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Smooth muscle – protein translocation and tissue function

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Abstract

Smooth muscle (SM) tissue is a complex organization of multiple cell types and is regulated by numerous signaling molecules (neurotransmitters, hormones, cytokines, etc.). SM contractile function can be regulated via expression and distribution of the contractile and cytoskeletal proteins, and activation of any of the second messenger pathways that regulate them. Spatial-temporal changes in the contractile, cytoskeletal or regulatory components of SM cells (SMCs) have been proposed to alter SM contractile activity. Ca^{2+} sensitization/desensitization can occur as a result of changes at any of these levels, and specific pathways have been identified at all of these levels. Understanding when and how proteins can translocate within the cytoplasm, or to and from the plasmalemma and the cytoplasm to alter contractile activity is critical. Numerous studies have reported translocation of proteins associated with the adherens junction and G protein-coupled receptor activation pathways in isolated SMC systems. Specific examples of translocation of vinculin to and from the adherens junction and protein kinase C (PKC) and 17 kDa PKC-potentiated inhibitor of myosin light chain phosphatase (CPI-17) to and from the plasmalemma in isolated SMC systems but not in intact SM tissues are discussed. Using both isolated SMC systems and SM tissues in parallel to pursue these studies will advance our understanding of both the role and mechanism of these pathways as well as their possible significance for Ca^{2+} sensitization in intact SM tissues and organ systems.

Smooth muscle, tissue that surrounds hollow organs, is one of the three major types of muscle in the body. Like all muscle, it has contractile proteins that can cause the cells to generate force and/or shorten. It also has a host of regulatory proteins which can function via numerous second messenger pathways to regulate contractile activity. The contractile proteins convey the force they generate to the cytoskeletal proteins at the cell membrane, and throughout the tissue via the extracellular matrix. Smooth muscle cells may or may not be electrically coupled, allowing individual cells or the entire tissue to be activated to generate force and/or shorten. While there are numerous similarities between smooth muscle tissues, the literature reports numerous differences between smooth muscle tissues that may be critical to their specific organ system function. SM tissue includes extensive extracellular matrix as well as multiple cell types in addition to SM cells. Gabella (Gabella, 1973, 1987, 2012) Somlyo's (Somlyo and Somlyo, 1968; Ashton et al., 1975; Somlyo et al., 1983) Bagby (Bagby, 1983), Small (Small, 1977, 1985; Small et al., 1992; Small and Gimona,

1998) and others have published structural studies of smooth muscle showing details of its organization from the subcellular to tissue level.

Functionally there are two major classes of smooth muscle. These include smooth muscle tissue that generates force relatively slowly with tissue activation but can maintain this force over an extended period of time (tonic smooth muscle), and smooth muscle tissue that generates force relatively quickly with tissue activation but does not maintain this force over time (phasic smooth muscle). This difference in function is of physiological interest because of its significance to tissue function, being specifically relevant for example to how pressure is maintained in the vascular system or the bladder vs. transient propulsive forces responsible for peristalsis in the digestive tract. This difference is also of clinical interest because of numerous pathological conditions that result from improper function of smooth muscles in these and other smooth muscle tissues. And finally, this difference is of academic interest because in spite of decades of effort we still do not know why some smooth muscle tissues are tonic while others are phasic, nor do we fully understand what regulates these different types of contractions. In tonic smooth muscle, the “latch state” has been coined to describe force maintenance at a sustained high level after initial force activation, despite decreases in $[Ca^{2+}]$; and myosin light chain 20 (MLC₂₀) phosphorylation to intermediate levels (Dillon et al., 1981; Dillon and Murphy, 1982; Hai and Murphy, 1989a). In contrast, MLC₂₀ phosphorylation levels do correlate with unloaded shortening velocity (Dillon et al., 1981; Dillon and Murphy, 1982). The mechanism underlying the latch state remains unknown. Several regulatory mechanisms have been hypothesized to explain differences between tonic and phasic contractions including: altered kinetics of phosphorylated vs. dephosphorylated myosin cross-bridges (Dillon et al., 1981; Hai and Murphy, 1989b), cytoskeletal remodeling (Pavalko et al., 1995; Mehta and Gunst, 1999; Gerthoffer and Gunst, 2001; Hu et al., 2007; Kim et al., 2008), calponin or caldesmon dependent actin- to-myosin cross-links (Sutherland and Walsh, 1989; Szymanski and Tao, 1997), second messenger pathway regulation of MLCK/MLCP activity (Jiang and Morgan, 1987; Sohn et al., 2001; Somlyo and Somlyo, 2003; Urban et al., 2003; Harnett et al., 2005; Ratz et al., 2005; Rattan et al., 2006; Poole and Furness, 2007), and the kinetic properties of actomyosin ATPase of different myosin isoforms (non muscle (NM) and smooth muscle (SM) myosin II isoforms) (Fuglsang et al., 1993; Kelley et al., 1993; Khromov et al., 1995; Khromov et al., 1998; Morano et al., 2000; Kovacs et al., 2003; Lofgren et al., 2003; Rosenfeld et al., 2003; Wang et al., 2003; Somlyo et al., 2004; Kovacs et al., 2007).

