Smooth muscle adherens junctions associated proteins are stable at the cell periphery during relaxation and activation

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Eddinger, Thomas J., Jessen D. Schiebout, and Darl R. Swartz. Smooth muscle adherens junction-associated proteins are stable at the cell periphery during relaxation and activation. Am J Physiol Cell Physiol 289: C1379–C1387, 2005. First published July 20, 2005; doi:10.1152/ajpcell.00193.2005.—This study was performed to determine the stability of the adherens junction (AJ)-associated proteins at the smooth muscle cell (SMC) plasma membrane during relaxing and activating conditions. Dog stomach, ileum, colon, and trachea tissues were stored in Ca2+-free PSS or regular PSS or were activated in 10 μM carbachol in PSS before rapid freezing. The tissues were subsequently sectioned and immersed using antibodies for vinculin, talin, fibronectin, and caveolin to determine their cellular distribution in these tissues under these conditions. In all four tissues and under all three conditions, the distribution of these four proteins remained localized to the periphery of the cell. In transverse tissue sections, the AJ-associated proteins formed a distinct punctate pattern around the periphery of the SMCs at the plasma membrane. These domains alternated with the caveolae (as identified by the presence of caveolin). In longitudinal tissue sections, the AJ-associated proteins formed continuous tracks or staves, while the caveolae remained punctate in this dimension as well. Caveolin is not present in the tapered ends of the SMCs, where the AJ-associated proteins appear continuous around the periphery. Densitometry of the fluorophore distribution of these proteins showed no shift in their localization from the SMC periphery when the tissues were relaxed or when they were activated before freezing. These results suggest that under physiologically relaxing and activating conditions, AJ-associated proteins remain stably localized at the plasma membrane.

vinculin; talin; fibronectin; caveolin; stomach; ileum; colon; trachea

CELL ADHESIONS GO BY A VARIETY of terms, including focal adhesions, focal complexes, adhesion plaques, dense plaques (in smooth muscle), and adherens junctions (AJs). They include a complex association of cytoskeletal, extracellular, and integral membrane proteins, kinases, phosphatases, GTPase modulators, and other enzymes that allow for cell-matrix, cell-cell, and cytoskeleton-contractile protein associations that bind or transmit force between these constituents (see Refs. 7, 11, 19, 33, 41, 44, 70). Integrins are single transmembrane-spanning receptors that connect the extracellular matrix to the cell cytoskeleton at the AJs (5, 33, 52). On the cytoplasmic face of the AJ, a large group of cytoskeletal proteins, including vinculin, talin, actin, filamin, calponin, tensin, α-actinin, and plecrtin are known to be associated with integrins, and these proteins are critical for the function of the AJ (55, 64). On the extracellular face, fibronectin is known to associate with integrins (46, 52). All of these cytoskeletal proteins are thought to be involved, along with other proteins, in the linking of actin filaments and/or stress fibers to the integral membrane proteins and ultimately to the extracellular matrix or to neighboring cells (20). Interdispersed between the AJs on the plasma membrane are caveolin-rich domains referred to as caveolae. Caveolae are invaginated membrane regions that are rich in glycosphingolipids, cholesterol, and numerous cell signaling molecules that are involved in endocytic and exocytic processes as well as in cell regulation (1). The exact function of all of these proteins is still not completely resolved.

All of these proteins have been studied in smooth muscle in an effort to understand its regulation and contraction. Talin and vinculin are two cytoskeletal proteins associated with the AJs on the cytoplasmic side of the membrane. Talin is a 230-kDa protein (49) that has been reported to associate with actin, vinculin, and β-integrins (9, 12, 51). Talin also has been reported to nucleate actin filament growth at the membrane (34, 35). Vinculin and metavinculin are splice variants of the same protein, and both are present in vertebrate smooth muscle (8, 25). In some smooth muscle tissue, metavinculin is actually present in higher concentrations than vinculin (24), but both of these proteins colocalize at dense plaques in smooth muscle cells (SMCs) (4). There is no known unique function for metavinculin, but a deficiency in the human heart can result in a cardiomyopathy (39).

