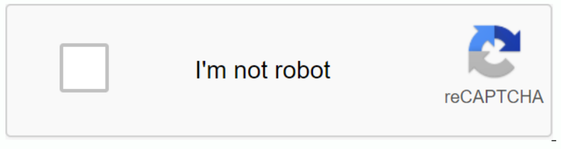

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Latrunculin a actin reorganization

The role of actin polymerization in the regulation of smooth muscle contractility was investigated in dog tracheal muscle strips. The effect of contractile activation on the content of monomeric globular (G)-actin was estimated using the method of DNase I inhibition. The G actin content was 30 % lower in extracts of muscle strips activated with 10-4 M acetylcholine (ACh) than in extracts from unstimulated muscle strips. The decrease in G-actin in response to contractile stimulation was prevented by latrunculin-A, a agent that prevents actin polymerization by binding to G-actin monomers. Inhibition of actinpolymerization by latrunculin-A markedly depressed force development in response to ACh but had no effect on ACh-induced myosin light chain (MLC) phosphorylation. Latrunculin also suppressed the length sensitivity of force during ACh-induced isometric contractions. The actin-capping agent cytochalasin-D also significantly inhibited force and caused only a slight decrease in MLC phosphorylation. Cytochalasin-D also inhibited force in α-toxin-permeabilized muscle strips that were activated either by Ca2+ or by ACh at constant pCa. No disorganization of smooth muscle cell ultrastructure was detected by electron microscopy or by immunofluorescence microscopy of muscles treated with either agent. The results suggest that the polymerization of actin is stimulated by the contractile activation of tracheal smooth muscle and that this actinpolymerization contributes directly to force development. In addition, actin filament remodelling contributes to the length sensitivity of tracheal smooth muscle contractility. Mongrel dogs (20-25 kg) were anaesthetized with pentobarbital sodium (150 mg kg−1i.v.) and killed by rapid exsanguination, approved by the Indiana University Animal Care and Use Committee. A 10-15 cm segment of the extrathoracic trachea was immediately removed and immersed in physiological saline (PSS) at 22°C of the following composition (mM): 110 NaCl, 3.4 KCl, 2.4 CaCl2, 0.8 MgSO4, 25.8 NaHCO3, 1.2 KH2PO4 and 5.6 glucose. The solution was aerated with 95% O2-5% CO2 to maintain a pH of 7.4. Rectangular strips of trachealis muscle 12-15 mm long and 2-3 mm wide dissected from the trachea after removal of the epithelium and connective tissue layer. Muscle strips were equilibrated for about 90 min after being mounted in a tissue bath and attached to a force transducer (Grass) at a dormant voltage of 2 g. The maximum active strength optimal length (Lo) was determined by increasing muscle length progressively during successive stimulations with 10−5 m acetylcholine (ACh) until the strength of active contraction reached a maximum. After the determination of Lo, muscle strips were maintained in PSS containing the vehicle (0,1 % DMSO), incubated in 1 h with cytochalasin-D (0,5, 1,0 or 10 μm), or incubated for 45 min with latrunculin-A (0,1, 0,5 or 1 μm) dissolved in 0,1 % DMSO. The strips were then stimulated with 10-4 M ACh for mine after which they were quickly frozen with liquid N2-cooled pliers for measuring MLC phosphorylation. Up to 14 muscle strips from a single trachea were studied simultaneously. Duplicate muscle strips were used for each measurement. The effects of cytochalasin-D and latrunculin-A lengthwise dependent on force and MLC phosphorylation were assessed in muscle lengths between Lo and 0.6Lo as follows. Muscle strips were contracted isometrically at the predetermined lengths repeatedly using 10−5 M ACh until they developed constant force at that length. They were then incubated with 1 μm cytochalasin-D or 0.5 μm latrunculin-A for 45 min. Strips were then stimulated with 10−5 m ACh for 5 min and contractile might be determined. In some experiments, strips were then frozen for the measurement of MLC phosphorylation. Frozen muscle strips were immersed in acetone containing 10% (w/v) trichloroacetic acid and 10 mM dithiothreitol (DTT) (acetone-TCA-DTT) cooled to -80°C with crushed dry ice. Strips were denished in acetone-TCA-DTT at room temperature and then washed with acetone DTT. Myosin light chains were extracted for 60 min in 8 M urea, 