free canine TSM were dissected free of all overlying connective tissue. For isometric and isotonic studies, TSM bundles measuring 0.25–0.5 mm in width and 0.5–1.0 mm in depth (total thickness of the smooth muscle layer alone) were fastened at either end in aluminum foil clips (Laser Services, Westford, MA). The clips held the muscles firmly as a flat sheet and were perforated for attachment to hooks. At equilibration (see below), reference length (Lref) of the tissues measured between 2.5 and 7 mm.

For isometric studies, the tissues were mounted in 15-ml (total volume) vertical organ baths (Easterbrooke, Winnipeg, MB). One clip was slipped over a stainless steel hook rigidly fastened at the bottom of the organ bath; the other end was attached to a Grass FT03 force transducer (Quincy, MA) by a short length of wire. The transducers were equipped with internal “red” springs to limit movement of the organ bath; the other end was fastened to a 300B lever arm that could measure both force output and length changes. The tissues were mounted horizontally in a water-jacketed chamber.

Excess K-H was removed by vacuum from the surface of the muscle. Gassed K-H solution was gravity fed at 1–2 ml/min through the bath. Tissues were equilibrated for at least 90 min (total volume) flow-through dip-tray muscle bath. After equilibration, tissues were contracted isometrically by using the Grass FT03 transducer (Quincy, MA) by a short length of wire. The transducers were equipped with internal “red” springs to limit movement of the organ bath; the other end was fastened to a 300B lever arm that could measure both force output and length changes. The tissues were mounted horizontally in a water-jacketed (37°C), 400 μl (total volume) flow-through dip-tray muscle bath. Gassed K-H solution was gravity fed at 1–2 ml/min through the bath. Excess K-H was removed by vacuum from the surface of the muscle chamber.

After equilibration, tissues were contracted isometrically by switching perfusion source to K-H solution containing 10⁻⁴ M ACh, and the maximal response was noted (Fmax). Lref and Fmax in response to ACh were then used as base parameters for force-oscillation protocols described below. Muscles were allowed to relax by reperfusing with K-H alone.

Protocol 1: Isotonic contractions in the absence or presence of force fluctuations. This protocol was developed to determine the effect of force fluctuations on shortening per se; it also tested the ability of canine TSM strips to be repeatedly contracted isotonically without decrement in maximal shortening and assessed the effects of jasplakinolide and latrunculin B on these responses. Twenty minutes after force reached baseline (resting tension), tissues were again exposed to 10⁻⁴ M ACh but were allowed to shorten isotonically against an afterload of 32% Fmax for 20 min. Length changes were noted. TSM strips were allowed to relax again to baseline. Twenty minutes later, muscles were reexposed to ACh and allowed to shorten against an afterload of 32% Fmax; however, during this second isotonic contraction, 0.2-Hz sine wave force oscillations of amplitude ±16% Fmax were superimposed on the mean load, and length changes were assessed. Muscles were oscillated by using Aurora Scientific Dynamic Muscle Control software. Muscle force and length outputs

In the presence of these agents, a cumulative ACh concentration-response study was performed (10⁻⁹ M to 10⁻⁴ M). All isotonic data in response to ACh were normalized to the contraction elicited during the last exposure of each muscle to 43 mM KCI-substituted K-H (19).

Concentration-response relationships also were obtained by using KCI-substituted K-H solution for TSM strips exposed to latrunculin B. Because 30 nM latrunculin B had such a profound effect on isometric contractile responses elicited by the muscarinic receptor agonist ACh, we exposed tissues to increasing concentrations of KCI (5.9–43.0 mM). KCI elicits contraction through voltage-dependent influx of extracellular calcium. Muscle strips were placed in the 15-ml organ baths containing 10 ml of K-H solution. Cumulatively, 10 additions of 0.5 ml of 127 mM KCI-substituted K-H solution (to a final volume of 15 ml) elicited a concentration-response relationship that reached a plateau at 43 mM KCI (17).

Auxotonic protocols. After equilibration, TSM strips were exposed for at least 45 min to latrunculin B (30 nM) or jasplakinolide (500 nM). These concentrations were chosen based on findings from isotonic studies; they were the concentrations at which the first significant effects on ACh concentration response studies were noted. Vehicle control tissues received equivalent concentrations of DMSO.