Fibronectin is a ubiquitous extracellular glycoprotein that can bind to itself, collagen, integrins, and numerous other extracellular proteins (46). Fibronectin is involved in external linkages for cell-matrix interactions and may be involved in the regulation of the cell via activation of the AJs (41, 47, 52).

Caveolae are distinct plasmalemmal microdomains that are identified by their unique morphology (clusters of flask-shaped invaginations of the plasma membrane), high concentrations of receptors and channels, and the presence of the protein caveolin (1, 45). Caveolin is an integral membrane protein that acts as a scaffolding protein to localize and regulate a wide range of kinases. Caveolae are interdispersed with AJs in an alternating pattern along the plasma membrane in which interactions between the receptors and channels in the caveolae and the neighboring AJs may be important in cell regulation and function (2, 36, 42, 55, 58).

The role and function of these AJ-associated proteins are not well defined. Smooth muscle AJs (dense plaques) are known to be present at the plasma membrane, where they alternate with caveolin (16, 42, 50, 60) and have been observed in a variety of tissues (16, 17). While vinculin is a major constituent of
AJs, it is not present in cytoplasmic dense bodies (21) but has been reported to be in equilibrium between cytosolic and cytoskeletal pools in chick embryo fibroblasts (38). Using tissue homogenization and fractionation, Kim et al. (37) reported that cholinergic stimulation of bovine trachea smooth muscle resulted in simultaneous increases in force and the recruitment of α-actinin, talin, and metavinculin (but not vinculin) from the cytosolic fraction to the cytoskeletal fraction. Opazo Saez et al. (43), using indirect immunofluorescence, also reported that cholinergic activation of canine tracheal SMCs resulted in the translocation of vinculin, talin, paxillin, and focal adhesion kinase (FAK) from the cytoplasm to the membrane. These results suggest that vinculin, talin, and other cytoskeletal proteins demonstrate dynamic SMC distribution upon activation and relaxation of the tissue. However, both talin and vinculin are reported to dissociate readily from focal adhesions in permeabilized cells and show high solubility in the presence of nonionic detergents (6). Thus it is not clear whether these AJ-associated proteins are stably localized at the cell periphery under physiological conditions.

In smooth muscle, mechanical plasticity, a deviation of muscle force or shortening behavior from that mandated by static isometric force-length curves (57) has been reported by researchers at a number of laboratories. Pratusevich et al. (48) reported force production that is length independent in canine airway SMCs, and subsequently Seow et al. (53) suggested a series-to-parallel transition in the filament lattice to explain this observation. Others have proposed a repositioning of actin filament anchorage to dense plaques and dynamic actin filament remodeling to explain these results (22, 28, 29, 40). Hai et al. (10) also reported smooth muscle plasticity in tracheal smooth muscle that appeared to be related to a “memory” of previous strain and length. In contrast, Wingard et al. (68) found excellent evidence for the conventional length-tension curve in porcine carotid tissue. Thus numerous possible mechanisms could result in mechanical plasticity in smooth muscle.

The purpose of the present study was to determine the dynamic range of distribution of vinculin, talin, fibronectin, and caveolin in intact smooth muscle tissue under reduced-Ca²⁺ PSS, normal PSS, and carbachol-activating PSS conditions. The results show that in several different smooth muscle tissues, the cellular distribution of these proteins does not change when the tissue is incubated in Ca²⁺-free PSS, in normal PSS, or in normal PSS activated with carbachol before freezing and immunostaining. Thus immunohistochemical results from intact tissue suggest that these proteins do not translocate to and from the cytoplasm and plasma membrane in normal physiological function.

