Cytoskeletal remodeling in differentiated vascular smooth muscle is actin isoform dependent and stimulus dependent

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Cytoskeletal remodeling in differentiated vascular smooth muscle is actin isoform dependent and stimulus dependent. Am J Physiol Cell Physiol 295: C768–C778, 2008. First published July 2, 2008; doi:10.1152/ajpcell.00174.2008.—Dynamic remodeling of the actin cytoskeleton plays an essential role in the migration and proliferation of vascular smooth muscle cells. It has been suggested that actin remodeling may also play an important functional role in nonmigrating, nonproliferating differentiated vascular smooth muscle (dVSM). In the present study, we show that contractile agonists increase the net polymerization of actin in dVSM, as measured by the differential ultracentrifugation of vascular smooth muscle tissue and the costaining of single freshly dissociated cells with fluorescent probes specific for globular and filamentous actin. Furthermore, induced alterations of the actin polymerization state, as well as actin decoy peptides, inhibit contractility in a stimulus-dependent manner. Latrunculin pretreatment or actin decoy peptides significantly inhibit contractility induced by a phorbol ester or an α-agonist, but these procedures have no effect on contractions induced by KCl. Aorta dVSM expresses α-smooth muscle actin, β-actin, nonmuscle γ-actin, and smooth muscle γ-actin. The incorporation of isoform-specific cell-permeant synthetic actin decoy peptides, as well as isoform-specific probing of cell fractions and two-dimensional gels, demonstrates that actin remodeling during α-agonist contractions involves the remodeling of primarily γ-actin and, to a lesser extent, β-actin. Taken together, these results show that net isoform- and agonist-dependent increases in actin polymerization regulate vascular contractility.

ACTIN MAKES UP ~20% of total protein content in smooth muscle (4). Its importance is highlighted by the fact that it is highly conserved throughout evolution and across species (4, 21). Six isoforms, separate gene products, are present in vertebrate muscles (60). Even among these actin isoforms, there is a large degree of sequence conservation, with over 95% homology at the protein level (61). Most sequence differences are clustered at the NH2-terminal region (21, 61), and the sequence differences in the NH2-terminal region of actin result in a small difference (0.02 units) in the isoelectric point of the isoforms (18), which allows the separation of the isoforms by two-dimensional gel electrophoresis. The NH2-terminal region of actin isoforms is also known to be involved in the binding of many actin-binding proteins (52) and has been suggested to result in isoform-specific functions, largely based on experiments performed in cultured cells (21, 27).

Since the realization that multiple actin isoforms can be expressed within a single cell type, considerable interest has focused on the question of whether such actin isoforms are differentially localized and perform different functions. Despite the importance of actin in diverse cellular functions, the exact functions and locations of actin isoforms have yet to be fully understood. North et al. (46) have reported that actin isoforms in chicken gizzard cells differ in their spatial localization and implied isoform-specific functions based their localization. In contrast, the Murphy laboratory (12) has reported a homogeneous distribution of actin isoforms in mammalian vascular smooth muscle (VSM). Whether the apparent discrepancy is due to tissue, species, or methodological differences has not been clear.

The cytoskeleton in differentiated VSM (dVSM) has generally been assumed to be largely static, performing a solely structural role, compared with the dynamic cytoskeleton of migrating, proliferative VSM cells (VSMCs) (56). However, a considerable literature on airway smooth muscle cells has documented a significant actin cytoskeletal remodeling, particularly in the setting of airway narrowing (32, 35, 41). Recently, a few laboratories have also suggested that actin cytoskeletal remodeling may occur to a significant degree in dVSM, specifically during stretch and hydrodynamic stimuli, which have been linked to changes in actin polymerization patterns (7, 40). However, conflicting results on agonist-induced cytoskeletal remodeling have been reported. Flavahan et al. (16) reported that α-agonists had no effect on actin remodeling, whereas Ohanian et al. (48) and Ward et al. (62) reported that α-agonists induced the tyrosine phosphorylation of adhesion plaque proteins expected to lead to cytoskeletal remodeling. Latrunculin and cytochalasin, agents known to decrease actin polymerization, have also been reported to inhibit agonist-induced contractility and tone maintenance (2, 3, 53, 54, 64, 66). However, it is difficult to discriminate between the possible nonphysiological effects of latrunculin and cytochalasin on force via a direct alteration of contractile filament content versus effects on individual signal transduction pathways involving cytoskeletal remodeling.

We report here, by a combination of cellular and biochemical approaches, a stimulus- and isoform-dependent cytoskeletal remodeling of dVSM. Furthermore, we show that alterations in the actin cytoskeleton caused by chemical agents or decoy peptides result in significant alterations in vascular tone in a stimulus-dependent manner.
MATERIALS AND METHODS