Study of regulation of contractile force in smooth muscle has occurred over the entire range of experimental models including from whole animal to isolated proteins or protein subunits. Many investigators choose to work in more isolated systems to minimize the complexities of *in vivo* smooth muscle function - including neuronal, hormonal, paracrine and autocrine factors. The use of smooth muscle tissue, isolated cells, cultured cells, and purified protein studies have been significant in advancing our understanding of regulation and function in smooth muscle tissues. As more and more work was done using isolated SMC model systems, we also learned more about how plastic smooth muscle cells are. Studies by numerous groups (for reviews see (Chamley-Campbell et al., 1979; Owens, 1995; Owens et al., 2004; Alexander and Owens, 2012; Campbell and Campbell, 2012)) have been

published reporting changes in structure and function of cultured cells leading to and resulting in better understanding of cell plasticity, replication, and contractile function. Our understanding of SMC ligands and receptors, contractile, cytoskeletal, and regulatory pathways were in some cases identified, confirmed, and/or better understood as a result of isolated/cultured SMC or cell component studies. Cultured SMC work will continue to be used experimentally because it allows greater control of experimental variables and confounding factors while providing relatively large numbers of “homogeneous” SMCs for experimentation (Owens, 1995).

On the converse side, an argument can be made that results from cultured SMC studies (any studies of SMCs removed from their three dimensional tissue organization) has greatly interfered with our understanding of SM tissues and their *in vivo* regulation and function. Cultured smooth muscle cells can be so unique relative to their *in vivo* tissue sources that they can have seemingly little or nothing in common. The most often observed changes from *in vivo* to cultured SMCs is a shift from a “contractile” to a “synthetic” phenotype. And while it is not completely clear what this exactly entails, as the names imply, there is a loss of ability of the SMCs to contract (a decrease of contractile myofilaments) with a significant increase in intracellular organelles for protein secretion (Golgi apparatus, ribosomes, rough endoplasmic reticulum, and mitochondria) (Mosse et al., 1985; Kocher and Gabbiani, 1986; Mosse et al., 1986; Kocher et al., 1991; Owens, 1995). By definition (muscle cells being able to generate force and or shorten), these cultured SMCs are no longer muscle cells. The extent to which this dedifferentiation from contractile to synthetic phenotypic switching occurs, the timing and duration, and the extent to which it can be reversed is highly variable – but appears in part to be dependent on the type of SM cells cultured, the density of plating, and if serum is included in the media. The shift in cultured SMCs to a synthetic phenotype includes a significant decrease in multiple contractile proteins (including SM α -actin, SM myosin, desmin, calponin, h-caldesmon, smoothelin, and vinculin) with a concurrent increase in many of the non-muscle isoforms of these proteins (Kocher and Gabbiani, 1986; Mosse et al., 1986; Kocher et al., 1991; Aikawa et al., 1995; Owens, 1995; Campbell and Campbell, 1997; van der Loop et al., 1997). There is a loss of contractility and a change in the intracellular distribution and organization of proteins in cultured SMCs from a variety of SM tissue sources (Chamley-Campbell et al., 1979; Chamley-Campbell and Campbell, 1981; Chamley-Campbell et al., 1981; Sas and Miller, 1988; Gabella, 1989; Thyberg et al., 1990; Halayko and Stephens, 1994; Eddinger et al., 2007; Campbell and Campbell, 2012). Curiously, changes in contractile protein expression in cultured SMCs do not always occur with changes in myofilaments (Campbell et al., 1989). Cultured rabbit aortic SMCs show a phenotypic modulation of contractile and cytoskeletal protein expression, and a reorganization of these proteins in the SMCS (Worth et al., 2001). The Campbells (Campbell and Campbell, 2012) reported that proteoglycan mediated interactions stabilize the contractile SM phenotype while destruction of these components can cause SMC dedifferentiation. And Owens (Owens et al., 2004) stated that they have “identified at least 15 cases of major differences in expression of SM promoter-enhancer genes in cultured SMCs versus *in vivo*.” Thus there appears to be abundant data suggesting differences in molecular, biochemical, physiological and morphological properties between *in vivo* SMCs and freshly isolated or cultured SMCs.