METHODS

Tissue preparation. Experimental procedures were approved by the Institutional Animal Care and Use Committees of the Medical College of Wisconsin and the Zablocki Veterans Affairs Medical Center. Tissues were harvested from dogs euthanized at the Milwaukee Veterans Affairs Medical Center after acute vascular studies had been performed on their rear legs. Immediately after the experiments, the animals were euthanized by administration of an overdose of anesthetic and KCl, and the trachea, stomach, ileum, and colon were removed from the animals and placed into cold PSS (in mM: 140.1 NaCl, 4.7 KCl, 1.2 Na2HPO4, 2.0 MOPS, pH 7.4, 0.02 Na2EDTA, 1.2 MgSO4, 1.6 CaCl2, and 5.6 glucose). The tissues were cleaned of blood, loose connective tissue, and in some cases the mucosa and stored in PSS or in Ca²⁺-free PSS (no added CaCl₂; 2.4 mM MgSO₄ and 0.5 mM EGTA) in the refrigerator overnight. The next day (after 12- to 16-h equilibration) some of the stored tissues were rapidly frozen in liquid nitrogen-cooled isopentane. Some tissue from the PSS incubation was placed into fresh PSS and warmed to 37°C for 1–2 h. This tissue was then activated with 10 µM carbachol or KPSS (PSS with 109 mM KCl and 70 mM NaCl in place of 140 mM NaCl) for 30–90 min and then rapidly frozen as described above. Tissues became shortened and/or reduced in diameter when placed in the activating solutions. All tissue was stored frozen until sectioned and immunoreacted. In addition, to rule out the possible nonphysiological consequences of cold temperature storage of the tissue on cytoskeletal proteins, some tissues were also warmed in PSS at 37°C for 1–2 h and then either frozen or relaxed in Ca²⁺-free PSS (at 37°C) and then frozen. Sections (5 µm thick) of the frozen tissues were cut on a Leica CM1900 cryostat, picked up on glass slides, and stored frozen for 1–3 days until immunoreactions were performed.

Reagents. The antibodies used were obtained from the following sources: talin (SD4), vinculin (VIN-11-5), and fibronectin (IST-3) were purchased from Sigma Chemical (St. Louis, MO); caveolin-1 was purchased from BD Biosciences/Pharmingen (San Diego, CA); Cy2 and Cy3 donkey anti-mouse or anti-rabbit secondary antibodies were purchased from Jackson ImmunoResearch (West Grove, PA); and phalloidin and DAPI were obtained from Molecular Probes (Eugene, OR). Antibody reactions were performed using standard procedures at room temperature. Frozen tissue sections (5 µm) picked up on glass slides were fixed with 2% paraformaldehyde for 10 min, permeabilized in 0.5% Triton X-100 for 10 min, and blocked with 5 mg/ml BSA for 1 h before being reacted with the primary antibody for 1 h and then the appropriate secondary antibody for 1 h. After reaction with the secondary antibody, the tissues were incubated in DAPI (0.5 µM), phalloidin (10–50 nM), or DAPI-phalloidin as appropriate for staining nuclei and/or filamentous actin. Multiple washes were performed after the primary and secondary incubations. Coverslips were mounted over the tissue sections using buffered 75% glycerol with 0.2% n-propyl gallate to minimize fading. All immunoreacting solutions were made in PBS-Tween 20 (in g/l: 8.0 NaCl, 0.2 KH₂PO₄, 1.15 Na₂HPO₄, 0.2 KCl, and 0.1% Tween 20, pH 7.4) with 0.1% BSA.

