Smooth Muscle Adherens Junctions associated proteins are stable at the cell periphery during relaxation and activation.

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Running Title: Smooth muscle cytoskeletal protein stability in contraction

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Abstract:

This study was performed to determine the stability of the adherens junctions 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 a Ca^{++}-free physiological salt solution (PSS), regular PSS, or activated in 10 μM carbachol in PSS prior to rapid freezing. The tissues were subsequently sectioned and immunoreacted using antibodies for vinculin, talin, fibronectin and caveolin to determine their cellular distribution in these tissues under these conditions. In all four tissues, in all three conditions, the distribution of these four proteins remains localized to the periphery of the cell. In transverse tissue sections the adherens junctions associated proteins form a distinct punctate pattern around the periphery of the SMC’s at the plasma membrane. These domains alternate with the caveoli (as identified by the presence of caveolin). In longitudinal tissue sections the adherens junctions associated proteins form continuous tracks or staves while the caveoli remain punctate in this dimension as well. Caveolin is not present in the tapered ends of the SMC’s, where the adherens junctions associated proteins appear continuous around the periphery. Densitometry of the fluorophore distribution of these proteins shows no shift in their localization from the SMC periphery when the tissues are relaxed, or activated prior to freezing. These results suggest that under physiologically relaxing and activating conditions the adherens junctions associated proteins remain stably localized at the plasma membrane.

Key words:

Vinculin, talin, fibronectin, caveolin, stomach, ileum, colon, trachea
Introduction:

Cell adhesions go by a variety of terms including focal adhesions, focal complexes, adhesion plaques, dense plaques (in smooth muscle) and adherens junctions. 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 cytoskeletal-contractile protein associations that bind or transmit force between these constituents (see 8, 11, 19, 33, 41, 44, 70). Integrins are single transmembrane spanning receptors that connect the extracellular matrix to the cell’s cytoskeleton at the adherens junctions (5, 33, 52). On the cytoplasmic face of the adherens junction a large group of cytoskeletal proteins including vinculin, talin, actin, filamin, calponin, tensin, α-actinin, and plectin are known to be associated with integrins, and critical for the function of the adherens junctions (55, 64). On the extracellular face, fibronectin is known to associate with integrins (46, 52). All these cytoskeletal proteins are believed to be involved, along with other proteins, in the linking of actin filaments/stress fibers to the integral membrane proteins and ultimately to the extracellular matrix or neighboring cells (20). Interdispersed between the adherens junctions (AJ) on the plasma membrane are caveolin rich domains referred to as caveoli. Caveoli 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 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 adherens junctions on the cytoplasmic side of the membrane. Talin is a 230 kDa protein (50) that has been reported to associate with actin, vinculin and β-integrins (9, 12, 51). Talin has also been reported to nucleate actin filament growth at the membrane (34, 35). Vinculin and metavinculin are splice variants of the same protein that are both present in vertebrate smooth muscle (6, 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 (3). There is no known unique function for metavinculin, but a deficiency in the human heart can result in a cardiomyopathy (99).

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 regulation of the cell via activation of the adherens junctions (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 concentration of receptors, 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 adherens junctions in an alternating pattern along the plasma membrane where interactions between the receptors and channels in the caveolae and the neighboring adherens junctions may be important in cell regulation and function (2, 36, 42, 55, 58).

The role and function of these adherens junction associated proteins is not well defined. Smooth muscle adherens junctions (dense plaques) are known to be present at
the plasma membrane where they alternate with caveolin (16, 42, 49, 60) and have been observed in a variety of tissues (16, 17). While vinculin is a major constituent of adherens junctions, it is not present at 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 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 smooth muscle cells results in 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 are dynamic in their smooth muscle cellular distribution upon activation and relaxation of the tissue. However, both talin and vinculin are reported to readily dissociate from focal adhesions in permeabilized cells and show high solubility in the presence of nonionic detergents (7). Thus it is not clear if these adherens junction 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 a number of labs. Pratusevich et al. (48) reported force production that is length independent in canine airway SM and subsequently Seow et al. (53) suggested a series-to-parallel transition in the filament lattice to explain this observation. Others have proposed repositioning of actin filament anchorage to dense plaques and dynamic actin
filament remodeling to explain these results (22, 28, 29, 40). Hai’s group (10) has also reported smooth muscle plasticity in tracheal SM, which appeared to be related to a “memory” of previous strain and length. In contrast, Murphy’s group (66) found excellent evidence for the conventional length-tension curve in porcine carotid tissue. Thus, there are numerous possible mechanisms that could result in mechanical plasticity in smooth muscle.