In the presence of these agents (above), a cumulative ACh concentration-response study was performed (10⁻⁹ M to 10⁻⁴ M). All auxotonic data in response to ACh were normalized to the contraction elicited during the last exposure of each muscle to 43 mM KCI-substituted K-H. ACh was removed from the organ bath with several rinses of fresh K-H. Then a final 43 mM KCI contraction was elicited (agents were not reintroduced); this response was compared with the pre-ACh response to 43 mM KCI.

Isotonic protocols. Our approach is modeled after that of Fredberg’s laboratory (13, 14), but it was modified to allow for comparison of results before and after pharmacological intervention. Where Fredberg et al. used increasing amplitudes of fluctuation from ±2 to 16% of the maximal isometric force elicited by ACh (Fmax) over several hours, we used a single amplitude of ±16% Fmax for 20 min. This protocol not only produced tissue relengthening similar to that of Fredberg et al. but also allowed for the comparison within the same strip of muscle before and after treatments. Strips of TSM were fixed to a 300B lever arm that could measure both force output and length changes. The tissues were mounted horizontally in a water-jacketed (37°C), 400 μl (total volume) flow-through dip-tray muscle bath. Gassed K-H solution was gravity fed at 1–2 ml/min through the bath. Excess K-H was removed by vacuum from the surface of the muscle chamber.

After equilibration, tissues were contracted isotonically by switching perfusion source to K-H solution containing 10⁻⁴ M ACh, and the maximal response was noted (Fmax). Lref and Fmax in response to ACh were then used as base parameters for force-oscillation protocols described below. Muscles were allowed to relax by reperfusing with K-H alone.
were acquired through a National Instruments PCI-6036E data-acquisition board; data were monitored and collected by using both the LabView-based Dynamic Muscle Control and ADInstruments PowerLab Chart software. After 20 min, the tissues were allowed to relax and then to equilibrate for 1 h in K-H solution containing either 30 nM latrunculin B, 500 nM jasplakinolide, or 0.3% DMSO (vehicle control). Hysteresivity (6) was measured by plotting stress-strain work loops of force oscillations and the resultant length oscillations. Then, after 1 h, the TSM strips were subjected to the above isotonic and isometric Fmax), tissues were exposed to 10

Protocol 2: Combined isotonic contractions with force fluctuations. Based on data derived from protocol 1 (see RESULTS), Protocol 2 was designed to streamline the experiment to decrease the total exposure time to DMSO and the toxins jasplakinolide and latrunculin B. Twelve 10-mM ACh and allowed to shorten isotonically against an afterload of 32% Fmax for 20 min. Then, without delay and during continued ACh exposure, force oscillations were superimposed (0.2 Hz and amplitude ±16% Fmax) for 20 min; thereafter, TSM strips were allowed to relax by switching to ACh-free K-H solution. All length changes were noted. Next, tissues were incubated for 1 h in K-H solution containing either 30 nM latrunculin B or 0.3% DMSO (vehicle control). After this equilibration period, the combined isotonic contraction with force fluctuation protocol was again performed in the continued presence of latrunculin B or vehicle. Length changes were compared for contractions performed before and after treatment and were expressed as %F0. Near the end of the second protocol, but before force fluctuations had stopped, some tissues were flash-frozen in liquid nitrogen, transferred to dry ice-chilled acetone (containing 5% TCA and 10 mM DTT), and stored at -80°C for subsequent protein extraction and Western blot analysis (see below). (The dip-tray organ bath was designed for rapid removal without disturbing the muscle strip or the force/length sensing lever arm. Typically, only 1 s elapsed from the time the bath was removed and the tissue was immersed in liquid nitrogen while still undergoing force fluctuations.) Other tissues were frozen for the estimation of G- and F-actin (see below).