Microscopy. Sections were observed using an Olympus IX70 inverted microscope with epifluorescence illumination. Digital images were obtained with a 16-bit Princeton Instruments (Princeton, NJ) charge-coupled device camera controlled through a PCI card board via IPLab for Windows (version 3.6; Scanalytics, Fairfax, VA) on a personal computer. Images were obtained using either a ×20 magnification/1.25 numerical aperture (NA) or ×100 magnification/1.3 NA oil-immersion objective and stored on the personal computer. Montages were assembled in Adobe Photoshop (version 6.0; Adobe Systems, San Jose, CA). Sections were viewed using a Zeiss confocal microscope (Axiovert 200 with LSM 5 Pascal software). No quantitative differences were observed in immunofluorescence distributions using these two different systems. All figures were obtained using the Olympus microscope. Histograms of fluorescence intensity (below camera saturation) of individual cells were obtained across the transverse tissue section using the IPLab software. Data from pairs of different cells (carbachol activated and relaxed in reduced Ca²⁺) were normalized to peak intensity and cell width using SigmaPlot software (version 8.0; Jandel Scientific, Corte Madera, CA) and replotted to allow for comparisons between cells. At least 4 animals were used for each tissue and antibody reported, with as many as 18 animals used to study the ileum and colon with some antibodies.

RESULTS

The cellular distribution of three proteins that are known to associate with integrins at the AJs was determined in four
different smooth muscle tissues. Rapidly frozen sections of the colon, ileum, stomach antrum, and trachea from dog were double immunoreacted with caveolin and vinculin (Fig. 1A), or with caveolin and talin (Fig. 1B), to determine the distribution of these intracellular cytoskeletal proteins (i.e., vinculin and talin). In addition, immunoreactions for caveolin and fibronectin (an extracellular matrix protein) (Fig. 1C) were also performed. Figure 1, A–C, left column, shows the distribution of these three AJ-associated proteins in these four smooth muscle tissues. The middle column in Fig. 1 shows the caveolin distribution for the same SMCs, and the right column shows merged images. For all four of these proteins, immunolabeling indicated that they were located at the cell periphery in a punctate arrangement. The vinculin, talin, and fibronectin were all located at the AJs and alternated with caveolae (identified by the presence of caveolin) as shown in the right column. Figure 1, A–C, shows that at the tapered ends of the SMCs, there was an increased presence of AJ-associated proteins and a decreased presence of caveolin. This was most extreme at the very ends of the SMCs, where caveolin was not observed and AJ-associated proteins appeared to be continuous around the entire cell periphery (arrows in Fig. 1, A–C). In the trachea, the SMCs were smaller than they were in the gut, and while the alternating punctate pattern of the AJ-related proteins and caveolin was still apparent, it was not as striking as it was in the larger gut SMCs. A more distinct difference between the ileal and colonic SMCs and the tracheal SMCs was that the fibronectin immunolabeling was always punctate and alternated with the caveolin around the periphery of the SMCs in the former two, while it always appeared to be almost continuous around the periphery of the SMCs in the tracheal smooth muscle tissue (Fig. 1C).

Sections of these smooth muscle tissues also were cut along the longitudinal axis of the SMCs so that the distribution of these proteins could be observed along the surface of the plasma membrane. Figure 2 shows high-magnification photomicrographs of several SMCs immunoreacted for caveolin (red) and vinculin (green) and merged into a single image. Similarly to the transverse sections (Fig. 1), vinculin was observed to alternate with the caveolin on the cell surface. The caveolin was in a discrete punctate pattern in the longitudinal sections (Fig. 2) as well as in the transverse sections (Fig. 1). The vinculin immunoreactivity appeared as tracks along the long axis of the cell, similar to barrel staves. These staves appeared to be continuous over much of the cell length. They were also observed to branch from and fuse to each other. When the SMC was sectioned at a slight tangent to the long axis of the cell (Fig. 2A), these vinculin staves were only on the periphery of the cell and did not appear in the center of the cell. Similar results were observed for talin and fibronectin immunolabeling (data not shown). In contrast to the AJ-associated