The purpose of this study was to determine the dynamic range of distribution of vinculin, talin, fibronectin and caveolin in intact smooth muscle tissue under reduced Ca\(^{++}\) physiological salt solution (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 a Ca\(^{++}\)-free PSS, normal PSS, or normal PSS and activated with carbachol prior to 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 Department of Veterans Affairs Medical Center. Tissues were harvested from euthanized dogs used at the Milwaukee VA Medical Center following acute vascular studies (on the rear leg). Immediately following the experiment, the animal was euthanized by an overdose of anesthetic and KCl and the trachea, stomach, ileum and colon were removed from the animal and put in cold physiological salt solution (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 a Ca⁺⁺-free PSS (no added Ca⁺⁺, 2.4 mM MgSO₄ and 0.5mM EGTA) in the refrigerator overnight. The following day (after 12-16 hours equilibration) some of the stored tissues were rapidly frozen in liquid nitrogen cooled isopentane. Some tissue from the PSS incubation was put in fresh PSS and warmed to 37°C for 1-2 hours. This tissue was then activated with 10 μM Carbachol or KPSS (PSS with 109 mM KCl and 70mM NaCl in place of the 140 mM NaCl) for 30-90 minutes and rapidly frozen as described above. Tissues were observed to shorten and/or reduce in diameter when placed in the activating solutions. All tissue was stored frozen until sectioned and immunoreacted. In addition, to rule out the possible non-physiological consequences of cold temperature storage of the tissue on cytoskeletal proteins, some tissues were also warmed in PSS at 37°C for 1-2 hours and then either frozen or relaxed in Ca⁺⁺-free PSS (at 37°C) and then frozen. Five μm thick sections of the frozen tissues
were cut on a Leica CM1900 cryostat, picked up on glass slides and stored frozen (1-3 days) until immunoreacted.

Reagents. The antibodies used were obtained from the following sources: Talin (8D4), Vinculin (VIN-11-5), and fibronectin (IST-3) from Sigma Chemical Co, St Louis Mo; Caveolin-1-BD from Biosciences/Pharmagen San Diego, CA; Cy2 and Cy3 Donkey anti mouse or rabbit secondaries- Jackson ImmunoResearch, West Grove, PA; Phalloidin and Dapi- Molecular Probes, Eugene, OR. Antibody reactions were done using standard procedures at room temperature. Frozen tissue sections (5 μm) picked up on glass slides were fixed with 2% paraformaldehyde for 10 minutes, permeabilized in 0.5% Triton X-100 for 10 minutes and blocked with 5mg/ml BSA for 1 hour prior to reacting with the primary antibody for one hour and then the appropriate secondary antibody for one hour. After the secondary antibody, the tissues were incubated in a DAPI (0.5 μM), Phalloidin (10-50 nM) or DAPI/Phalloidin as appropriate for staining nuclei and/or filamentous actin. Multiple washes were used following the primary and secondary incubations. Cover slips 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 [(in g/liter: NaCl 8.0, KH₂PO₄ 0.2, Na₂HPO₄ 1.15, KCl 0.2,).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 taken with a 16bit Princeton Instruments (Princeton, NJ) CCD camera, controlled through a PCI board via IPLab for
Windows on a PC (Ver. 3.6, Scanalytics; Fairfax, VA). Images were taken using either a 60x (1.25 NA) or 100x (1.3 NA) oil lens and stored on the PC. Montages were assembled in Adobe Photo Shop (6.0, Adobe Systems Inc. San Jose, CA). Sections were also 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 in this text were obtained using the Olympus microscope. Histograms of fluorescence intensity (below camera saturation) for individual cells were taken across the transverse tissue section using the IP lab software. The data from pairs of different cells (carbachol activated and relaxed in reduced Ca\(^{++}\)) were normalized to peak intensity and cell width in Sigma Plot (8.0, Jandel Sci., Corte Madera, CA) and re-plotted to allow comparisons between cells. At least 4 animals were used for each tissue and antibody reported here, with as many as 18 animals being used for ileum and colon for some antibodies.
Results