A note should be included here to mention a distinction between freshly isolated SMCs and cultured SMCs. While cultured SMCs can and do include at some point in culturing all the changes described in the preceding paragraph, freshly isolated cells have been described as “contractile” and “differentiated”, implying that they are similar (identical?) to SMCs in intact tissues. This would seem to distinguish freshly isolated SMCs studies from cultured SMC studies as being more relevant and perhaps having direct “translational” application – an important and necessary current component for funding. However, it remains unclear whether the reported “cultured” SMC changes occur during cell dissociation or in early cell culture (Thyberg et al., 1990; Bowers and Dahm, 1993). In addition, and as discussed below, there are numerous changes that occur during the physical separation of isolating SMCs, resulting in differences in protein localization that are present in freshly isolated SMCs prior to culture. Thus while freshly isolated SMCs are indeed still “contractile”, the extent to which this contraction and any of the many other “differentiated” characteristics of SMCs in tissues may have been altered in freshly isolated SMCs is far from understood. Acknowledging the potential implications of this issue will lead to studies that will allow us to appreciate the relevance and limitations of using these different experimental approaches for understanding *in vivo* SMC function.

From the above discussion one may conclude that isolated/cultured SMCs are unique relative to their phenotype *in vivo*. Exactly when and how this change occurs is not clear. However, the most common method for isolating SMCs from SM tissues include some variation of enzymatic digestion of the extracellular matrix with one or more proteases, interspersed with variable mechanical perturbations and centrifugations. Thus, during cell isolation potentially every stimulus SMCs are ever exposed to are activated simultaneously or sequentially (mechanical, neuronal, paracrine, endocrine, and altered extracellular matrix). These include the same factors that may cause SMC de/-differentiation *in vivo* (Owens, 1995; Hungerford and Little, 1999; Owens et al., 2004; Alexander and Owens, 2012). Worth (Worth et al., 2001), in their study of phenotypic modulation of SMCs in culture concluded by saying: “Since the cytoskeleton acts as a spatial regulator of intracellular signaling, reorganization of the cytoskeleton may lead to realignment of signaling molecules, which, in turn, may mediate the changes in function associated with SMC phenotypic modulation.” Thus while we know what we have done to the smooth muscle tissue to get isolated cells for experiments or culture, we do not know which of these factors, or to what extent each of these factors is involved in causing/allowing SMC dedifferentiation. Nor do we know the biological pathways that bring these changes about, or the extent to which, or how, phenotypic dedifferentiation can be reversed. As mentioned by Alexander and Owens (Alexander and Owens, 2012), we need to understand the critical cues affecting SMC de/differentiation and the signaling and molecular pathways driving gene expression for the contractile and synthetic SM phenotypes. Altering the extracellular environment (by tissue digestion and cell isolation) can cause cytoskeletal reorganization in addition to contractile changes in SMCs (Zigmond, 1996) and this may be responsible in part for the changes observed in cultured SMC function (Janmey, 1998).