Fig. 1. Fluorescent photomicrographs of transverse sections of smooth muscle tissue from colon, ileum, stomach antrum, and trachea (rows 1–4, respectively). A–C: left column shows immunoreactions for vinculin, talin, and fibronectin, respectively. Caveolin immunoreactions are shown in the middle column, and merged images of both fluorophores are shown in the right column. In all tissues, these three adherens junction (AJ)-associated proteins demonstrated an alternating punctate pattern with caveolin that is localized at the cell periphery. Arrows in right columns in A–C indicate the small-diameter tapered ends of smooth muscle cells (SMCs) in which only the AJ-associated proteins were present.
proteins, while observed primarily at the plasma membrane, caveolin occasionally showed some immunoreactivity throughout the cell (arrows in Fig. 2A) that most often seemed to be near the nucleus and may have been a result of caveolin in the nuclear membrane or in the perinuclear endoplasmic reticulum and the Golgi. Figure 3 shows transverse (Fig. 3A) and longitudinal (Fig. 3B) sections of dog ileum triple labeled for vinculin (green), caveolin (red), and DAPI (blue). When nuclei are present in the section, they appear as blue spheres near the center of the cell. Caveolin appears punctate along the length of the cell, similar to its pattern of localization in the transverse sections. A shows cells that were sectioned slightly tangential to their long axes and demonstrates that vinculin was restricted to the cell periphery, while caveolin showed limited immunoreactivity in the center of the cell (arrows).

The ability of the cell to transmit force to adjacent cells and maintain tissue integrity is dependent on the presence of functional AJs. To determine the stability of the AJs in intact tissues during different physiological states, smooth muscle tissues were placed into either Ca\(^{2+}\)/H\(_{1001}\)-free PSS or normal PSS at 4°C overnight. They were then rapidly frozen the next day. A subset of tissues was warmed in 37°C PSS for 1–2 h and then either frozen or relaxed in Ca\(^{2+}\)-free PSS and then activated with 10 \mu M carbachol or KPSS for 30–90 min before the tissues were rapidly frozen for immunoprocessing. Figure 4, A–C, shows transverse sections of the longitudinal layer of the colon immunoreacted with vinculin, talin, or fibronectin and double labeled with caveolin. The left column in Fig. 4 shows the AJ-associated protein, the middle column shows caveolin, and the right column shows a merged picture of the two channels. The AJ-associated proteins demonstrated a punctate pattern that was localized to the plasma membrane of the individual SMCs and alternated with caveolin localization, similarly to Fig. 1 (right column, merged channels). These specific patterns were not affected by Ca\(^{2+}\) depletion or activation with 10 \mu M carbachol (Fig. 4, A–C; compare top, middle, and bottom rows). Qualitatively similar results were also obtained for the ileum, stomach antrum, and trachea (data not shown). Some cells (arrowheads in Fig. 4, A–C) demonstrated caveolin immunoreactivity within the SMC core, but this was not observed for the AJ-associated proteins. Caveolin appeared to be absent at the tapered ends of the SMCs, where these cytoskeletal proteins became continuous around the cell periphery rather than demonstrating the punctate pattern observed near the middle of the cell (arrows in Fig. 4, A–C).

In additional studies, tissues were warmed to 37°C for 1–2 h and then either frozen or relaxed in Ca\(^{2+}\)-free PSS and then...
Fig. 4. Fluorescent photomicrographs of transverse sections of longitudinal smooth muscle tissue from colon. A–C: left columns were immunoreacted for vinculin, talin, and fibronectin, respectively. Caveolin immunoreactions are shown in the middle columns, and merged images of both fluorophores are shown in the right columns. The top row shows tissue that was relaxed in EGTA-buffered, Ca\(^{2+}\)-free PSS at 4°C overnight. The middle row shows tissue that was incubated in normal PSS at 4°C overnight. The bottom row shows tissue that was activated in 10 μM carbachol for approximately 1 h at 37°C after 2-h incubation in PSS at 37°C. No differences were observed in the distribution of these proteins using these three protocols. Arrowheads (right columns) show instances in which caveolin appears to be present in the center of a cell, and arrows indicate the small-diameter tapered ends of SMCs in which only the AJ-associated proteins were present.
frozen. Immunohistochemistry performed with these tissues showed a peripheral punctate pattern of the AJ-associated proteins and caveolin that was the same as that of the tissues stored at 4°C in PSS or in Ca²⁺-free PSS overnight (data not shown).