20 mM Tris, 22 mM glycine and 10 mM DTT. Proteins were separated by glycerol-urea polyacrylamide gel electrophoresis and blotted on nitrocellulose. MLCs were specifically labeled with polyclonal rabbit anti-myosin light chain 20 antibodies. The primary antibody was detected with 125I-labeled recombinant Protein A (New England Nuclear). Unphosphorylated and phosphorylated bands of myosine light chains were localized on nitrocellulose membranes by autoradiography. Tapes were cut out and counted in a gamma counter. Background counts were subtracted and fractional phosphorylation was calculated as the ratio of phosphorylated myosin light chains to total myosin light chains. A modification of the method of Kitazawa et al (1989) was used to permeate the muscle strips. Muscle strips (0.1-0.2 mm wide and 7-10 mm long) were incubated at 22°C in a relaxing solution composed of (mM): 8.5 Na2ATP, 4 K-EGTA, 1 DTT, 10 sodium creatine phosphate, 20 imidazole, 8,9 magnesium acetate, 100,5 potassium acetate and 1 mg−1 creatine phosphokinase (Sigmakatt. No C 3755; 310 U mg−1). After 20 minutes, the strips were then incubated in the same solution with the addition of α-toxin (350 u ml−1) (Calbiochem), plus 0.1 mg ml−1 phosphocuminkinase and 1 μm leupeptin (a protease inhibitor) for a further 20-25 min. Intracellular Ca2+ stores were depleted by incubation of strips in 10 μm calcium ionofor A23187 in relaxing solution. An algorithm for Fabiato & Fabiato (1979) was used to calculate the composition of relaxing or contracting solutions containing free Ca2+ from pCa 9 to pCa 5. For the measurement of isometric force, permeabilized muscle strips were mounted in tissue baths and attached to the Gould GM-2 force transducer. In each experiment, permeabilisation of the strips was verified by contracting the muscles with solution at pCa 5.The content of G-actin in smooth muscle strips was estimated by measuring inhibition of DNase I activity by G-actin (Blikstad et al. 1978). Muscle strips were subjected to treatment with ACh, latrunculin or cytochalasin-D and then frozen and powdered during liquid N2. Powdered powder was transferred to dry ice-cooled centrifuge tubes for the extraction of protein. After the addition of 800 μl extraction buffer, the sample was rapidly vortexed. The extraction buffer (pH 6.9) contained 60 mM Pipes, 25 mM Hepes, 10 mM EGTA, 2 MgCl2, 0,5 % Triton X-100, 0,1 mM DTT, 0,5 mM phenylmethylsulfonylfluoride (PMSF) and protease inhibitors (0,01 mg ml−1 each of chymotrypsin, leuptinep, aprotinin and pepstatin). The test was then held at 4°C for 5 min after which it was centrifuged at 14000 g for 8 min. The supernatant was then transferred to another tube for measuring G-actin content. The DNase I inhibition analysis was performed at 25°C. The same time rate for protein extraction and G-actdetermination was maintained for each sample. One millilitre of DNA solution (100 μg veal milling DNA dissolved in 0.1 M Tris-HCl (pH 7.4), 4 mM MgSO4 and 1.8 mM CaCl2) was added to 10 μl DNase I solution (1 μg enzyme in 0.05 M Tris-HCl (pH 7.4), 0.01 mM PMSF, and 0.1 mM CaCl2). The production of DNA oligonucleotides due to hydrolysis of DNA was then monitored by recording hyperchromicity at 260 nm as a function of time using a Beckman UV spectrophotometer. The concentration of G-actin in different volumes (5-30 μl) of muscle extract was determined using several aliquots from each muscle extract sample. Extract samples were mixed with DNase I solution for 10 s before the addition of DNA solution and reaction rate was followed for up to 3 min. Muscle extract volumes were chosen to allow 30-70% inhibition of DNase activity. DNase activity was also measured in the presence of the same volume of sample buffer without the addition of muscle extract. The concentration of G-actin in the muscle extract causing 50 % inhibition of DNase activity was estimated from a standard curve determined using known amounts of purified actin. The concentration of protein in each sample of muscle extract was estimated by using a standard microprotein analysis (Pierce). The G-actin content of the muscle extract was then normalized as a fraction of soluble protein. The accuracy of the analysis in smooth muscle extract was