This leads me to propose that comparison of cytoskeletal, contractile, and regulatory proteins between SMCs in intact SM tissues and isolated/cultured SMCs should be a

valuable means of increasing our understanding of smooth muscle function and the biological pathways involved in de/differentiation. The premise of this article is not to argue that either whole tissue or freshly isolated/cultured SMC experiments are appropriate or inappropriate, but to argue that they should be used in combination to advance our understanding of SM regulation and function and the pathways involved in de/differentiation. Smooth muscle cells are at some level a product of their environment, and when that environment is dramatically altered or removed as in the process of isolating SMCs, there are bound to be consequences. For the remainder of this manuscript, I will discuss two proposed regulatory pathways (changes in cytoskeletal reorganization and a second messenger pathway for Ca^{2+} sensitization) from SM tissues vs. isolated/cultured cells as examples of where there is currently discord between published results in the literature. I propose that these differences suggest a way to advance our understanding of SM regulation and function. The process involved in preparation of freshly isolated or cultured SMCs removes the extracellular matrix through which integrins communicate and mechanically couple SMCs with each other in the tissue. This results in cellular (anatomical and physiological) changes in these isolated SMCs relative to their *in vivo* situation. Measurement of SMC functions in these isolated SMCs that are related to or affected by these changes may or may not be relevant to *in vivo* SMC tissue function. However, studying these various perturbed cellular functions in isolated SMCs may be beneficial in increasing our understanding of the extent to which de/differentiation can occur, the pathways regulating de/differentiation of SMCs, and resulting changes in SMC function as it moves between differentiated and dedifferentiated states.

I will begin with a hypothesis that adherens junction protein association/dissociation is a physiological mechanism of contractile regulation in SMCs (Opazo Saez et al., 2004). SM cells in tissues are imbedded in an extracellular matrix and are connected to each other physically (structural and in some cases electrical). One of the major proteins involved in the structural connections is the transmembrane protein integrin. The composition of the extracellular matrix is communicated to the cell via the integrin heterodimer combinations with specific binding affinities (Hynes, 2002; Brakebusch and Fassler, 2003), which can affect the organization of the adherens junction (Zaidel-Bar et al., 2007), protein composition, and ultimately cell function (for review see (Wolfenson et al., 2013)).

These adherens junctions complexes play a major role in transmitting the intracellular force generated by the contractile proteins to the plasma membrane and ultimately to the extracellular matrix and adjacent cells. They are localized to specific regions in the plasma membrane and have been referred to by multiple names including adherens junctions, dense plaques, adhesion plaques, focal adhesions, focal complexes, cell-matrix adhesions, and adhesomes (and while these names technically may not be completely interchangeable, I will use adherens junctions generically in this document). Adherens junctions, as sites of attachment for thin filaments, are responsible for transmitting force from the intracellular contractile filaments to the membrane, and from the cell to the cell matrix/neighbor cells. They are extremely complex associations of extracellular, intramembranous, and intracellular structural proteins and enzymes (including kinases, phosphatases, GTPase modulators, proteoglycans, lipids and carbohydrates) (Geiger, 1983; Clark and Brugge,

1995; Burridge and Chrzanowska-Wodnicka, 1996; Zamir and Geiger, 2001; Hynes, 2002; Miranti and Brugge, 2002b; Martinez-Lemus et al., 2003; Palazzo et al., 2004; Eddinger et al., 2005; Zaidel-Bar et al., 2007; Martinez-Lemus et al., 2009). Schematics of varying detail, including reported components of adhesion junctions and their complexity have been published (Horwitz, 1997; Zamir and Geiger, 2001; Miranti and Brugge, 2002a; Zaidel-Bar et al., 2007; Zaidel-Bar and Geiger, 2010). But if this were not complicated enough, in addition to acting as mechanotransducers, integrins also function as cell receptors which are capable of conveying signals not only from outside the cell to the inside (outside-in) but also from inside the cell to the outside (inside-out). Integrins are believed to be involved in migration, proliferation, differentiation, extracellular matrix protein expression, activation of growth factors, cell survival and apoptosis (Zamir and Geiger, 2001; Hynes, 2002; Zaidel-Bar and Geiger, 2010).