The cellular distribution of three proteins that are known to associate with integrins at the adherens junctions 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 caveolin and talin (Fig 1B), to determine the distribution of these intracellular cytoskeletal proteins (vinculin and talin). In addition, immunoreactions for caveolin and fibronectin (an extracellular matrix protein, Fig 1C) were also done. The left column in each figure (Fig 1 A-C) shows the distribution of these three adherens junction associated proteins in these four smooth muscle tissues. The middle column shows the caveolin distribution for these same smooth muscle cells and the right column shows a merged picture. For all four of these proteins, the immunolabelling indicates that they are located at the cell periphery in a punctuate arrangement. The vinculin, talin, and fibronectin are all located at the adherens junctions and alternate with the caveolae (as identified by the presence of caveolin) as shown in the right column. In all three figures (Fig 1 A-C), one can observe that at the tapered ends of the smooth muscle cells (SMC) there is an increased presence of the adherens junctions associated proteins and a decreased presence of the caveolin. This is most extreme at the very ends of the SMC’s where caveolin is not observed and the adherens junctions associated proteins appear to be continuous around the entire cell periphery (arrows in Fig 1 A-C). In the trachea, the smooth muscle cells are smaller than they are in the gut, and while the alternating punctate pattern of the adherens junctions related proteins and caveolin is still apparent, it is not as striking as it is in the larger gut smooth muscle cells. A more distinct difference between the ileal and colonic SM and the
tracheal SM is that the fibronectin immunolabeling was always punctate and alternate with the caveolin around the periphery of the SMC’s in the former two while it always appears almost continuous around the periphery of the SMC’s in the tracheal SM tissue (Fig 1C).

Sections of these smooth muscle tissues can also be cut along the longitudinal axis of the SMC’s so that the distribution of these proteins can be observed along the surface of the plasma membrane. Figure 2 shows high magnification micrographs of several SMC’s immunoreacted for caveolin (red) and vinculin (green) and merged for a single picture. Similar to the transverse sections (Fig 1), the vinculin is observed to alternate with the caveolin on the cell surface. The caveolin is in discrete punctae pattern in the longitudinal sections (Fig 2) as well as the transverse sections (Fig 1). The vinculin immunoreactivity appears as tracks along the long axis of the cell, similar to barrel staves. These staves appear to be continuous over much of the cells length. They can also be observed to branch and fuse with each other. When the SMC is sectioned at a slight tangential to the long axis of the cell (Fig 2A) one can see that these vinculin staves are only on the periphery of the cell, and do not appear in the center of the cell. Similar results were observed for talin and fibronectin immunolabelling (data not shown). The caveolin, in contrast to the adherens junctions associated proteins, while primarily observed at the plasma membrane, does occasionally show some immunoreactivity throughout the cell (arrows, Fig 2A) that seems to most often be near the nucleus and may be a result of caveolin in the nuclear membrane or the perinuclear endoplasmic reticulum and Golgi. Fig, 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 can be seen at the periphery of the cells and also at the edge of the nuclei. In addition, in some cells caveolin immunoreactivity is observed in the center of a SMC even when no nuclei are observed in the section (Fig 2A).

The ability of the cell to transmit force to adjacent cells, and maintain tissue integrity is dependent on the presence of functional adherens junctions. To determine the stability of the adherens junctions in intact tissues during different physiological states, smooth muscle tissues were placed in either a Ca\(^{++}\) free PSS or normal PSS solutions at 4°C overnight. They were then rapidly frozen the following day. A subset of tissues was warmed in 37°C PSS for 1-2 hours and then activated with 10 μM carbachol or KPSS for 30-90 minutes prior to rapidly freezing the tissues for immuno-processing. Figures 4A-C show transverse sections of the longitudinal layer of the colon immunoreacted with vinculin, talin, or fibronectin and double labeled with caveolin. In all three figures, the left column shows the adherens junction associated protein, the middle shows caveolin, and the right-shows a merged picture of the two channels. The adherens junctions associated proteins show a punctuate pattern that is localized to the plasma membrane of the individual SMC’s and is alternate with the caveolin localization, similar to that shown in figure 1 (right columns, merged channels). These specific patterns are not affected by Ca\(^{++}\) depletion or activation with 10 μM Carbachol (Fig 4A-C, compare top, middle and bottom rows). Qualitatively similar results were also obtained for the ileum, stomach antrum, and trachea (data not shown). In some cells (arrowheads, Fig 4A-C), there is caveolin immunoreactivity within the SMC core, but this is not observed for the adherens
junction associated proteins. The caveolin appears to be absent at the tapered ends of the smooth muscle cells where these cytoskeletal proteins become continuous around the cell periphery rather than their punctuate pattern observed near the middle of the cell (arrows, Fig 4A-C).