confirmed by adding known amounts of purified G-actin to samples of muscle extract and verifying that this resulted in predicted increases in inhibition of DNase I activity. In separate experiments, the proportion of G-actin was estimated to total actin in tracheal muscle strips by quantifying the actin content of the supernatant of muscle extract relative to actin found in total muscle homogenates, provided that most of the actin of the supernatant was in the form of G-actin. Proteins in and in total muscle homogenates were separated by 15 % sodium dyl sulfate-polyacrylamide gel electrophoresis, transferred to nitrocellulose, and blocked with a 5 % solution of fat-free dry milk. The membranes were probed with mouse monoclonal G-actin antibody (Sigma) and then with horseradish peroxidase and anti-mouse mouseglobulin (Amersham) for visualization of chemiluminescence. Actin in supernatant and total muscle homogenate was quantified by densitometry and calculated as a ratio for determining the proportion of G-actin to total actin. Muscle strips were established at room temperature in 2% glutaraldehyde in 0.075 M cacodylat containing 1.2 mM calcium and 4.5% sucrose at pH 7.4. The tissues were postfixed in 2% osmium in the 0.1 M cacodylate buffer for 2 h, rinsed with buffer and in blocks stained with saturated aqueous uralate acetate for 90 min. They were then dehydrated in graduated alcohols and embedded in Spurr's rets. Longitudinal section 60-70 nm thick was then cut with a Sorwall MT5000 ultramicrotome with muscle tissue oriented longitudinal parallel to the edge of the Dupont diamond microtom knife. The tissue sections were picked up on uncoated grids and seen at a magnification of ×10,000 under the electron microscope. Tracheal muscle strips (1 mm wide, 10-20 mm long) were transferred to 5 ml dissociation solution (composition, mM: 125 NaCl, 4.7 KCl, 0.25 CaCl2, 1.0 MgCl2, 10 Hepes, 0.25 EDTA, 10 D glucose, 10 taurine, pH 7), with added collagenase (400 U ml−1, type I), papain (30 U ml−1, type IV) bovine serum albumin (1 mg ml−1) and DTT (1 mM). All the enzymes were from Sigma. The strips were then maintained in a 37°C shaking bath at 80 min oscillations. After 40 min, the strips were washed several times with Hepes-buffered saline (composition, mM: 130 NaCl, 5 KCl, 1.0 CaCl2, 1.0 MgCl2, 20 Hepes, 10 D-glucose, pH 7.4) and gently triturated to rid individual smooth muscle cells (Halayko et al. 1996). Dissociated cells had to settle on duvets for 40 min. Dissociated smooth muscle cells adhering to cover ropes were incubated in Hepes buffered saline containing 0,1 % DMSO, 10 μm cytochalasin-D, or 1 μm latrunculin for 45 min after which they were fixed with 3,7 % (v/v) paraformaldehyde in phosphate buffered saline solution (composition, mM: 137 NaCl, 4,3 Na2HPO4, 1,4 KH2PO4, 2,7 KCl, pH 7,4) for 10 min. The cells were washed three times in Tris buffered saline (TBS) containing 50 mM Tris, 100 mM NaCl, 0.1% NaN3 and then permeabilized for 2 min with 0.2% Triton X-100 dissolved in TBS. The cells were rinsed again three times in TBS and tagged with rhodamin-phalloidin (1:400, Molecular Probes) at room temperature. After 25 min the cells were washed again to remove excess label and cover tie was mounted on slides with 10-15 μl mounting medium containing antifade agents (gelvatol with glycerol, n-propyl gallate and sodium azide). Cells were then seen using a BioRad 1024 MRC laser confocal and seen with a × 100 oil immersion target (numeric aperture 1.4). Comparisons between different groups were performed by one-way analysis of variance or Kruskal-Wallis one-way analysis of variance. Differences between the two groups were analyzed by Student's t-test or Dunn's method. Statistical analysis was performed using SigmaStat software. Values of n represent the number of experiments used to obtain each value. P< 0.05 was considered significant. Force and MLC phosphorylation in response to 10−4 M ACh were assessed in muscle strips treated for 45 min by 0.0.1, 0.5 or 1 μm of latrunculin-A, a agent that binds to monomeric actin and prevents its assembly to actinfilament (Fig. 1A and B). Force was significantly reduced in all latrunculin-A treated muscle strips compared to that in untreated muscle