In wandering cells inactive integrins may be distributed throughout the plasmalemma as individual monomers or heterodimers. With activation, integrins migrate together to form localized aggregates of proteins that form the adherens junctions. In cells that are part of tissues, such as SMCs, some significant proportion of the integrins will be activated and in clusters forming adherens junctions that are always present. These localized punctate adherens junctions have been reported by many researchers as alternating with caveolae (caveolin) within the plasmalemma, as demonstrated with double labeling for proteins associated with these two structures (Gabella, 1979; Small, 1985; North et al., 1993; Tanaka et al., 2001; Eddinger et al., 2005) and (Fig 1). Publications in the literature using either cell fractionation to separate proteins from the soluble (cytosolic) and insoluble (membrane/cytoskeletal) fractions, or immunohistochemistry of isolated cells (Taggart et al., 1999; Kim et al., 2004; Opazo Saez et al., 2004) have reported that there is a shift in some of the integrin associated proteins (vinculin, talin, paxillin, FAK, α -actinin and metavinculin) from the cytosol to the membrane with activation, suggesting that this recruitment may be part of the contractile regulation in SMCs.

The idea of association/dissociation of adherens junction associated proteins to integrins at the plasmalemma as a means of regulating force transmission from the myofilaments to the membrane (and ultimately the adjacent SM cells in the SM tissue) is well developed (for review see) (Zaidel-Bar and Geiger, 2010). However, it remains unclear to what extent data derived from isolated cells (that are normally in tissues) supporting the “switchable integrin adhesome” model are consistent with data from these cells in their *in vivo* environment. We examined this idea and reported 1) that association/dissociation of adherens associated proteins (vinculin, talin, fibronectin) with the adherens junction does not occur with relaxation and activation in intact SM tissues and 2) while there can be a dissociation of these adherens junction associated proteins from the adherens junction in freshly isolated SMCs, activation of these cells does not consistently result in their re-association with the adherens junctions at the plasmalemma.

In a host of species (dog, rabbit, swine) and a range of SM tissues (trachea, ileum, colon, stomach) immunohistochemistry done on cryosections of intact SM tissues shows that proteins associated with integrins at adherens junctions (vinculin, talin, fibronectin) are always present preferentially at the plasma membrane in a punctate pattern that alternates

with caveolin (Eddinger et al., 2005, 2007). This observation is consistent with earlier reports of an alternating punctate distribution of these two plasma membrane domains (Bagby, 1983; North et al., 1993; Small, 1995; Tanaka et al., 2001; Kawabe et al., 2004). This primarily peripheral distribution of these proteins does not change with relaxation or activation of the tissue (Eddinger et al., 2005). However, it is altered to a quasi-uniform distribution throughout many SMCs following SM cell isolation, and this distribution does not return to a primarily peripheral distribution with CCh or PDBu activation (Eddinger et al., 2007) and (Fig 2 –PDBu stimulation shown). This leaves us with the question of why specific adherens junction associated proteins are relatively uniformly distributed throughout the cytoplasm of freshly isolated SMCs while they are primarily localized at the adherens junctions in SMCs of intact tissues?