Protocol 3: Jasplakinolide and latrunculin B combined studies. Protocol 3 was designed to test the hypothesis that stabilization of actin filaments with jasplakinolide could prevent the effect of latrunculin B on force fluctuation-induced muscle relengthening. For this protocol, tissues were equilibrated, and Fmax was determined as outlined above. Untreated muscles were then stimulated with ACh and allowed to contract for 20 min against a steady load of 32% Fmax and then for another 20 min against 32 ± 16% Fmax oscillating load, as during protocol 2. Thereafter, TSM strips were incubated with 500 nM jasplakinolide for 1 h, and the contraction sequence was carried out again in the continued presence of the actin filament stabilizer. However, after completion of this second contraction sequence, the tissues were allowed to relax by switching to ACh-free K-H containing 500 nM jasplakinolide. Once baseline relaxed tone was again achieved, the tissues were further incubated in 30 nM latrunculin B (and continued 500 nM jasplakinolide) for 1 additional h. A third combined contraction sequence was then performed in the presence of both latrunculin B and jasplakinolide.

Western analysis of myosin light chain 20 phosphorylation. Proteins from sham-(n = 3) and latrunculin B-treated (n = 3) muscles that had been frozen at the end of the second oscillation (Protocol 2) were extracted as described previously (21). Denatured proteins were separated by polyacrylamide gel electrophoresis (Invitrogen, NuPage 4–12% gels), transferred to Immobilon-P polyvinylidene fluoride membranes, and probed for phosphorylated (W. T. Gerhoffer and nonphosphorylated (Santa Cruz) 20-kDa myosin light chain (MLC20). Phosphorylated and nonphosphorylated proteins were detected on separate gels by using Pierce SuperSignal West Pico chemiluminescent substrate, and blot intensities (volumes) were calculated by using a BioRad densitometer and software.

Quantification of G- and F-actin. Two pairs of tissues from two dogs that had been sham or latrunculin B treated were embedded in optimum cutting temperature compound, frozen, and sectioned (4–5 μm). Sections (3 from each tissue) were fluorescent stained for G- (Oregon green DNs and F-actin (Texas red phalloidin). For each section, G- and F-actin were visualized and digitized (1,024 × 1,024 pixels) by using an Olympus ix70 inverted FLUOVIEW confocal (aperture 2) microscope with the use of a protocol modified from Wang et al. (30). Oregon green and Texas red fluorescence were quantified by using Photoshop 6.0 software.

Data analysis. Results from different groups were compared by using Student’s t-test for paired or unpaired data or ANOVA as appropriate.

RESULTS

Isometric studies. The effects of 100, 300, and 500 nM jasplakinolide were assessed (Fig. 1, A, B, and C). No significant shift in the concentration-response relationship to ACh was observed for the lesser concentrations of jasplakinolide; however, at 500 nM, jasplakinolide significantly blunted the contractile response of muscles compared with volume-matched vehicle (0.5% DMSO) control tissues (Fig. 1C). Maximal responses to ACh were reduced from 147.4 ± 11.9% (vehicle control) to 117.7 ± 8.1% (43 mM KCl) in the presence of jasplakinolide. The effects of 30 and 100 nM latrunculin B were assessed. In a concentration-dependent manner, latrunculin B significantly attenuated force production in response to ACh compared with volume-matched vehicle (DMSO) control tissues (Fig. 1D). The greater concentration of latrunculin B not only attenuated force production by the smooth muscle strips but also significantly shifted the concentration-response relationship to ACh to the right. To ensure that these concentrations of latrunculin B were not nonselectively affecting muscarinic receptor-mediated contractile responses, the effect of this agent on KCl-elicited contraction was assessed (Fig. 1E). Latrunculin B significantly reduced contractile response elicited by increasing concentrations of KCl-substituted K-H solution.

Auxotonic studies. Auxotonic shortening contractile responses for 12 TSM strips from four dogs were assessed in the presence of 500 nM jasplakinolide or 30 nM latrunculin B and compared with vehicle (DMSO) control tissues. Although these concentrations of agents at least tended (if not significantly) to reduce force elicited by ACh (Fig. 1 and above), none of these interventions affected auxotonic shortening of these muscles in response to muscarinic agonist (Fig. 2).

Isotonic studies. In general, whether tissues were studied using protocol 1 or protocol 2, the net effects of force oscillation were similar for TSM strips treated with latrunculin B and/or jasplakinolide. Latrunculin B (30 nM) had profound effects on relengthening on superimposition of force oscillations compared with DMSO vehicle- or jasplakinolide-treated (500 nM) TSM strips. (Representative tracings for protocols 1 and 2 are shown in Fig. 3.)