strips. The reduction in force increased when the concentration of latrunculin was increased from 0.1 to 1.0 μm. Latrunculin-A had no effect on MLC phosphorylation in ACh-stimulated or unstimulated muscles. Force and MLC phosphorylation in response to 10-4 M ACh were also assessed in five muscle strips incubated for 1 h in 0,0,5, 1,0 or 10 μm cytochalasin-D (Fig. 1C and D). Force development in response to ACh was significantly depressed in all muscle strips treated with cytochalasin-D compared to that in untreated muscle strips. The magnitude of the effect of cytochalasin-D on force in response to ACh increased with increases in the concentration of cytochalasin. Cytochalasin-D significantly reduced ACh-induced MLC phosphorylation below obtained in untreated muscle strips; however, the effect of cytochalasin-D on MLC phosphorylation was small and was not dose dependent. Cytochalasin-D had no effect on MLC phosphorylation in unstiulated muscle strips. Permeabilized muscle strips were used to evaluate whether the effects of cytochalasin-D on force were due to effects on Ca2+ signaling. Permeabilized muscle strips were incubated in 0.1, or 10 μm cytochalasin-D in ca2+-free buffer (pCa 9) for 15 min. Strips were then contracted isometrically with construction solution at pCa 5 or with 10−4 M ACh at pCa 7. Force development in response to an increase in Ca2+ was significantly depressed in muscle strips treated with 1 or 10 μm cytochalasin-D compared to that in untreated muscle strips (Fig. 2). Contractions induced by ACh at constant Ca2+ (pCa 7) were also significantly depressed in muscle strips treated with either 1 or 10 μm cytochalasin-D.Muscle strips were set at lengths of 0.6Lo, 0.8Lo or Lo and then incubated for 45 min in PSS with or without 1 μm cytochalasin-D or 0.5 μm latrunculin. The strips were then stimulated by 10-5 M ACh for 5 min after which they were frozen for measurement of MLC phosphorylation. Figure 3 illustrates the effect of treatment with cytochalasin and latrunculin on force and MLC phosphorylation for contractions induced at Lo, 0.8Lo or 0.6Lo. Values shown for power are normalized to force at Lo in the untreated muscle strips. In latrunculin-treated muscle strips, active force in response to ACh was not significantly different in muscle strips contracted at 0.6Lo, 0.8Lo or Lo (Fig. 3A), indicating that latrunculin inhibited the length of the sensitivity of muscle force (P< 0.05). Thus, inhibition of force by latrunculin was greater at longer muscle lengths: at Lo, latrunculin inhibited force by 49.2 ± 2.0% (n = 18); at 0.8Lo, stunted latrunculin force by 34.7 ± 4.8% (n = 8); while at 0.6Lo, force was inhibited by only 25.2 ± 3.0% (n = 18). Differences in the effect of latrunculin on force at different muscle lengths were statistically significant. However, cytochalasin had no effect on the length sensitivity of muscle power. Cytochalasin inhibited force by 33.4 ± 2.7% at Lo and by 35.2 ± 8.3% at 0.6Lo (n = 8-10) (Fig. 3C). MLC phosphorylation was significantly lower in muscle strips isometrically contracted at 0.6Lo compared to Lo in all muscle strips regardless of treatment (Fig. 3B and D), demonstrating that the length sensitivity to MLC phosphorylation was not affected by either cytochalasin or latrunculin. Treatment with cytochalasin resulted in a small but significant depression of MLC phosphorylation at both Lo and 0.6Lo.The tension and strain experienced by muscle cells both increase when the muscle is contracted at longer muscle lengths. We therefore evaluated whether the greater inhibitory force through latrunculin at longer muscle lengths results from the effect of the increased active tension or the longer muscle length itself (Fig. 4). In each experiment, a pair of muscle strips were contracted in different lengths (Lo and 0.6Lo) using concentrations of ACh that resulted in similar levels of tension (Fig. 4C and D). Another pair of strips were contracted at the same muscle length (Lo) using concentrations of ACh that resulted in different levels of tension (Fig. 4A and B). In strips contracted to different levels of tension at the same length, the percentage inhibition of muscle force by latrunculin was not significantly different (Fig. 