As presented above, the integral membrane protein integrins are receptors and mechanotransducers. As such, integrins are affected by and communicate changes to and from the extracellular matrix and the cytoplasm, as well as conveying forces generated in the SMCs or applied to the SMCs in the tissue. This is critical at the tissue level where the SMCs may be required to constitutively maintain pressure within, or cause propulsion along, hollow organs. Changes to the extracellular matrix, or the association of a cell to the extracellular matrix (as for wandering cells), can result in integrin signaling which can result in cytoskeletal changes, migration, cell proliferation, etc. And while there can be ongoing temporal changes inside or outside of the SMCs of SM tissues physiologically, there are no published data that I am aware of showing major changes under physiological conditions in the extracellular matrix on a contraction to contraction time scale. In fact most SM tissues are generating some level of contractile force at all times. Functional, fully associated adherens junctions would thus be required for this force to be transmitted to the plasmalemma and neighboring cells. In order to isolate SMCs enzymatic digestion is used, and as would be predicted there is evidence of proteolysis of the extracellular matrix. As we reported (Eddinger et al 2007) and (Fig 3) enzymatic digestion of SM tissues does not alter caveolin (an intracellular protein) which appears relatively unchanged immunohistologically following papain digestion (Fig 3a), while the immunohistochemical reactivity of fibronectin is significantly decreased by this procedure (Fig 3b). At higher magnification (Fig 4), one observes that the normal peripheral punctate distribution of caveolin (Fig 4a) is not eliminated by papain digestion procedures for isolating SMCs, but does become more uniform along the plasmalemma (fig 4B) suggesting some alteration of the proteins at the plasmalemma as a result of this enzymatic treatment. Serial sections of dog SM tissues (Fig 5; left panel set is dog longitudinal colon SM and right panel set is dog stomach body) following papain digestion as used for SMC isolation, immunoreacted for vinculin (5a), fibronectin (5c) or talin (5e) and double labeled with caveolin (Fig 5 right panels b, d, f) show that only fibronectin immunoreactivity (an extracellular protein) is reduced while all the others (intracellular proteins) remain unaltered. It is also observed that the papain proteolysis occurs from the outside of the SM fascicles towards their centers as would be predicted based on diffusion of the papain into the tissue and fascicles. At this point in the SMC cell isolation procedure (after enzymatic digestion but prior to separation of the SMCs, Fig 5) even though there has been a significant alteration of the extracellular matrix (as noted by the decreased fibronectin immunoreactivity) the intracellular proteins vinculin and

talins remain tightly associated with the adherens junctions in a punctate pattern along the plasmalemma. However, following mechanical separation of SMCs the distribution of intracellular adherens junction associated proteins (vinculin and talin) fall into a bi-modal distribution with approximately half of the cells showing this same punctate peripheral distribution and the other half showing a quasi-uniform distribution throughout the SMC cytoplasm (Eddinger et al., 2007) and (Fig 2 – only vinculin shown). The assumption would be that either the enzymatic digestion of fibronectin and other extracellular matrix proteins and/or the altered association of fibronectin with the integrins and mechanical strain from SMC separation alters the binding affinity of integrin for vinculin and talin and they are then free to diffuse throughout the cytoplasm. Treatment of the cells with saline solutions without Ca^{2+} , or various agonists to activate the cells to contract, does not significantly alter this bimodal distribution for vinculin and talin (Fig 2) and (Eddinger et al., 2007).

This significant alteration of adherens junction associated proteins vinculin and talin to a quasi-uniform distribution in freshly isolated SMCs, relative to their seemingly uniform and consistent peripheral and punctate distribution when in tissues, provides a fortuitous opportunity to study the function, regulation and physiological impact of the adherens junction in SMCs. When and how these two proteins are dissociated from the adherens junction during SMC isolation and the functional significance of this change can be compared between various intact tissues and isolated cells. In addition, one can examine what other components of the adherens junction are also dissociated along with vinculin and talin. And perhaps more significantly, studies like Opazo Saez (Opazo Saez et al., 2004) can be done to determine what is required to get these proteins to return to the adherens junction including the pathway(s) involved, and in which order, where, when, how this occurs. And while this may or may not be directly related to regulation of SMC contraction in intact SM tissues, increasing our understanding of the adherens junctions role in mechanical coupling, regulation and communication is important in multiple systems, and is critical in development and many diseases.