Tissue preparation and force measurements. All procedures were performed according to protocols approved by the Institutional Care and Use Committee. As previously described (37), ferrets (Marshall Farms, North Rose, NY) were euthanized by an overdose of chloroform, and the aorta was quickly removed from animals and dissected in oxygenated (95% O2:5% CO2) physiological salt solution (PSS) consisting of (in mM) 120 NaCl, 5.9 KCl, 1.2 NaH2PO4, 25 NaHCO3, 11.5 dextrose, 1 CaCl2, and 1.4 MgCl2 (pH 7.4). The aorta was carefully dissected to remove all connective tissue and the endothelium under a dissecting microscope. The aorta was cut into circular rings (~3 mm wide), which were then attached to a force transducer and stretched to ~1.3× their resting length (a resting tension of ~2.0 g; previously determined to estimate optimal length). The rings were allowed to equilibrate at 37°C for 1 h in oxygenated PSS and were then stimulated for up to 10 min with 51 mM KCl-PSS (51 mM of NaCl replaced with KCl in PSS). After the rings were allowed to reach maximal, steady-state force, the depolarizing solution was washed three times with PSS. The rings were then allowed to relax for 1 h before beginning the experiment. The aorta rings were quick-frozen by immersion into dry ice-acetone slurry containing 10 mM dithiothreitol. The concentrations of agonist used here were chosen to be exactly those used in our past studies using the same tissue and thus allow direct comparison. The concentrations used are also maximally effective concentrations and allow us to make accurate measurements with the techniques used in this article.

Differential ultracentrifugation of homogenates for ratios of filamentous- and globular-actin. The protocol was according to manufacturer’s instructions (Cytoskeleton, Denver, CO) with some modifications. Each muscle strip (~20 mg wet weight) was placed into a glass homogenizer tube (Kimble Kontes, Vineland, NJ) and quickly homogenized by a motorized tissue grinder in 200 μl of lysis buffer (37°C): 50 mM PIPES (pH 6.9), 50 mM NaCl, 5 mM MgCl2, 5 mM EGTA, 5% (vol/vol) glycerol, 0.1% Nonidet P-40 (-NP-40), 0.1% Triton X-100, 0.1% 2-mercapto-ethanol, 0.001% antifoam C with 100 mM ATP and a cocktail of protease inhibitors; and 0.4 mM tsoyl arginine methyl ester, 1.5 mM leupeptin, 1 mM pepstatin A, and 1 M benzamidine (Cytoskeleton). Tissue homogenates were placed on ice until all samples are completed and then incubated at 37°C for 10 min. Tissue homogenates were transferred to a prewarmed (37°C) ultracentrifuge and spun at 150,000 g for 1 h at 37°C to separate the globular (G)-actin (supernatant) and filamentous (F)-actin fractions (Beckman, Fullerton, CA). The pellets were resuspended with the same amount of volume of ice-cooled distilled water containing 10 μM cytochalasin D. The pellets were dissolved by trituration with a pipette and left on ice for 1 h, vortexing every 15 min for 1 min to dissociate F-actin. Resuspended solutions were centrifuged at 2,300 g for 5 min at 4°C. The second supernatants (F-actin) were collected, and all samples were diluted with appropriate loading buffer and boiled for 5 min. The samples were stored at ~80°C until further separation by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting.

Immunoblotting. Quick-frozen tissue samples were homogenized and analyzed via immunoblotting as previously published (37). Briefly, the homogenized samples were boiled in Laemmli sample buffer. The samples were separated using SDS-PAGE and transferred to a polyvinylidene difluoride membrane (Millipore). Protein transfer was confirmed by staining the membrane with Ponceau S red. The membrane was stained with an appropriate primary antibody overnight at 4°C. After extensive wash with Tris-buffered saline-Tween 20, the membrane was stained with an appropriate secondary antibody. Densitometry of the actin bands was performed with the software for an Odyssey infrared imaging system (LI-COR Biosciences).

Single cell isolation from aorta. Cells were freshly enzymatically dissociated from aorta tissue by a previously published method (37). Briefly, the aorta was carefully dissected to remove all connective tissue and the endothelium under a dissecting microscope. About 100 mg (wet weight) of aorta tissue was then cut into 2×2-mm pieces and placed into a silicon-coated flask containing 7.5 ml Ca2+, Mg2+-free Hanks’ balanced salt solution (HBSS), ~100 U/ml type II collagenase ( Worthington), ~1.0 U/ml elastase (Roche Diagnostics), and 0.2% BSA (Sigma). The flask was secured in a shaking water bath (rate, ~100 cycles/min; and temperature, ~34°C) under an atmosphere of 100% oxygen for 80 min. The tissue pieces were filtered on a nylon mesh (pore size, ~0.5 mm) and washed with ~12 ml Ca2+, Mg2+-free HBSS, and 0.2% BSA. The wash buffer containing the dissociated cells was collected and poured over glass coverslips on ice under an atmosphere of 100% oxygen. Cells were allowed to adhere to the coverslips for ~1 h. After the first digestion, three additional digestions were performed with 50% less elastase for ~20–30 min. After each of these additional digestions, the tissue pieces were filtered and washed, and the dissociated cells were allowed to adhere to the coverslips for ~1 h. The coverslips were washed with Ca2+, Mg2+-free HBSS, and 0.2% BSA, and Ca2+ and Mg2+ were then re-introduced to the (DMSCs) by gradually adding CaCl2 and MgCl2 into the medium surrounding the coverslips until they reached concentrations of 1 mM and 1.2 mM, respectively. In one coverslip of each batch, it was confirmed that the cells were capable of shortening in sucrose-free PSS. Other coverslips were put in a PSS containing 300 mM sucrose, which increases the toxicity of the solution and prevents shortening directly at the cross bridge level (20), allowing use of the coverslips for imaging. Sucrose HBSS has been shown to cause no change in resting or activated Ca2+ levels, PKC signaling, ERK activation time courses, cytoskeletal structure, or resting cell length in differentiated smooth muscle cells (dSMCs) (10, 28, 31, 50, 51).