Protocol 1: Isotonic contractions in the absence or presence of force fluctuations. Before incubation with vehicle, latrunculin B, or jasplakinolide, similar isotonic and isometric re-
Responses were observed among the three groups, including ACh-elicited Fmax, maximal isotonic shortening, maximal shortening with force oscillations, or spontaneous lengthening (Table 1 and Figs. 4 and 5). Latrunculin B did not significantly affect spontaneous lengthening, but it markedly augmented lengthening in response to force oscillation (Fig. 5). Latrunculin B-treated tissues demonstrated a 30.6 ± 8.4% increase in oscillation-induced lengthening compared with pretreatment (Fig. 5); in some instances, TSM strips lengthened to beyond Lref (Fig. 3). Vehicle control tissues showed only a 4.8 ± 1.3% increase in force fluctuation-induced lengthening during the posttreatment protocol (Fig. 5). Latrunculin B also increased hysteresivity by 28.8 ± 9.0% over pretreatment values, whereas control tissues modestly decreased hysteresivity during the second isotonic contraction with oscillation by 2.9 ± 3.5% (Fig. 5). Jasplakinolide had minimal effects on all parameters measured post- vs. preincubation (Figs. 4 and 5).

Protocol 2: Combined isotonic contractions with force fluctuations. Tissues treated with latrunculin B again demonstrated enhanced relengthening during force oscillation and increased hysteresivity compared with control TSM strips (Fig. 6). Force fluctuation-induced relengthening increased by 80.7 ± 25.3% for latrunculin B-treated tissues (Fig. 6); in contrast, vehicle-treated muscles demonstrated small decreases in this parameter (post- vs. pretreatment). Furthermore, latrunculin B caused a
31.8 ± 13.5% increase in tissue hysteresivity compared with control TSM strips, which decreased hysteresivity by -4.2 ± 4.5% during the second isotonic contraction (Fig. 6). Neither maximal isotonic shortening nor spontaneous relengthening was affected by either vehicle or treatment with latrunculin B (Fig. 6).

The increased relengthening observed for latrunculin B was not associated with any decrease in phosphorylation of MLC20. In fact, relative to vehicle-treated control TSM strips (1.024 ± 0.084, n = 3), latrunculin B-treated muscles demonstrated a significant increase in relative MLC20 phosphorylation (2.196 ± 0.314, n = 3; P = 0.023).

Also, latrunculin B-treated tissues demonstrated significantly greater (P = 0.022) G-actin-to-F-actin ratios (0.381 ± 0.066) vs. sham-treated control tissues (0.282 ± 0.047), as determined by image analysis of Oregon green vs. Texas red fluorescence.

Protocol 3: Jasplakinolide and latrunculin B combined studies. Prior treatment with jasplakinolide prevented the latrunculin B-induced increases in relengthening in response to force oscillation and hysteresivity observed in protocol 2 studies (compare Figs. 6 and 7). No differences were observed before and after latrunculin B in the continued presence of jasplakinolide, including maximal isotonic shortening and spontaneous lengthening (Fig. 7).

DISCUSSION

Previously, we hypothesized that the length of contractile actin filaments should play an important role in determining the plastic behavior of contracted airway muscle (2, 28). Our rationale was that, on stress-induced periodic lengthening of muscle (as during deep breathing) with short actin filaments, myosin filaments might have to undergo parallel to series rearrangement in order for cross-bridge formation and force generation to continue. By decreasing the number of force-generating units in parallel, such stress-induced lengthening would decrease force, and the muscle would respond with relengthening. In contrast, stress-induced periodic lengthening of muscle with longer actin filaments might allow for continued parallel arrangement of force-generating units, despite muscle lengthening, and would maintain force and would blunt the relengthening with force fluctuations. Data from Pratusevich et al. (23) and Seow et al. (26) have already demonstrated...
that series-to-parallel or parallel-to-series rearrangements of contractile units in contracting airway smooth muscle can occur, so the process we postulate during force oscillation seems not an unreasonable possibility.