The second area to be addressed relates to second messenger pathways and how they may sensitize SM to cause an increased contraction. Evidence for contractile regulation, via a spatial-temporal signaling network (Ratz, 1999; Urban et al., 2003), beginning with activation of receptors and subsequent second messenger pathway events originating at the plasma membrane and ultimately altering actomyosin interactions in the interior of the cell have been hypothesized, tested, and expanded for the past 30 years (for reviews) (Somlyo and Somlyo, 2003; Ratz et al., 2005; Murthy, 2006). Variable regulation of myosin light chain kinase (MLCK) and myosin light chain phosphatase (MLCP) to regulate myosin light chain 20 (MLC_{20}) phosphorylation appears to be involved in Ca^{2+} sensitization of SM contraction, and is one of the multiple proposed hypotheses to explain the different contractile properties of phasic and tonic SM contractions. A specific downstream pathway reported to regulate MLC_{20} phosphorylation is G-protein coupled receptor activation, which is proposed to be directly involved in SM Ca^{2+} sensitization via the protein kinase C – C-kinase activated PP1 inhibitor protein of 17 kDa ($\text{PKC}\alpha$ – CPI-17) pathway. This has been reported to decrease MLCP activity resulting in maintained/elevated MLC_{20} phosphorylation and thus maintain/increase contractile force (Eto et al., 1995; Li et al., 1998; Kitazawa et al., 2000; Kitazawa et al., 2003). Expression, spatial-temporal

localization, and redistribution of PKC α and CPI-17 with tissue activation have been proposed as mechanisms of Ca²⁺ sensitization of SM contraction (Somlyo and Somlyo, 2003; Somlyo et al., 2004; Ratz et al., 2005; Murthy, 2006). Castagna (Castagna et al., 1982) were the first to report phorbol esters directly activate PKC and Kraft (Kraft et al., 1982) were the first to report a translocation of PKC from the soluble cytosolic fraction to the insoluble membrane fraction with phorbol ester stimulation in EL4 mouse thymoma cells. Measurement of PKC activity from isolated insoluble (membrane/cytoskeletal) and soluble (cytosolic) SM tissue fractions with and without previous agonist stimulation showed an increase in PKC activity in the insoluble (membrane/cytoskeletal) fraction and led the authors to conclude that the PKC translocated from the cytosol to the membrane (Haller et al., 1990; Secrest et al., 1991). It is unclear how the authors ruled out activation of PKC already at the membrane as an alternate reason for this increase in PKC activity at the membrane, but this is not discussed. Numerous additional labs (including my lab) have used direct observation of PKC cellular location using immunofluorescence microscopy to show a shift in location of PKC from the cytosol to the membrane following isolated SMC agonist stimulation (Ibitayo et al., 1999; Taggart et al., 1999; Bitar et al., 2002; Li et al., 2002; Eddinger et al., 2007; Nelson et al., 2008) (Fig 6). There is also a single report in the literature of PKC translocation in the opposite direction. In unstimulated isolated SMCs from the toad stomach PKC is reported to be localized primarily at the plasma membrane and with cholinergic stimulation PKC redistributes to the cytosol and ultimately associates with the contractile filaments (Meininger et al., 1999).

Based on this large body of data showing spatial-temporal redistribution of PKC from the cytosol to the plasma membrane occurs with activation in isolated SMCs, investigators infer that this also occurs and is a (the) mechanism whereby PKC-CPI-17 regulates MLCP activity in intact SM tissues (i.e. – PKC is a cytosolic protein that, upon tissue activation, translocates to the plasmalemma where it is activated and then translocates back to the cytosol where it activates CPI-17 which decreases MLCP activity and thus affects MLC₂₀ phosphorylation levels). In contrast to this, we have reported that PKC α has a preferential peripheral localization at the plasma membrane in unstimulated SMCs in tissues and this does not change with agonist activation (Zhang et al., 2013) and (Fig 7). However, in freshly isolated SMCs, PKC α appears in a quasi-uniform distribution throughout the cell. Thus it appears that similar to the change in the primarily peripheral (plasmalemmal) distribution of vinculin and talin at the adherens junctions in intact SM tissues to a quasi-uniform cytoplasmic distribution following SMC isolation, PKC α also redistributes from a primarily peripheral (plasmalemmal) distribution to a quasi-uniform cytoplasmic distribution following SMC isolation. Curiously, the quasi-uniform cytoplasmic distribution of PKC α changes back to a primarily peripheral plasma membrane distribution in freshly isolated SMCs with PDBU stimulation, but not with carbachol stimulation (Zhang et al., 2013) and (Fig 6 – PDBu stimulation shown). Neither PDBU nor carbachol stimulation causes vinculin to revert back to its primarily peripheral plasma membrane distribution from a quasi-uniform distribution in freshly isolated SMCs (Eddinger et al., 2007).