Cell staining and imaging. All stimulated and unstimulated dSMCs were fixed with 4% paraformaldehyde for 10 min in 100 mM sodium phosphate buffer. The fixative was quenched by washing the cells with 0.1 mM glycine in 1% BSA HBSS. The cell membranes were then permeabilized using 0.1% Triton X-100 in 1% BSA HBSS. Nonspecific binding sites were blocked by incubating the cells with 1% goat serum in 0.05% Triton X-100 and 1% BSA HBSS. After blocking, the cells were incubated with the desired primary antibody diluted in 2% goat serum, 0.05% Triton X-100, and 1% BSA HBSS. Cells were incubated with the appropriate secondary antibody diluted in HBSS. Cells were washed six times with HBSS and mounted with FluorSave (Calbiochem). Cells were costained with DNP I (Alexa Fluor 488; 300 nM) and phalloidin (Alexa Fluor 568; 1:50 dilution) for 20 min. Laminuculin B (30 nM) was used for pretreatment for 45 min. Images of immunostained dSMCs were taken with a Kr/Ar laser (BioRad Radiance 2000) scanning confocal microscope equipped with a Nikon X-60 (numerical aperture (NA), 1.4) oil immersion objective. Images were recorded with Laser Sharp 2000 (Bio-Rad) software. In all cases, it was confirmed that there was no detectable background fluorescence by staining cells in the absence of a primary antibody. In addition, for all colabeling experiments, it was confirmed that there was no detectable cross talk between fluorescent labels by exchanging excitation/emission filters on single-labeled cells.

Digital confocal microscopy for phalloidin-to-DNase I ratio. Ratio measurements for phalloidin-to-DNase I ratios followed a modification of previously published methods (17). Phalloidin has been shown to be highly specific for F-actin, and DNase I was used to identify G-actin and discriminate it from F-actin (34). Images were acquired using a Kr/Ar laser (Bio-Rad Radiance 2000) scanning confocal microscope equipped with a Nikon X-60 (NA, 1.4) oil immersion objective. All images were digitally scanned at 166 lines/s. Kalman averaged three times with minimal pinhole size, and quantified with Bio-Rad Laser Sharp 2000 for Windows NT. For each cell, the single central nucleus was first located at the widest and thickest part of the cell. In previously published studies, nuclear staining confirmed that visual inspection gives accurate identification of the nuclear location.

Digital confocal microscopy for phalloidin-to-DNase I ratio.
(29). The position of the nucleus was especially obvious in the current study, since the phallolidin staining was characteristically decreased in the nuclear region. Others have also noted that nuclear stains give an identical image to that seen in the bright field image of fixed/permeabilized cells (26). The stage was moved so that the nucleus was positioned to one side of the field of view. We used center optical sections and drew each box to extend from cell edge to cell edge. We used three regions of interest (ROIs) per cell in relatively straight tail regions and excluded the nucleus area. In most cases, we covered the majority of the straight, nonnuclear space (Fig. 2A, inset). The average integrated intensity per pixel within each ROI was determined for each wavelength. The ratio of the value for phallolidin was divided by that for DNase I. The ratio values for the three ROIs for each cell were averaged, and the final number was taken as an index of the ratio of F-actin-to-G-actin (F/G ratio) for that cell as previously described (34). One advantage of the phallolidin/DNase I method used here is that the relative abundance of structures too small to resolve by confocal microscopy (such as G-actin monomers) can be quantitated by integrating the fluorescent signal.

Two-dimensional gel electrophoresis. Two-dimensional gel electrophoresis was performed following previously published methods (15, 46, 47). Briefly, samples were homogenized in a buffer containing 9.5 M Urea, 2.0% NP-40, 5.0% β-mercaptoethanol, 1.6% amphotolines (pH 4–6.5), and 0.4% amphotolines (pH 3–10). The isoelectric focusing gels were cast according to O’Farrell’s method (47) except that the ampholyte mixture was restricted to the 4–6.5 intervals [9.2 M Urea, 4% acrylamide, 2% NP-40, 1.6% amphotolines (pH 4–6.5), 0.4% amphotolines (pH 3–10), 0.01% ammonium persulfate, and 0.1% N,N,N′,N′-tetramethylethylenediamine]. The gels were allowed to polymerize overnight at room temperature and were prefocused at room temperature using 20 mM NaOH (anode solution) and 20 mM phosphoric acid (cathode solution) for 15 min at 200 V, 15 min at 300 V, and 15 min at 400 V. Isoelectric focusing was performed for 3 h at 400 V and 15 to 16 h at 500 V using Hoefer Scientific tube gel equipment. The second-dimension gels were run as a regular SDS-PAGE. The first-dimension tube gel was transferred to the slab gel by running buffer containing 250 mM Tris, 1.92 M glycine, and 1% SDS.