In additional studies tissue were warmed to 37°C 1-2 hours and then either frozen, or relaxed in Ca^{++} free PSS and frozen. Immunohistochemistry done on these tissues showed the same peripheral punctate pattern of the adherens junction associated proteins and caveolin as the tissues stored at 4°C in PSS or Ca^{++} free PSS overnight (data not shown).

In order to quantify the distribution of these proteins under these different physiological conditions, we looked at fluorophore intensity histograms. Figures 5A&B are high magnification transverse micrographs of tissue sections from colon longitudinal smooth muscle following 10 μM carbachol activation (top panel) or Ca^{++} free PSS (middle panel). As in the other figures, the cytoskeletal proteins (vinculin – fig 5A, and talin – fig 5B, both green) are always localized near the periphery of the cell and alternating with the caveolin (red in both fig 5A&B). In each panel, a line has been 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 (panel C in fig 5A&B). As shown for vinculin and talin (Fig 5A&B respectively, histogram), their relative distribution does not change between the Ca^{++} reduced and carbachol activated conditions. The decay function of the decrease in fluorescence intensity as one moves away from the cell periphery to the center of the cell is not different between the relaxed and activated cells (nor is the peak to valley ratio).
Discussion

Recent reports of cytoskeletal proteins translocating to the plasma membrane upon activation of smooth muscle tissue or SMC’s raises the question of the physiological significance of such gross movement of cytoskeletal proteins. This study was done in an effort to determine the localization of these adherens junction associated proteins in intact tissues and the extent to which translocation may occur with tissue relaxation and activation. 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 adherens junctions. In the vascular system where the tissue is under constant load and SMC shape changes are minimal, economy may be most relevant. However, in the digestive track where loads are highly variable and tissue and SMC shape changes can be extreme, the ability to accommodate large changes in cell length (changing tissue diameter), and range of motion may be of greater concern. This paper looked at three regions of the gut where tissue shape changes can be extreme, as well as the trachea where translocation of cytoskeletal proteins have been reported to occur upon tissue/cell activation (37, 43) to determine its possible physiological relevance.

The adherens junctions are known to be a complex arrangement of transmembrane, intracellular and extracellular proteins. While over 50 proteins have been reported to be associated with these structures (70), the exact role for all of these proteins is not completely understood. In the literature there are reports of numerous cytoskeletal proteins being localized to the adherens junctions in smooth muscle. α-actinin (54), fibronectin (42), filamin (14, 56), integrins (42), metavinculin (25), paxillin (61), plectin (90x709)
talin (13, 15, 63), and vinculin (21, 42, 54) are some of the more commonly cited. These proteins are observed to be located at the cell periphery in a punctate pattern in transverse sections of smooth muscle that alternate with the caveoli (18, 42, 54, 60, this paper). In longitudinal sections of smooth muscle the pattern of these proteins appears as “stacks” (60), “ribs” (42, 54), or “staves” (this paper).

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

There are also reports of cytoskeletal proteins translocating within cells as a result of stimulation. Beckerle et al., (4) has reported talin redistribution from the cytoplasm to the adhesion plaque in platelets following activation. Opazo Saez et al., (43) reported the translocation of cytoskeletal proteins (vinculin, talin and paxillin), as well as focal adhesion kinase to the cell membrane with agonist activation of isolated SMC’s. Kim et al., (37) also reported translocation of α-actinin, talin and meta-vinculin (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), are localized to the cell periphery at the
adherens junction. They are located in a punctate pattern around the cells periphery in an
alternating pattern with caveolin (Fig 1) as has been reported by others (42, 60). This
pattern appears stable independent of tissue activation. A reduction of intracellular Ca\(^{++}\)
by tissue incubation in a Ca\(^{++}\) free PSS (no added Ca\(^{++}\) and 0.5mM EGTA) at 4\(^{o}\)C
overnight or carbachol activation (30-90 minutes) following 1-2 hours at 37\(^{o}\)C prior to
freezing had no effect on the cellular distribution of these proteins (Fig 4). Fluorophore
intensity histograms of vinculin and talin (Fig 5A&B) distributions in relaxed and
activated smooth muscle show no differences. In both the Ca\(^{++}\) free and activated
conditions these proteins are localized at the cell membrane and show an identical decay
function in fluorescence intensity going from the cell periphery towards the center of the
cell (Fig 5A&B, bottom panels).
To our knowledge, this is the first time that these protein distributions have been
analyzed in tissues following relaxation (Ca\(^{++}\) reduction) and activation using
immunohistochemistry. These results suggest that the vinculin, talin and fibronectin are
stably associated at the adherens junctions in gut and trachea SMC’s in relaxed and
activated conditions. Thus in these intact tissues, association of these proteins at the
adherens junctions appears to be physiologically important. While no other authors that
we are aware of have directly examined the localization of these proteins in tissues with
relaxation and activation, there are numerous reports of localization of these proteins in
smooth muscle and they are always found at the plasma membrane (references 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 the smooth muscle tissue was stored in adversely affects the
cytoskeletal system (including microtubules), and thus affects the results reported here. However, other studies we have performed do not show changes in microtubule pattern or staining intensity when freshly isolated SMC’s are cold treated, and microtubule depolymerization favors Rho Kinase activation which would favor modest tissue activation suggesting that this is not the case (unpublished results). In addition, the PSS sample serves as a control for the influence of re-warming on microtubule status (both regular PSS and Ca\(^{++}\) free PSS samples were treated the same). Finally, in experiments where tissues were warmed in PSS at 37\(^{\circ}\)C for 1-2 hours and then either relaxed in Ca\(^{++}\)-free PSS or activated with carbachol (all at 37\(^{\circ}\)C) and then frozen, the adherens junctions associated proteins remain at the periphery, independent of treatment.