Therefore, we undertook this study to test the hypothesis that actin filament length is a regulator of the mechanical plasticity of contracted airway smooth muscle. Contractile filament maintenance is a dynamic process in which actin monomers are added to or removed from filament ends and in which actin-severing proteins cut actin filaments internally. Each of these processes is regulated through one or more mechanisms (reviewed in Ref. 2), and the combined influences of all of these processes determine mean actin filament length. Because latrunculin B binds and sequesters actin monomers, it prevents further polymerization of actin (16); treatment with this agent should affect actin incorporation but not depolymerization or actin severing and should result in shorter contractile actin filaments. Conversely, jasplakinolide stabilizes actin filaments by binding along the filament length (10), thereby inhibiting mechanisms that, in its absence, would have favored actin filament shortening. Based on the effects that these two well-characterized agents have on actin, we used them as pharmacological probes to manipulate filament length in our studies. Differences in actin length were presumably induced by these agents before contraction, but might have become greater on contraction, as Mehta and Gunst (16) have shown that actin polymerization is stimulated by contractile activation.

There are several significant and novel findings from our studies. First, even though latrunculin B had no effect whatsoever on maximal isotonic shortening of TSM strips (discussed below), it markedly augmented relengthening in response to force fluctuations and tissue hysteresivity (Figs. 4–6). One possible mechanism for increased relengthening with latrunculin B treatment was a nonspecific reduction in the activation state of these tissues. However, based on Western analysis of protein extracts from TSM strips, MLC phosphorylation was greater with latrunculin B treatment compared with control tissues. These data differ from those of Mehta and Gunst (16), who showed no change in MLC phosphorylation when tissues were treated with latrunculin A. Their results were obtained from canine TSM strips contracted isometrically.

Figure 4. Protocol 1: Effect of latrunculin B, jasplakinolide, and vehicle on force and shortening of canine TSM. Neither 30 nM latrunculin B (n = 6 strips from 6 dogs), 500 nM jasplakinolide (n = 3 strips from 3 dogs), nor DMSO (vehicle; n = 6 strips from 6 dogs) had any effect on Fmax, relative maximal isotonic shortening, or relative isotonic shortening with superimposed force oscillations when pretreatment (shaded bars) and posttreatment (open bars) were compared. Also, maximal isotonic shortening was similar in the presence and absence of superimposed 32% Fmax force oscillations among or before and after treatments. Values are means ± SE.
for 5 min using ACh. In contrast, our latrunculin B-treated tissues were frozen after 40 min of isotonic shortening without and then with sinusoidal force oscillations. The effects of latrunculin B on cross-bridge dynamics during force fluctuations are unknown, but our data demonstrating similar or greater MLC phosphorylation with treatment argue for at least comparable muscle activation vs. vehicle-treated control tissues.

Second, although jasplakinolide treatment by itself had no effect on isotonic shortening or force-oscillation-induced re-lengthening (Figs. 4 and 5), this actin filament stabilizing agent completely prevented latrunculin B-induced increases in hysteresivity and force oscillation-induced re-lengthening (Fig. 7). By preventing the addition of actin monomers to actin filaments, latrunculin B could have exerted its effect either by shortening actin filaments or by interfering with coupling between the actin cytoskeleton and focal adhesions, which apparently requires actin polymerization near the focal adhesions (16). Our results with jasplakinolide speak against the...
latter possibility. While jasplakinolide should have protected already-formed actin filaments, it should not have prevented latrunculin B from sequestering G-actin monomers and inhibiting further actin polymerization. As such, the reversal of latrunculin B-enhanced tissue relengthening by jasplakinolide could not have stemmed from an effect at the cytoskeleton-focal adhesion interface. Rather, the more likely scenario is that jasplakinolide obviated the latrunculin B effect on relengthening by eliminating its ability to modulate actin filament length. In fact, when we analyzed fluorescence images of tissues stained with Oregon green DNase (G-actin) and Texas red phalloidin (F-actin), we found a significantly greater amount of green vs. red fluorescence for latrunculin B- compared with sham-treated smooth muscle strips, indicating reduced filamentous actin. Together, these results strongly implicate actin filament length as a key determinant of the mechanical plasticity of contracted airway smooth muscle. Interestingly, actin filament stabilization by itself did not alter maximal shortening, spontaneous lengthening, or relengthening in response to force oscillation (Figs. 4, 5, and 7). Perhaps conditions within the normal contracting airway myocyte already favor actin filament stability so that the potential for jasplakinolide to enhance actin filament stability even further is limited.