Synthesis of NH2-terminal peptide of actin isoforms. Five tetra- or penta-peptides representing the NH2–terminal actin isoform sequence were synthesized by solid-state peptide synthesis using fluorenylmethoxycarbonyl chemistry. The isoforms, acetylated at their NH2-termini, were α-skeletal muscle actin, Ac-DEDE; γ-smooth muscle actin, Ac-EEETT; γ-cytosplasmic actin, Ac-EEIEIA; β-cytosplasmic actin, Ac-DDDIA; and α-smooth muscle actin, Ac-EEED. All isoforms were synthetically coupled to a TAT protein transduction domain comprising residues YGRKKRRQRRR.

To add a fluorescent label to the peptide, a bridge consisting of the sequence GGG-β-alanine-FITC was manually added as a side chain at the first lysine residue of the TAT sequence. The ε-amino group of this residue was protected during the initial synthesis by the acid labile 4-methyltrityl (MTT) group. The MTT was selectively removed from the peptide by five consecutive 1-min treatments with 2% trifluoroacetic acid (TFA) plus 3% tri-isopropylsilane, leaving the peptide still attached to the resin with all other reactive side chains still protected. The three glycine residues and the β-alanine were added to this site with capping by acetic anhydride carried out after coupling each bridge residue. The FITC was coupled to the amino-group of the β-alanine. The peptides were purified to >95% using reversed-phase HPLC.

Loading of NH2-terminal peptide of actin isoforms. An NH2-terminal FITC tag was added to monitor loading efficiency, and a TAT sequence (GYGRKKRRQRRR) was added to facilitate loading (1). α-Skeletal muscle actin peptides were used as a negative control peptide. Aorta tissue was loaded with the peptides as following protocol. At the beginning of all experiments, the tissue was challenged with 51 mM KCl-PSS to test for viability and to normalize for differences in the size or strength of each strip. As Kitazawa and his colleagues mentioned in their publications (11, 33, 63), most blood vessels show an increase in the amount of contraction observed to KCl challenges with time during the experiment. The data in this study were normalized to the first KCl challenge at the start of the day, and thus the response to a later KCl challenge was generally greater than 100% of the initial challenge. The KCl-PSS was washed out several times with PSS. The aorta tissues were allowed to relax for 1 h. The aorta tissues were then incubated for 3 h with 50 μM of each peptide in PSS aerated in oxygen. The peptide solution was changed with fresh peptide solution every hour. A parallel experiment without peptide in the solution was used as a sham. After the incubation period, the tissues were stimulated with 12-deoxyphorbol-13-isobutyrate-20-acetate (DPBA; 10 μM, for 20 min), phenylephrine (PE; 3 μM, for 10 min), or KCl-PSS (51 mM, for 10 min). The tissues, after peptide loading and extensive washing, are visually inspected, before proceeding with the experiment, for the yellowish-green color, confirming the successful loading of the FITC-labeled peptide. Details of this method are published in past studies from our laboratory using the same tissue and successfully loading TAT-peptides and peptides with similar TAT-like sequences (17, 24, 37).

Reagents. Jaspaklinidol, phallolidin, and DNase I were purchased from Molecular Probes (Eugene, OR). Latrunculin B was purchased from A. G. Scientific (San Diego, CA). A G-actin/F-actin assay kit (BK 037) was purchased from Cytoskeleton. DPBA was purchased from LC Laboratories (Woburn, MA). Pharmalytes for isoelectric focusing were purchased from Amersham Pharmacia Biotech AB (Uppsala, Sweden). Paraformaldehyde was purchased from Electron Microscopy Sciences (Hatfield, PA). General laboratory reagents used from analytical grade or better were purchased from Sigma (St. Louis, MO) and Bio-Rad (Hercules, CA).

Antibodies. The following antibodies were used: rabbit polyclonal G-actin antibody (1:500, 0.5 mg/ml; Cytoskeleton); mononclonal anti-α-smooth muscle actin (clone 1A4; 1:10,000, 4.4 mg/ml) and mononclonal anti-β-actin (clone AC-15; 1:150, 1.6 mg/ml) (Sigma); sheep anti-actin, γ-polycylonal antibody (1:1,000; Chemicon); Alexa Fluor 488 goat-anti mouse IgG (2 mg/ml), Alexa Fluor 560 goat-anti-mouse IgG (2 mg/ml), Alexa Fluor 488 donkey anti-sheep IgG (1:2,500, 2 mg/ml), Alexa Fluor 680 goat-anti mouse IgG (2 mg/ml), and Alexa Fluor 680 donkey anti-sheep IgG (1:2,500, 2 mg/ml; Molecular Probes); and IR-Dye 800 goat anti-rabbit IgG (1.0 mg/ml), IR-Dye 800 goat anti-mouse IgG (1.0 mg/ml), and IR-Dye 700 goat anti-mouse IgG (1.0 mg/ml; Rockland). The concentration of antibodies is in stock.

Data analysis. All values given in the article are means ± SE. Differences between means were evaluated using a Student’s t-test. Statistical significant differences were taken at the P < 0.05 level. The n values given represent numbers of cells or animals used in each experiment.