Using high speed centrifugation, Kim et al., (37) reported that cholinergic activation of bovine trachea strips results in a shift of \(\alpha\)-actinin, talin and metavinculin (but not vinculin) from the cytoplasmic to cytoskeletal/membrane fraction. Peak force in this tissue was observed with 1\(\mu\)M carbachol and 80% of peak force was obtained with 0.1\(\mu\)M carbachol. However, while the shifts from the cytoplasmic to the cytoskeletal/membrane fractions were significant, they required activation with 1-10\(\mu\)M carbachol (1-2 orders higher concentration than that required for 80% peak force). Thus the tissue can generate at or greater than 80% of peak force without significant shifts in the cellular distribution of these proteins. This leaves in question the physiological significance of this movement from the soluble (cytoplasmic) to insoluble (cytoskeletal) fraction with cholinergic activation.
Using immunohistochemistry, Opazo Saez et al., (43) reported that vinculin, paxillin, talin and focal adhesion kinase (FAK) are all evenly distributed throughout the cytoplasm of freshly isolated tracheal SMC’s. When these cells are activated with 10 μM Ach, the distribution changes to one where these proteins are primarily at the cell periphery. They concluded that this shift is physiologically relevant and critical for force generation and/or shortening. The results of this paper showing that two of these proteins (vinculin and talin) do not translocate to the membrane upon cholinergic activation are inconsistent with their results. The difference can not be explained by tissue differences as dog tracheal smooth muscle was also used in this study. The difference between activation with ACh by Opazo Saez et al., (43) and the use of carbachol in this study does not seem like a reasonable explanation. It is possible that the enzymatic and mechanical disruption of the tissue for the SMC isolation used by Opazo Saez et al., (43) may have altered the adherens junctions leading to disassociation of these proteins. Subsequent activation of the cells could then lead to reformation and organization of the adherens junctions.

There is a growing body of literature suggesting 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 based on overall cell length and past activation history. This might in part explain the ability of SM tissue to generate high tensions 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 nearly doubling of the myosin filament density of this tissue, of which only a portion can be explained by shrinkage or other changes (23,26). This same group subsequently reported that the increase in filaments was approximately 23% in the anococcygeus but was not observed in the taenia coli, suggesting tissue differences (69). This is similar to the results of Watanabe et al. (65) reporting 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 (66 for example) found evidence for the conventional length-tension curves in smooth muscle, other studies (30), have reported a lack of a unique length-tension relationship in single SMCs. As cited 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 adherens junction associated proteins (43) would be another mechanism allowing for mechanical plasticity in smooth muscle. The results of this study suggest that this last option is inconsistent with a physiological relevant explanation for mechanical plasticity in intact smooth muscle. This work does not rule out however, the possibility of dynamic changes in the association of filamentous actin with the adherens junctions.

In conclusion, the role of the adherens junctions associated proteins vinculin, talin and fibronectin appears to be of such import that they remain “intact” in smooth muscle tissues in relaxed and activated conditions. Changes to the cytoskeleton that would allow
for the extreme range of shortening possible in smooth muscle would most likely take place elsewhere in the system, perhaps at the association of the thin filaments to the adherens junctions associated proteins, arrangement of the think and thin filaments, or changes in thin and thick filament lengths.
Figure Legends:

Figure 1.