4B). However, when strips were contracted in different lengths to comparable levels of tension, inhibition of muscle power by latrunculin was much greater at the longer length. This indicates that increased muscle length rather than greater tension is the primary stimulus for increased sensitivity of muscle power to latrunculin during isometric contraction at longer lengths. We used DNase I inhibition analysis to assess the effects of contractile activation and muscle length on G-actin content (Fig. 5A). Strips were assayed for G-actin after being maintained at either 0.6Lo or Lo without contractile stimulation (unstiulated), or after 5 min isometric contraction with ACh on either Lo or 0.6Lo (stimulated). At both muscle lengths, stimulation with ACh caused the G-actin content to decrease significantly by about 30%. Mean G-actin content of muscles at 0.6Lo was slightly higher than at Lo, but the difference was not statistically significant. The level of G-actin in unstimulated muscle strips was similar at Lo and at 0.6Lo.Vi also evaluated the effects of cytochalasin (10 μm) and latrunculin (1 μm) on the G actincontent of muscle strips stimulated by ACh. Latrunculin prevented the reduction of G-actin during stimulation with ACh; G-actin levels in latrunculin-treated muscle strips stimulated with ACh were similar to those in unstimulated strips. Latrunculin had no effect on G-actin in unstimulated strips. However, cytochalasin did not prevent the reduction of G-actin resulting from contractile activation (Fig. 5B). In separate experiments, we estimated that G-actin represented 31 ± 1.1% (n = 4) of the muscle's total actin content. Thus a 30 % decrease in G-actin content represents polymerization of approximately 10 % of the total actin in the cell. Sections of tracheal smooth muscle strips were seen under the electron microscope to determine the effects of treatment with 10 μm cytochalasin-D or 1 μm latrunculin-A on the ultrastructure of muscle cells and the organization of contractile filaments (Fig. 6). No differences in contractile filament organization or ultrastructure could be detected in electron micrographs taken from strips treated with cytochalasin or latrunculin compared to untreated muscle strips. We also evaluated the effects of cytochalasin and latrunculin on cell morphology and F-actin fluorescence in isolated dissociated tracheal smooth muscle cells. Unstimulated cells were incubated in 10 μm cytochalasin-D or 1 μm latrunculin for 45 min, and then solid and stained with rhodamine-phalloidin. No significant differences in cell morphology or F-actin fluorescence were detected among untreated, cytochalasin-treated and latrunculin-treated smooth muscle cells (Fig. 7). The results of this study show that the contractile activation of tracheal smooth muscle causes a decrease in the content of G-actin, and that inhibition of actinfilament polymerization inhibits force development. These results suggest that actin polymerization is stimulated by the contractile activation of tracheal smooth muscle, and that this polymerization is necessary for force development. Our results also show that actin filament dynamics are sensitive to muscle length; latrunculin has a greater inhibitory effect on force development at a long muscle length than at a short muscle length. Thus, the regulation of actin dynamics can contribute to the length dependent on smooth muscle contractility. Our data clearly show that the inhibition of contractile force by agents inhibiting actin filament polymerization is not the result of disruption of signal pathways that regulate myosin light chain phosphorylation, or from the disorganization of smooth muscle cell ultrastructure. The results of this study are consistent with our hypothesis that stimulation initiates strain-sensitive signaling pathways that regulate the attachment and polymerization of actinfilaments in smooth muscle cells, and that this contributes to the longitudinal regulation of smooth muscle contractility. These studies provide several lines of evidence that contractile activation stimulates actin polymerization in tracheal smooth muscle. The contraction of the tracheal muscle with acetylcholine reduces the content of G-actin by approximately 