RESULTS

Contractile agonists increase the F/G ratio in dVSM. F/G ratios were determined by two methods. First, F/G ratios were measured by quantifying results from the differential centrifugation of homogenates of dVSM (Fig. 1). Latrunculin, an agent known to associate with G-actin, thereby preventing actin polymerization (44), and phallolidin, an agent known to stabilize F-actin (9), were used as positive controls by directly adding them to homogenates of unstimulated aortic strips. As shown in Fig. 1, A and B, the addition of phallolidin significantly increased the F/G ratio, indicating that the technique measures changes in the F/G ratio when they do occur. Interestingly, the addition of latrunculin causes no significant change in the F/G ratio.

Stimulation with the phorbol ester DPBA or the α-agonist PE to aortic tissues increases the F/G ratio in a consistent and
Latrunculin (Lat; 10^(-6)M) or phalloidin (Phal; 4 μM)-stimulated aorta strip homogenates. A: immunoblot of actin in homogenates of ferret aorta. F- and G-actin were probed with a pan-actin antibody in unstimulated, phorbol ester 12-deoxyphorbol-13-isobutyrate-20-acetate (DPBA; 10 μM) or phenylephrine (PE; 10 μM)-stimulated aorta strip homogenates. Latrunculin (Lat; 10 μM) or phalloidin (Phal; 4 μM) was directly added to homogenates. B: F/G ratio from densitometry of actin bands. *P < 0.05; **P < 0.01, compared with unstimulated. Average is ± SE (n = 4–6).

Fig. 1. Differential ultracentrifugation of differentiated vascular smooth muscle (dVSM) indicates agonist-dependent changes in ratio of filamentous (F)-actin to globular (G)-actin (F/G ratios). A: immunoblot of actin in homogenates of ferret aorta. F- and G-actin were probed with a pan-actin antibody in unstimulated, phorbol ester 12-deoxyphorbol-13-isobutyrate-20-acetate (DPBA; 10 μM) or phenylephrine (PE; 10 μM)-stimulated aorta strip homogenates. Latrunculin (Lat; 10 μM) or phalloidin (Phal; 4 μM) was directly added to homogenates. B: F/G ratio from densitometry of actin bands. *P < 0.05; **P < 0.01, compared with unstimulated. Average is ± SE (n = 4–6).

To confirm that the stimulus-dependent modula-

tion of actin polymerization state modulate contrac-
tility in a stimulus-dependent manner. To test the hypothesis that the net increases of actin polymerization described above cause changes in contractility, we pretreated live aortic smooth muscle tissue with either latrunculin or jasplakinolide. As shown in Fig. 3A, steady-state tonic contractile force in response to DPBA is significantly inhibited by pretreatment with latrunculin. In contrast, the average tonic contractile force response to DPBA is slightly increased by jasplakinolide pre- treatment, but the difference is not statistically significant.

The above results could simply be the nonspecific result of latrunculin-induced alterations of cellular cytoskeleton content. However, we performed the same experiment in preparations activated by 51 mM KCl-PSS, and neither latrunculin nor jasplakinolide pretreatment has any significant effect on force generation (Fig. 3B). These results also point to a difference in the mechanism by which KCl-PSS increases net actin polymerization, since this must involve a latrunculin-insensitive mechanism, i.e., one that is not affected by the sequestering of G-actin monomers.

It is worth noting that the treatment of aortic tissues with latrunculin or jasplakinolide alone has no detectable effect on intrinsic basal tone, even though the ferret aorta has previously been shown to display an active, intrinsic, basal tone (23). This further supports the selectivity of these effects.

**Inhibition of net polymerization by latrunculin is agonist dependent.** To confirm that the stimulus-dependent modula-

tions of contractility caused by latrunculin or jasplakinolide pretreatment are paralleled by changes in actin polymerization, we pretreated freshly dissociated dVSMCs with the same agents as above and quantitated the effect on F/G ratios. Figure 4A shows illustrative images of individual freshly dissociated dVSMCs under different conditions. Although considerable cell-to-cell variability was noticed, when the results were quantitated (Fig. 4B) by ratiometric analysis, it was found that latrunculin alone causes no significant change in the phalloi-
din-to-DNase I staining ratio. However, latrunculin pretreatment significantly inhibits both PE- and DPBA-induced increases in the phalloidin-to-DNase I ratios.

In contrast, when 51 mM KCl is used as a stimulus, pretreatment with latrunculin causes no significant inhibition of the KCl-induced increase in the F/G ratio (Fig. 4B). Thus these results are consistent with the fact that latrunculin pretreatment has no significant effect on the depolarization-induced increases in contractility (Fig. 3) and, furthermore, point to a difference in the mechanism by which KCl depolarization, versus DPBA, causes a net increase in actin polymerization with the former being latrunculin insensitive and the latter being latrunculin sensitive.

Multiple actin isoforms coexist in dVSM. Whether actin isoforms are differentially distributed within individual smooth muscle cells remains controversial. Thus we first determined the actin isoform subtype composition in intact ferret aorta tissue. Homogenates of intact aorta tissues display three predominant spots at 42 kDa, when run on two-dimensional gels (Fig. 5A). These three spots were confirmed to be α-smooth muscle actin, β-cyttoplasmic actin, and γ-actin (from acidic to basic; Fig. 5A) by mass spectrometry (Tufts University Core Facility, Boston, MA). γ-Smooth muscle actin and γ-cyttoplasmic actin cannot be separated by two-dimensional gels, but the relative amounts of proteins in the three spots were quantified by densitometry after staining the two-dimensional gels with Proteom IQ Blue. The major actin component in aorta is α-smooth muscle actin (~66.2% of total actin), followed by β-cyttoplasmic actin (~21.1% of total actin), and γ-actin (~12.7% of total actin). This result is consistent with a previous study on vascular actin isoform composition from different species (15).