Fluorescent micrographs of transverse sections of smooth muscle tissue from colon, ileum, stomach antrum and trachea (rows 1-4 respectively). In Panels A-C the left columns are immunoreacted for vinculin, talin and fibronectin respectively. Caveolin immunoreactions are shown in the middle column and a merged picture of both fluorophores is given in the right column in all panels. In all tissues, these three adherens junctions associated proteins are observed in an alternating punctuate pattern with caveolin that is localized at the cell periphery. Arrows in right columns of panels A-C point to the small diameter tapered ends of SMC’s where only the adherens junctions associated proteins are present.

Figure 2.

Fluorescent micrographs (panels A and B) of longitudinal sections of smooth muscle tissue from the circular muscle of the colon, double immunoreacted for vinculin (green) and caveolin (red). The vinculin immunoreactivity appears as staves along the periphery of the cell running with the long axes of the cell. They appear continuous over long distances (as opposed to the punctuate pattern observed in transverse sections). The caveolin is also primarily at the periphery of the cell, alternating in position with the vinculin. The caveolin appears punctuate along the length of the cell, similar to its pattern of localization in the transverse sections. In panel A, cells have been sectioned on a slight tangential to their long axes showing that the vinculin is restricted to the cell periphery while the caveolin does show limited immunoreactivity in the center of the cell (arrows).
Figure 3. Triple labeled fluorescent micrographs of transverse (A) and longitudinal (B) sections of smooth muscle tissue from the ileum. In both panels, vinculin is shown in green, caveolin in red and nuclei (DAPI) in blue. In both planes of section (panels A&B) caveolin immunoreactivity can be observed at the periphery of the nucleus (when it is present in the plane of the section) or sometimes in the center of the cell even when the nucleus is not present. Vinculin and caveolin can be seen in their alternating pattern around the periphery of all the cells. In panel B, cells cut at their surface show the staves of vinculin along their periphery while those cut through their center show no immunoreactivity for vinculin.

Figure 4.

Fluorescent micrographs of transverse sections of longitudinal smooth muscle tissue from colon. In Panels A-C the left columns are immunoreacted for vinculin, talin and fibronectin respectively. Caveolin immunoreactions are shown in the middle column and a merged picture of both fluorophores is given in the right column in all panels. The top row is from tissue that was relaxed in an EGTA buffered Ca\(^{++}\)-free PSS solution at 4\(^0\)C overnight. The middle row is from tissue that was incubated in normal PSS solution at 4\(^0\)C overnight and the bottom row is from tissue that was activated in 10 \(\mu\)M carbachol for approximately one hour at 37\(^0\)C following 2 hours incubation in PSS at 37\(^0\)C. No differences were observed in the distribution of these proteins by these three protocols. Arrowheads (right columns, all panels) show instances where caveolin appears present in the center of a cell and arrows point to the small diameter tapered ends of SMC’s where only the adherens junctions associated proteins are present.
Figure 5.

High magnification fluorescent micrographs of transverse sections of longitudinal smooth muscle tissue from colon immunoreacted for vinculin (panel A, green) or talin (panel B, green) and caveolin (red, both panels). The vinculin/talin and caveolin reactivity is observed primarily at the cell periphery in an alternating punctuate pattern. Row A is from tissue that was activated in 10 μM carbachol for approximately one hour at 37°C following 2 hours incubation in PSS at 37°C. Row B is from tissue that was relaxed in an EGTA buffered Ca²⁺-free PSS solution at 4°C overnight. A line was drawn across one cell from each of the activated and relaxed tissues and a fluorophore intensity histogram along each of these lines (for the green channel) is shown in row C. The peak intensity of the line for each cell and the width of each cell were normalized to aid comparison. The intensity distribution of the fluorophore for vinculin/talin (panels A/B respectively) from each cell shows identical distribution across the cell indicating that vinculin/talin is not being recruited to or from the membrane during tissue activation.
Acknowledgements

We thank PS Clifford and JJ Hamann at the Medical College of Wisconsin for allowing us to harvest dog tissues at the end of their experiments.

Grants

This study was supported by the National Heart, Lung and Blood Institute Grant RO1HL-62237.
References


Fig 2

A. Dog Colon 100x Caveolin and Vinculin
B. Dog Colon 100x Caveolin and Vinculin
Fig 3
Fig 4A
Fig 4B
Fig 4C