To quantify the distribution of these proteins under these different physiological conditions, we studied fluorophore intensity histograms. Figure 5, A and B, shows high-magnification transverse photomicrographs of tissue sections from colon longitudinal smooth muscle after 10 μM carbachol activation (Fig. 5, Aa and Ab) or Ca²⁺-free PSS (Fig. 5, Ab and Bb). As in the other figures, the cytoskeletal proteins (vinculin, Fig. 5Aa, and talin, Fig. 5Bb; both green) were always localized near the periphery of the cell and alternated with caveolin (red in Fig. 5, A and B). In each image, a line is drawn across a SMC and the histogram of the intensity of the fluorophore of the respective cytoplasmic protein from a cell in the activated tissue and one in the relaxed state is plotted (Fig. 5, Ac and Bc). As shown for vinculin and talin (Fig. 5, A and B, respectively, histograms), the relative distribution did not change between the Ca²⁺-reduced and carbachol-activated conditions. The decay function of the decrease in fluorescence intensity with increasing distance away from the cell periphery to the center of the cell was not different between the relaxed and activated cells, and neither was the peak-to-valley ratio.

DISCUSSION

Recent reports of cytoskeletal proteins translocating to the plasma membrane upon activation of smooth muscle tissue or SMCs raise the question of the physiological significance of such gross movement of cytoskeletal proteins. The present study was conducted in an effort to determine the localization of these AJ-associated proteins in intact tissues and the extent to which translocation may occur with tissue relaxation and activation. The stability of the cytoskeletal attachments seems economically desirable, while regulation of a range of motion in smooth muscle tissues may necessitate active turnover of the...
AJs. In the vascular system, where tissue is under constant load and SMC shape changes are minimal, economy may be most relevant. However, in the digestive tract, where loads are highly variable and tissue and SMC shape changes can be extreme, the ability to accommodate large changes in cell length (i.e., changing tissue diameter) and range of motion may be of greater concern. We studied three regions of the gut in which tissue shape changes can be extreme, as well as the trachea, in which the translocation of cytoskeletal proteins has been reported to occur upon tissue or cellular activation (37, 43), to determine the possible physiological relevance of the translocation of cytoskeletal proteins.

The AJs are known to be a complex arrangement of transmembrane, intracellular, and extracellular proteins. Although >50 proteins have been reported to be associated with these structures (70), the exact roles of all of these proteins are not completely understood. In the literature, there are reports of numerous cytoskeletal proteins being localized to the AJs in smooth muscle. α-Actinin (54), fibronectin (42), filamin (14, 56), integrins (42), metavinculin (25), paxillin (61), plectin (60, 66, 67), talin (13, 15, 63), and vinculin (21, 42, 54) are some of the commonly cited examples. These proteins are observed to be located at the cell periphery in a punctate pattern in transverse sections of smooth muscle in alternation with the caveolae (Refs. 18, 42, 54, 60; present study). In longitudinal sections of smooth muscle, the pattern of these proteins appears as stacks (60), ribs (42, 54), or staves (present study).

The morphology, stability, and regulation of the proteins associated with the AJs, as well as the entire cell, have been reported to undergo dynamic changes (see Ref. 70). These observations are generally reported in cultured cells, but remodeling of and/or translocation to and/or from the AJ also has been reported in tissues. For example, Taggart et al. (59) reported movement of receptor-coupled excitation molecules PKC, RhoA, and Rho kinase to the cell membrane in isolated SMCs with agonist stimulation, and Urban et al. (62) reported that RhoA kinase translocates to the membrane with K⁺ depolarization of arterial smooth muscle.