To determine whether the subcellular distribution of actin is isoform dependent in dVSMCs, we stained freshly dissociated aorta cells with isoform-specific antibodies for α-smooth muscle actin, β-cyttoplasmic actin, and α-γ-actin (Fig. 5B). The α-smooth muscle actin is present in longitudinally or slightly tangentially oriented filamentous bundles (arrows) in the freshly isolated dVSMCs (top) in a manner similar to that shown previously for myosin-containing contractile filament bundles (50). In contrast, β-cyttoplasmic actin was prominent in the cell cortex and in shorter punctuate spots (arrows) through-
out the cell (middle). Unfortunately, there is no available entirely specific anti-γ-antibody. We found that the commonly used commercially available polyclonal γ-antibody (Chemi-con, Temecula, CA) recognizes, on two-dimensional gels, both α- and γ-actin isoforms (data not shown). When cells are stained with this antibody, the distribution of staining includes some longitudinal filaments (arrows) expected from α-smooth muscle actin cross-reactivity (Fig. 5B, bottom), but a clear discrimination of the γ-actin staining from that due to α-smooth muscle actin could not be determined.

Isoform-specific NH₂-terminal actin peptides depress contractile force. To further test whether actin isoforms modulate the contractile function of smooth muscle, we synthesized NH₂-terminal decoy peptides of actin isoforms (Table 1) coupled to a cell permeation TAT tag sequence and introduced them to intact dVSM. It has previously been reported that the NH₂-terminal α-smooth muscle actin tetrapeptide inhibits the formation of stress fibers and wrinkles on silicone substrate in an isoform-specific manner in cultured cells (6, 22). The NH₂-terminal actin sequences are also known to include sequences involved in the association with many actin-binding proteins, and since considerable evidence in nonmuscle cells indicates that, despite the large degree of sequence conservation, actin displays isoform-specific functions, we synthesized all of the relevant NH₂-terminal peptides as putative decoy peptides. We also made the α-skeletal muscle peptide as a negative control to test the specificity of the approach.

As expected, the α-skeletal muscle actin peptide has no significant effect on the steady-state contractile force induced by either DPBA or PE (Fig. 6). The introduction of the β-cytoplasmic actin peptide also has no significant effect on either DPBA- or PE-induced smooth muscle contraction. However, the contractile force induced by DPBA is significantly inhibited by the introduction of α-smooth muscle, γ-smooth muscle, and γ-cytoplasmic actin peptides (Fig. 6A). Interestingly, the contractile force induced by PE is significantly inhibited by the introduction of γ-smooth muscle, and γ-cytoplasmic actin peptides, but not in α-smooth muscle actin (Fig. 6B).

In contrast, in the same experimental protocol, but with preparations activated by KCl-PSS, none of these peptides has any significant effect on force generation (Fig. 6C), further pointing to selectivity in the action of these peptides. There was also no detectable decrease in intrinsic basal tone with any of the peptides.

Contractile agonists induce cytoskeletal remodeling in an actin isoform-dependent manner in dVSM. The above results indicate that smooth muscle function can be regulated in an actin isoform-dependent manner. To further test the hypothesis that actin remodeling is actin isoform dependent, we used differential centrifugation combined with immunoblots. The F/G ratio of α-smooth muscle and β-cytoplasmic actin was obtained by using either anti-α-smooth muscle actin or anti-β-cytoplasmic actin antibodies to probe the samples. As shown in Fig. 7, the α-smooth muscle actin population shows no significant DPBA- or PE-induced changes in the F/G ratio (Fig. 7A). In contrast, stimulation with DPBA increases the F/G ratio of β-cytoplasmic actin significantly (Fig. 7B). However, the β-specific remodeling is far less pronounced than the preceding results on F/G ratios for total actin (Fig. 1B), which suggests that the γ-isoforms may remodel to a greater degree than other isoforms. It is also of note that the α-smooth muscle isofrom appears to be the least dynamic isoform during contractile responses.

We could not directly apply the same method to the γ-actin isoform, because of the lack of availability of a truly γ-specific actin antibody. Instead, we chose to run the F-actin sample on two-dimensional gels. The less abundant, G-actin samples on two-dimensional gels were not measurable because of lower signal-to-noise ratios of the bands. However, we were able to get reliable results comparing the F-actin samples from the γ-spots of two-dimensional gels. Samples were cut from individual gels and transferred to the same membrane so that they could be directly compared. The data are expressed as the densitometry of the γ-spot as a fraction of the total of the unstimulated actin spots. When analyzed in this way, as shown in Fig. 7C, the γ-F-actin population shows significant increases with PE stimulation. Taken together, these data point to isoform-specific roles in signaling pathways activated by different
stimuli in dVSM, with γ-actin being the most dynamic, especially in the presence of PE.