30%. This represents the polymerization of about 10 % of the total cellular actin (Fig. 5). In addition, both latrunculin and cytochalasin inhibit force development. Cytochalasin-D and latrunculin-A are both inhibitors of actin polymerization, although they act through different mechanisms. Cytochalasin caps existing actinfilaments, preventing their growth on the tagged end, while latrunculin binds to actin monomers and prevents their assembly in filamentous actin (Cooper, 1987; Coue et al. 1987). At the optimal muscle length, Lo, we found that both cytochalasin-D and latrunculin-A depressed ACh-induced force development in a dose-dependent manner. Latrunculin also prevented the decrease in G-actin content in response to contractile stimulation. This is consistent with its inhibitory effect on actinpolymerization by inactivation of G-actin monomers. In the presence of cytochalasin, ACh induced a decrease in G-actin content. Actinpolymerization in the presence of cytochalasin may have resulted from the accelerated nucleation of new actinfilament caused by capping of existing filaments (Saito & Karaki, 1996; Sugidachi et al. 1998). Several investigators have reported that force development through cytochalasin in other smooth muscle tissues has been inhibited (Adler et al. 1983; Obara & Yabu, 1994; Saito et al. 1996; Tseng et al. 1997), although the effects of latrunculin on smooth musculature have not previously been reported. In endothelial cells, fibroblasts and platelets, cytochalasin inhibits Ca2+ signaling (Bourguignon et al. 1993; Ribeiro et al. 1997; Holda & Blatter, 1997), suggesting that actinpolymerization is necessary for Ca2+ signaling in these cells. If this was also true in smooth muscle tissues, the effects of cytochalasin or latrunculin on force development can be accounted for by an inhibition of Ca2+-calmodulin-dependent myosin light chain phosphorylation. However, we found that while latrunculin-A caused a marked inhibition of force development, it had no effect on ACh-induced myosin light chain phosphorylation (Fig. 1). Although cytochalasin-D caused a slight depression of ACh-induced MLC phosphorylation, the decrease was insufficient to take into account the marked inhibition of force that occurred at higher concentrations of this agent (Fig. 1). Further, cytochalasin-D inhibited force in α-toxin-permeabilized muscle strips that were activated either by an increase in pCa or by the addition of ACh at constant pCa (Fig. 2). This indicates that cytochalasin was still effective at inhibiting force development even when intracellular Ca2+ concentration was experimentally maintained. Our results thus show that inhibition of force development caused by cytochalasin-D and latrunculin-A in the dog tracheal muscle does not result from effects on signaling pathways leading to Ca2+ dependent MLC phosphorylation. These findings therefore suggest that actin polymerization plays a direct role in force development in smooth muscle that is independent of signaling events that regulate phosphorylation of myosin light chains. The results of previous studies examining the mechanism of the effects of cytochalasin on smooth muscle contraction are contradictory. Tseng et al. (1997) reported that in bovine tracheal smooth muscle, inhibition of force was caused by cytochalasin-B, a less specific agent than cytochalasin-D, associated with a decrease in myosin light chain phosphorylation. They concluded that the inhibitory effect of cytochalasin on force was due to inhibition of Ca2+ signaling and MLC phosphorylation caused by disruption of the actin cytoskeleton. However, Saito et al found. (1996) that cytochalasin-D inhibited contractions induced by norepinephrine and K+ in rat aorta smooth muscle without affecting intracellular Ca2+ or MLC phosphorylation. In line with this, Obara & Yabu (1994) found no effect of cytochalasin-B on voltage-dependent Ca2+ currents and membrane potentials in taenia coli smooth muscle. Our current results show that smooth muscle contraction is inhibited by latrunculin, which has no effect on myosin light chain phosphorylation, provides unequivocal evidence that inhibition of actin polymerization per se inhibits smooth muscle contraction. The activation