There also have been reports of cytoskeletal proteins translocating within cells as a result of stimulation. Beckerle et al. (3) reported talin redistribution from the cytoplasm to the adhesion plaque in platelets after activation. Opazo Saez et al. (43) reported the translocation of cytoskeletal proteins (vinculin, talin, and paxillin) as well as FAK to the cell membrane with agonist activation of isolated SMCs. Kim et al. (37) also reported translocation of α-actinin, talin, and metavinulcin (but not vinculin) in smooth muscle tissues with cholinergic stimulation.

Freshly frozen tissues from the gut and trachea showed that three cytoskeletal proteins (vinculin, talin, and fibronectin) were localized to the cell periphery at the AJ. They were located in a punctate pattern around the cell periphery in an alternating pattern with caveolin (Fig. 1) as also reported by others (42, 60). This pattern appeared to be stable and independent of tissue activation. A reduction of intracellular Ca²⁺ by tissue incubation in Ca²⁺-free PSS (no added Ca²⁺ and 0.5 mM EGTA) at 4°C overnight or by carbachol activation (30–90 min) after 1–2 h at 37°C before freezing had no effect on the intracellular distribution of these proteins (Fig. 4). Fluorophore intensity histograms of vinculin and talin (Fig. 5, A and B) in relaxed and activated smooth muscle also showed no differences in intracellular distribution. In both the Ca²⁺-free and activated conditions, these proteins were localized at the cell membrane and showed an identical decay function in fluorescence intensity from the cell periphery toward the center of the cell (Fig. 5, Ac and Bc).

To our knowledge, our present study represents the first time that these protein distributions have been analyzed in tissues after relaxation (Ca²⁺ reduction) and activation using immunohistochemistry. These results suggest that vinculin, talin, and fibronectin are stably associated at the AJs in gut and trachea SMCs in relaxed and activated conditions. Thus, in these intact tissues, the association of these proteins at the AJs appears to be physiologically important. While no other authors of whom we are aware have examined the localization of these proteins directly in tissues with relaxation and activation, numerous reports of the localization of these proteins in smooth muscle have been published, and these proteins are always found at the plasma membrane (see references cited at start of discussion).

Immunolocalization of these proteins to the plasma membrane with specific antibodies leaves little room for alternative explanations. There is a possibility that the cold temperature in which the smooth muscle tissue was stored adversely affected the cytoskeletal system (including microtubules) and thus might have affected our results reported herein. However, other studies that we have performed did not show changes in microtubule pattern or staining intensity when freshly isolated SMCs were cold treated, and microtubule depolymerization favors Rho kinase activation, which would favor modest tissue activation, suggesting that freezing isolated SMCs did not affect our results (Swartz DR and Zhang D, unpublished results). In addition, the PSS sample served as a control for the influence of rewarming on microtubule status (both regular PSS and Ca²⁺-free PSS samples were treated in the same manner). Finally, in experiments in which tissues were warmed in PSS at 37°C for 1–2 h and then either relaxed in Ca²⁺-free PSS or activated with carbachol (all at 37°C) and then frozen, the AJ-associated proteins remained at the periphery, regardless of treatment.

Using high-speed centrifugation, Kim et al. (37) reported that cholinergic activation of bovine trachea strips resulted in a shift of α-actin, talin, and metavinculin (but not vinculin) from the cytoplasmic to the cytoskeletal membrane fraction. Peak force in this tissue was observed with 1 μM carbachol, and 80% of peak force was obtained with 0.1 μM carbachol. However, while the shifts from the cytoplasmic to the cytoskeletal membrane fractions were significant, they required activation with 1–10 μM carbachol, a concentration 1–2 orders of magnitude higher than that required for 80% peak force. Thus the tissue can generate >80% of peak force without significant shifts in the cellular distribution of these proteins. This leaves open the question regarding the physiological significance of movement from the soluble (cytoplasmic) to the insoluble (cytoskeletal) fraction with cholinergic activation.