DISCUSSION

In the present study we show, for the first time, that the actin cytoskeleton of dVSM undergoes remodeling during contrac-
tile activation in an actin isoform-dependent and stimulus-
dependent manner. It is generally accepted that VSM contractility is regulated both by mechanisms that regulate actin-
activated myosin ATPase activity, via changes in the
phosphorylation state of the 20-kDa myosin light chain (55), as
well as by mechanisms that regulate the availability of actin to
interact with myosin, via the action of inhibitory actin-binding
proteins such as caldesmon (43). It has also been proposed, but
is more controversial, that signaling pathways exist by which
the actin cytoskeleton is remodeled during agonist activation
and that this might modulate vascular contractility (7, 49).

Cytoskeletal remodeling has previously been reported to
occur in a few VSM studies (48, 58). However, what has not
been clear is exactly how, or whether, cytoskeletal remodeling
modulates contractility of smooth muscle. Some groups have
suggested that cytoskeletal remodeling may alter the transmis-
sion of cross-bridge generated force to the cell membrane in
airway smooth muscle (19). Other groups have suggested that
it modulates force maintenance or optimizes the energetic cost
of tension in the VSMC (25).

Using a method of differential centrifugation to quantify the
F- and G-actin, we found that the F/G ratio of dVSM is agonist
sensitive. Our results are qualitatively and quantitatively con-
sistent with the F/G ratios, reported by Gunst and colleagues
(65) for airway smooth muscle. Previously, Tang and Tan (58)
reported similar increases in F/G ratios in VSM stimulated by
m-agonist norepinephrine but lower absolute F/G ratios of
dVSM compared with our values. To confirm these results and
also to develop a method that allows better time resolution, we
have also quantitated F/G ratios, for the first time in dVSMCs,
by costaining with fluorescently labeled phalloidin and DNase
I in freshly enzymatically dissociated single dVSMCs. Quali-
tatively similar agonist-dependent results are obtained with both methods; however, it is important to emphasize that the absolute phalloidin-to-DNase I ratios are also affected by staining intensity, photomultiplier nonlinearity, and confocal settings and thus are not quantitatively identical to the F/G ratios obtained by differential centrifugation. Even though the two methods cannot be directly compared in a quantitative manner, the qualitative results in both cases clearly indicate that certain contractile stimuli induce a significant net increase of actin polymerization in dVSMCs.

We have shown that three stimuli, KCl, PE, and DPBA, as well as an actin filament stabilizer, jasplakinolide, increase actin polymerization, as measured by F/G ratios. However, latrunculin, a stabilizer of G-actin, caused no significant change in the unstimulated tissue F/G ratio. These results suggest that an endogenous protein (for example, capping proteins, G-actin binding proteins, etc.) may stabilize G-actin.

**Table 1. Actin isoform-specific peptides**

<table>
<thead>
<tr>
<th>Actin Isoform</th>
<th>Peptide</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-Smooth muscle actin</td>
<td>Ac-EEED-FITC-TAT</td>
</tr>
<tr>
<td>β-Cytoplasmic actin</td>
<td>Ac-DDDDIA-FITC-TAT</td>
</tr>
<tr>
<td>γ-Smooth muscle actin</td>
<td>Ac-EEEETT-FITC-TAT</td>
</tr>
<tr>
<td>γ-Cytoplasmic actin</td>
<td>Ac-EEEIA-FITC-TAT</td>
</tr>
<tr>
<td>α-Skeletal muscle actin</td>
<td>Ac-DEDE-FITC-TAT</td>
</tr>
<tr>
<td>Acetylation</td>
<td>Acetylation</td>
</tr>
</tbody>
</table>

TAT, GYGRKKRRQRRR; Ac, acetylation.

Fig. 5. Actin isoforms in aorta. A: 2-dimensional gel of aorta homogenate stained with Coomassie blue. B: images of illustrative dVSMCs immunostained with isoform-specific actin antibodies. Scale bars, 10 μm. IEF, isoelectric focusing.

**Fig. 6. Decoy actin peptides inhibit contractility in an isoform-dependent manner.** Steady-state force in the presence of DPBA (10 μM; A), PE (3 μM; B), or KCl (51 mM; C) in the presence of peptides or sham treated (Table 1) is shown. Contractile responses were normalized to force to a challenge with KCl physiological salt solution (PSS; 51 mM KCl) at the beginning of each experiment. *P < 0.05, effects of peptides compared with sham (Sham). Average is ± SE (n = 3–6). A-SM, α-smooth muscle actin; beta, β-cytoplasmic actin; G-SM, γ-smooth muscle actin; G-CYT, γ-cytoplasmic actin; A-SK, α-skeletal muscle actin.
so that the addition of latrunculin has no effect. However, latrunculin pretreatment is able to prevent stimulus-induced increases in the F/G ratio, suggesting that a stimulus-induced signal transduction pathway results in the liberation of free G-actin separate from the putative endogenous G-actin binding protein (40).