of cells such as platelets, neutrophils and fibroblasts results in a rapid polymerization of monometric globular (G) actin to filamentous (F) actin (Cano et al. 1991; Symons & Mitchison, 1991; Hartwig, 1992; Iwig et al. 1995). This polymerization takes place primarily at the tagged end of the actinfilament, where the filament links to transmembrane integrins (Cooper, 1987, 1991; Schafer & Cooper, 1995). In these cells, actinpomerization is regulated by the availability of free tagged ends of actinfilaments as well as by the pool of available actinmonomers. Most actin filament tagging ends are not freely available in dormant cells (Hartwig, 1992; Hug et al. 1995) as they are blocked from elongation through roof proteins such as gelsolin and CapZ (Barkalow et al. 1996). These proteins are also found in differentiated smooth muscles (Pollard & Cooper, 1986; Schafer & Cooper, 1995). The activation of platelets and other non-muscle cells causes uncapping of actinfilament, which increases the number of free jagged ends and allows actinpolymerization to continue (Symons & Mitchison, 1991; Hartwig, 1992, 1995; Nachmias et al. 1996). In platelets, the interaction between capping protein gelsolin with actin may be inhibited by PIP2, a by-product of phosphatidyl inositol breakdown that is also produced in smooth muscle in response to stimulation (Janmey, 1994). Roofs of the barbed ends of actinfilamentcan be connected to the detachment of the filament from the diaphragm (Carlier, 1998). Cytochalasin-D binds to the tagged (fast-growing) ends of actinfilament and acts as a roof protein (Cooper, 1987). As the binding of cytochalasin is not regulated by PIP2, actin filaments limited by cytochalasin do not become uncapped during activation of the cell. Thus, they do not undergo polymerization on the tagged end, and they must not attach to the integrin complex and engage in force transfer during cell activation. Thus, the effects of cytochalasin on force in smooth muscle may be related to its ability to block the attachment of actin filaments to the integrin complex, as well as its ability to prevent filament polymerization at the tagged end. In contrast, latrunculinen does not interfere with uncapping or attachment of filamentous actin to the integrin complex, but prevents actinpolymerization by complexing with free actin monomers and thus inactive pool of monomeric actin (Coue et al. 1987). A striking finding of our study was that the inhibitory effect of latrunculin on contractile force was length sensitive, suggesting that mechanical strain may stimulate the degree of actin polymerization. The force inhibition caused by latrunculin was greatest when the strips were activated at the longest length, Lo, and at least when the muscles were activated at 0.6Lo. As a result, in the presence of latrunculin, there were no significant differences in force development at muscle lengths between 0.6Lo and Lo in response to acetylcholine (Fig. 3). Myosine light chain phosphorylation is cross-disciplinary in smooth muscle, and the length sensitivity of contractile activation has been proposed as a mechanism to take into account longitudinal modulation of contractility (Rembold & Murphy, 1990; Mehta et al. 1996). However, latrunculin did not affect the length sensitivity of myosine chain phosphorylation; thus the differences in its effect on contraction on different muscle lengths could not have resulted from a suppression of the mechanosensitivity of myosinactivation. We observed a slight difference in the mean levels of G-actin in muscles activated with ACh at Lo and 0.6Lo using DNase inhibition analysis, but this difference was not statistically significant (Fig. 5A). This suggests that differences in the amount of actin polymerized at different lengths may be small and difficult to discriminate with the help of this analysis. Alternatively, mechanical strain may affect the dynamics of actinpolymerization rather than the total amount of G-actin polymerized to filaments during contractile activation. We evaluated differences in the sensitivity of contractions at Lo and 0.6Lo against inhibition by were directly related to the difference in muscle length or to the differences in active tension at these