Using immunohistochemistry, Opazo Saez et al. (43) reported that vinculin, paxillin, talin, and FAK were all evenly distributed throughout the cytoplasmic to the cytoskeletal membrane fraction. Peak force in this tissue was observed with 1 μM carbachol, and 80% of peak force was obtained with 0.1 μM carbachol. While the shifts from the cytoplasmic to the cytoskeletal membrane fractions were significant, they required activation with 1–10 μM carbachol, a concentration 1–2 orders of magnitude higher than that required for 80% peak force. Thus the tissue can generate >80% of peak force without significant shifts in the cellular distribution of these proteins. This leaves open the question regarding the physiological significance of movement from the soluble (cytoplasmic) to the insoluble (cytoskeletal) fraction with cholinergic activation.
translocate to the membrane upon cholinergic activation are inconsistent with the results reported by Opazo Saez et al. The difference cannot be explained by tissue differences, because dog tracheal smooth muscle also was used in this study. The difference between activation with ACh by Opazo Saez et al. and the use of carbachol in the present study does not seem like a reasonable explanation. It is possible that the enzymatic and mechanical disruption of the tissue for SMC isolation used by Opazo Saez et al. (43) may have altered the AJs, leading to dissociation of these proteins. Subsequent activation of the cells could then have led to reformation and organization of the AJs.

An increasing body of literature suggests that the contractile filaments in smooth muscle are not static but assemble and disassemble with activation, relaxation, and/or changes in mechanical load. For example, if the thick and/or thin filaments within SMCs were not as stable as striated thick and thin filaments, they could assemble and disassemble more readily. This would allow the filamentous system to reorganize on the basis of overall cell length and activation history. This might explain in part the ability of smooth muscle tissue to generate high tension over a significantly wider range of muscle lengths than striated muscle. There are reports of increased birefringence in rat anococcygeus muscle with activation that was shown to correlate with a near-doubling of the myosin filament density of this tissue, of which only a portion can be explained by shrinkage or other changes (23, 26). Researchers from the same group subsequently reported that the increase in filaments was ~23% in the anococcygeus but was not observed in the taenia coli, suggesting tissue differences (69). This finding is similar to the results described by Watanabe et al. (65), who reported an increase in myosin filaments in rat anococcygeus but not in guinea pig taenia coli.

The well-established length-tension relationship of skeletal muscle (27, 31, 32) may not be directly applicable to smooth muscle. While many groups (see, e.g., Ref. 68) have found evidence for the conventional length-tension curves in smooth muscle, other investigators (30) have reported a lack of a unique length-tension relationship in single SMCs. As mentioned in the introduction, this could be a result of changes in the arrangement of the contractile filaments (53), a repositioning of actin filaments relative to dense plaques (22, 28, 29, 40), or a “memory” of previous strain and length (10). Translocation of AJ-associated proteins (43) might be another mechanism that allows for mechanical plasticity in smooth muscle. The results of the present study suggest that the latter option is inconsistent with a physiologically relevant explanation for mechanical plasticity in intact smooth muscle. This study does not rule out the possibility of dynamic changes in the association of filamentous actin with the AJs, however.

In conclusion, the role of the AJ-associated proteins vinculin, talin, and fibronectin appears to be of such import that these proteins remain intact in smooth muscle tissues under relaxed and activated conditions. Changes to the cytoskeleton that would allow for the extreme range of shortening possible in smooth muscle most likely takes place elsewhere in the system, perhaps at the association of the thin filaments to the AJ-associated proteins, the arrangement of the thick and thin filaments, or changes in thin and thick filament lengths.

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SMOOTH MUSCLE CYTOSKELETAL PROTEIN STABILITY IN CONTRACTION