Third, after initial isotonic shortening, there was modest spontaneous relengthening of our maximally ACh-activated canine TSM strips during sustained contraction against a steady afterload of 32% F$_{\text{max}}$ (Figs. 3–7). In previous studies in isometrically contracted canine TSM, our laboratory (21) demonstrated that force is maintained without decrement in the presence of 100 μM ACh for tens of minutes to hours. Perhaps isotonically contracted tissues are more susceptible to fatigue than isometrically contracted tissues, or perhaps spontaneous lengthening is a manifestation of parallel-to-series rearrangement of contractile units within the myocytes (26) or reflects dynamic changes in actin filament-focal adhesion linkage (8). Fourth, the initial maximal shortening observed during ACh stimulation was similar whether or not a ±16% F$_{\text{max}}$ force oscillation was superimposed on the mean load (Fig. 3 and Table 1), even though the subsequent relengthening during sustained contraction was greater in the presence of load fluctuation. Equivalent maximal shortening with or without force perturbation was not expected, but this observation clearly indicates that the mechanism that initially shortens TSM may be separate from that which maintains the tissue at its shortened length. Perhaps this dissociation is a manifestation of contributions from normal and slowly cycling cross bridges (9).

A fifth remarkable observation was that latrunculin B and jasplakinolide exhibited profoundly different effects among isometric, auxotonic, and isotonic contractions of canine TSM strips (Figs. 1, 2, 4, and 5). Latrunculin B (30 nM) significantly reduced ACh- and KCl-elicted isometric force by ~50% of that observed for vehicle (DMSO) control tissues (Fig. 1, D and E). Jasplakinolide (500 nM, Fig. 1C) also caused significant reductions in isometric force but of lesser magnitude than latrunculin B. However, these same concentrations of these agents did not affect auxotonic shortening at all (Fig. 2). Although lower concentrations of latrunculin B and jasplakinolide were used in isotonic studies, they did not cause any significant effect on shortening (Figs. 3–7), but latrunculin B did have profound effects on lengthening with oscillation and hysteresivity (Figs. 5 and 6). Differences between isometric and isotonic responses are not without precedent. TSM from immune-sensitized dogs demonstrate increased isotonic shortening velocity and capacity compared with muscle strips from littermate control animals, yet no differences in F$_{\text{max}}$ could be found between these two tissues when isometric, tetanic parameters were analyzed (12). Similar differences between isometric and isotonic responses were observed in passively and sham-sensitized human seventh-generation airways (20), and abnormal contraction dynamics have been established in single smooth muscle cells from lung biopsies of asthmatic vs. nonasthmatic subjects (15).

There are some limitations to the interpretation of our data. We did not measure actin filament length directly; rather, we presumed that actin filaments would be stabilized with jasplakinolide and that G-actin sequestration with latrunculin B would lead to altered polymerization/depolymerization dynamics and reduced actin filament length (16). However, we did not directly quantify soluble and filamentous actin compartments by using DNase and phalloidin staining, respectively, and found a greater ratio of G- to F-actin in latrunculin B-treated tissues, suggesting less filamentous actin in these muscles compared with control strips. Less filamentous actin could be the result of either fewer or shorter filaments. Our data could not differentiate between these two possibilities; however, based on known effects of jasplakinolide and latrunculin B, the latter possibility is the more plausible. Also, our data could not differentiate between effects on contractile apparatus-associated or cytoskeletal actin filaments. Finally, we cannot rule out the possibility that latrunculin B directly alters cross-bridge dynamics during force oscillations superimposed on isotonic contractions (5, 6, 8, 13), although we know of no mechanism that suggests this should occur. However, it is interesting to speculate that latrunculin B might indirectly alter cross-bridge dynamics through shorter actin filaments, despite increased MLC phosphorylation.

It is tempting to speculate that increased actin filament length contributes to the apparently less compliant behavior of smooth muscle on deep inspiration within the airways of asthmatic subjects. As noted above, actin filament length is regulated through multiple pathways, many of which respond to external stimuli, including inflammatory stressors such as cytokines and growth factors known to be present in asthmatic airways (3). Perhaps the inflammatory environment of the asthmatic airway leads to abnormally long actin filaments in asthmatic airway myocytes that impart a reduced ability to relengthen with deep inspiration. If so, then perhaps a novel strategy for asthma therapy might be to restore the failed compliance of asthmatic airway smooth muscle.

**REFERENCES**