muscle lengths. Our results indicated that muscle length rather than tension is the primary determining factor of latrunculin sensitivity (Fig. 4). These results are consistent with our recent report that muscle length rather than tension is the stimulus for the longitudinal increases in tyrosine phosphorylation of paxillin and focal adhesion kinase during contractile activation of tracheal smooth muscle (Tang et al. 1999). These proteins have been involved in the signal pathway for cytoskeletal remodeling in non-muscle cells (Burridge & Chrzanowska-Wodnicka, 1996; Shyy & Chien, 1997; Schmidt, 1998; Glogauer et al. 1998). Taken in the sum, our observations suggest a mechanosensitive process in smooth muscle in which externally applied strain activates an integrin-mediated signaling pathway leading to actin filament polymerization and cytoskeletal reorganization. Actin polymerization in smooth muscle cells may involve prolongation of existing actin filaments and/or nucleation of new actin filaments. This could allow the smooth muscle cell to adapt the arrangement of its contractile apparatus to externally forced changes in the shape of the cell. In tracheal smooth muscle, contractile activation at different lengths results in persistent differences in stiffness suggesting differences in cell structure (Gunst et al. 1995; Gunst & Wu, 1996; Gunst, 1999). In our study, the effects of cytochalasin-D contrasted with those of latrunculin in that cytochalasin inhibited force development proportionally during contraction at all muscle lengths while latrunculin is inhibited proportionally more at long muscle lengths (Fig. 3). The absence of an effect of cytochalasin at length the sensitivity of active force has previously been reported for bovine tracheal muscle (Youn et al. 1998). The difference in the inhibitory effect of latrunculin and cytochalasin on the length sensitivity of active force can be interpreted in terms of the mechanisms of action of these agents. Latrunculin prevents the polymerization of new actinfilaments or the extension of existing filaments. This should prevent the trunk-sensitive rebuilding of actinfilaments, although existing filaments could continue to participate in power transmission. In contrast, cytochalasin caps existing F-actin filaments and may therefore inhibit their attachment and participation in force transmission (Carlier, 1998). Only the actinfilament left without a cytochalasin-D roof could continue to participate in power transmission. Therefore, in the presence of cytochalasin, force development at all muscle lengths should be proportionally inhibited. We used electron microscopy and immunofluorescence microscopy to evaluate the effects of cytochalasin and latrunculin on actin filament integrity and ultrastructure of tracheal smooth muscle. Electron micrographs of muscle strips treated with cytochalasin or latrunculin showed no differences in contractile filament organization or ultrastructure as compared to untreated muscle strips (Fig. 6). We also found no significant differences in morphology of untreated, cytochalasin-treated or latrunculin-treated smooth muscle cells stained for F-actin using rhodamine-phalloidin (Fig. 7). Thus, the effects of these agents on force is unlikely to result from the disruption of actin filament integrity or disorganization of the contractile apparatus. In conclusion, our findings suggest that the contractile activation of tracheal smooth muscle stimulates actin polymerization, and that this actin polymerization contributes directly to force development. The polymerization of actin in response to contractile stimuli may modulate the organization or length of actin filaments as well as the attachment of actin filaments to the cell membrane for the transfer of force. Inhibition of force by inhibitors of actinpolymerization is not due to disruption of signaling pathways leading to myosin light chain phosphorylation, or from the disorganization of cell structure. Actin polymerization can also play an important role in regulating the length sensitivity of force development. We are grateful to Dr. Simon Atkinson for his advice and help with DNase inhibition analysis. 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