Interestingly, latrunculin was able to prevent only PE- and DPBA-induced increases in F/G ratios and not those induced by the depolarization of VSM. Although there are some studies that show either PE or DPBA stimulation on smooth muscle also induces membrane depolarization (13, 45), these results strongly suggest that a different mechanism of the regulation of the F/G ratio is being recruited by KCl-induced depolarization. Others have reported that depolarization can activate Rho/ROCK pathways that differ from the PKC-dependent pathways activated by phorbol esters and PE (42, 59), and this may point to distinct mechanisms by which actin remodeling may be regulated in dVSM.

The fact that latrunculin pretreatment causes a significant inhibition of the DPBA-induced contraction in parallel with the inhibition of actin polymerization links the observed cytoskeletal remodeling with an effect on contractility. One might conclude that the effects of latrunculin are due a nonspecific disruption or enhancement of the cytoskeleton, causing nonspecific effects on all types of smooth muscle contraction. However, the fact that in preparations activated by KCl-induced depolarization, neither latrunculin nor jasplakinolide had any effects on force generation, argues against this possibility.

It is worth noting that jasplakinolide had a small effect on the F/G ratio in cells but no significant effect on active force development in the intact tissue preparation. We assume that this can be explained by the fact that multiple redundant pathways regulate contractile force, whereas the F/G ratios directly measure a single pathway, so that we could not detect a change in the former case but could see a significant, but small change in the latter case. However, the possibility also exists that there could be some differences in function between the intact tissue and the dissociated cells (14).

We have previously reported a differential subcellular distribution of α-smooth muscle and β-cytoplasmic actin in portal vein VSMCs (50). North et al. (46) have also reported a differential distribution for β-cytoplasmic actin versus the unidentified (presumably γ-smooth muscle) actin isoform present in actomyosin-containing contractile bundles in chicken gizzard smooth muscle. A distinct population of actin filaments has also been reported by using immunoprecipitation for an anticalponin antibody to demonstrate the existence of an actin filament population lacking caldesmon (36). According to the model proposed by North et al. (46), nonmuscle β-actin in gizzard cells is localized in dense bodies and longitudinal channels linking consecutive dense bodies and membrane-associated dense plaques and lying along intermediate filaments. In contrast, Stromer et al. (57) have argued against any actin isoform-specific domains in gizzard or VSM. In the present study, we found that α-smooth muscle actin is present in longitudinally or slightly tangentially oriented filamentous bundles and β-actin is prominent in the cell cortex and in punctate spots. These results are consistent with previous reports from Parker et al. (50) and qualitatively consistent with those of the North et al. study (46). The lack of availability of

Fig. 7. Isoform-specific changes in F/G ratios in differential ultracentrifugation samples. F- and G- actin probed in immunoblots for α-smooth muscle actin (A) or β-cytoplasmic actin (B) in unstimulated DPBA (10 μM) or PE (10 μM)-stimulated aorta. C: γ-actin in the F fraction after immunoblotting with γ-actin antibody after 2-dimensional separation of parallel homogenates. *P < 0.05, compared with unstimulated. Average is ± SE (n = 3–5).
a truly specific anti-γ-actin antibody prevented us from imagining its subcellular distribution. However, differential centrifugation results indicate that the γ-actin isoform is the most dynamic actin and, since in nonmuscle cell types, cortical actin has been shown to be the site of the most dynamic actin turnover (8), one might suggest that the γ-actin in the dVSM cell fills this role.

In this study, for the first time, we introduced actin isoform-specific decoy peptides coupled to a cell permeation TAT tag sequence into dVSM. We observed a significant inhibition of dVSM contractility in a peptide-specific manner and in a stimulus-dependent manner. Negative results obtained with the skeletal α-actin sequence in these dVSM cells and confirm the specificity of the approach. The lack of a significant effect of skeletal muscle actin in these dVSM cells and confirm the skeletal stimulus-dependent manner. Negative results obtained with sequence into dVSM. We observed a significant inhibition of specific decoy peptides coupled to a cell permeation TAT tag.

We were somewhat puzzled by the effect of the α-smooth muscle peptide to inhibit phorbol ester-induced contractions but not PE-induced contractions. Since phorbol esters are nonphysiological stimuli, this may point to a difference in the mechanism of contraction caused by this stimulus versus the more relevant α-agonist PE. However, these results are also in contrast with the fact that we saw no change in the smooth muscle α-actin F/G ratio in the presence of any stimuli with differential centrifugation. The reason for this difference is not clear at present.

To further test the idea that actin remodeling is isoform dependent, with a major role played by the γ-isoform, we also analyzed results from differential centrifugation. Isoform-specific probing of the F- and G-actin fractions shows no evidence of involvement of α-smooth muscle actin in cytoskeletal remodeling. However, γ-actin does show significant isoform-dependent remodeling during PE contractions. Similarly, Mauss et al. (39) have shown that Ieal smooth muscle, but not VSM, contractility is inhibited by botulinum C2 toxin. This toxin ADP-ribosylates G-actin of the cytoskeleton on smooth muscle contraction. In summary, the results presented here indicate that the actin cytoskeleton of dVSMCs remodels in a stimulus-dependent manner by increasing net actin polymerization during contraction. Furthermore, we identify γ-actin as the most dynamically remodeled actin within the dVSMC cytoskeleton, and thus we predict that gamma actin, despite its lesser abundance, may play a critical role in the regulation of smooth muscle contractility.

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