Conventional PKC's are defined by their requirement of phosphatidylserine, diacylglycerol (DAG) and Ca²⁺ for activation (Newton, 1995; Mochly-Rosen and Gordon, 1998; Newton, 2001, 2010). DAG levels are increased with stimulation by G-coupled protein receptor

activation which in turn can activate phospholipase C (PLC). PLC hydrolyzes the plasma membrane phosphatidylinositol 4,5-bisphosphate (PIP₂) to inositol 1,4,5-trisphosphate (IP₃) and DAG. DAG is a lipid, and thus is assumed to remain at the plasma membrane. IP₃ on the other hand is a small soluble molecule that is proposed to diffuse freely within the cytosol where it may bind to IP₃ receptors on the sarcoplasmic reticulum (SR), releasing Ca²⁺ from the SR and thus increasing cytosolic [Ca²⁺]. If, as reported, DAG and phosphatidylserine are required for activation of PKC α , and both of these are restricted to the plasma membrane, then PKC needs to be at the plasma membrane to be activated. Our data from SMCs in intact tissue shows that PKC α is preferentially at the plasma membrane before tissue activation, remains there with activation, and stays there following activation (measured to 30 minutes, (Zhang et al., 2013)). Based on these data, downstream effector proteins would need to move to the activated PKC α at the plasma membrane rather than the PKC α translocating into the cytoplasm. In fact when we looked at the cellular distribution of CPI-17, PKC α substrate, in intact SM tissues this is exactly what we observe. CPI-17 is quasi-uniformly distributed throughout SMCs in non-stimulated SMCs in tissue and becomes primarily peripherally localized to the plasma membrane when the SM tissue is activated (Zhang et al., 2013). Following SM tissue activation CPI-17 co-localizes with PKC α at the plasma membrane (Fig 8), where both of these proteins co-localize with caveolin (Zhang et al., 2013). The CPI-17 was not observed to redistribute back throughout the cytoplasm following up to 30 minutes of stimulus activation with either carbachol or PDBU (Zhang et al., 2013). These results are consistent with those of Sakai (Sakai et al., 2005) who reported that CPI-17 moves from a cytosolic fraction to a membrane fraction with cholinergic stimulation of rat bronchial SM, and that this redistribution is maintained for the duration of the stimulation (out to 20 minutes in their study). In addition, CPI-17 was found to be phosphorylated only in the membrane fraction. These data suggest that CPI-17 does not move back to the cytoplasm on a time scale that is relevant for the Ca²⁺ sensitization observed physiologically in SM tissue experiments. An alternate explanation is that the regulation of MLCP via CPI-17 requires MLCP to translocate to the plasma membrane to interact with CPI-17 which is primarily localized there following tissue activation. Spatial-temporal redistribution of MLCP to the plasma membrane would remove it from the contractile myosin filaments that are distributed throughout the cytoplasm thereby preventing MLCP from dephosphorylating myosin light chain 20, resulting in a larger and or longer lasting contraction. With proper resources, this hypothesis would be fairly straight forward to test in SMCs in intact tissues. Morgan and coworkers (Shin et al., 2002) have shown that MLCP does in fact translocate from a cytosolic to a peripheral distribution in freshly isolated SMCs. PGF_{2 α} stimulation of freshly isolated SMCs causes MYPT1 (MLC targeting subunit of MLCP) phosphorylation, and translocation of MLCP to the plasma membrane where MYPT1 dissociates from the catalytic subunit (PP1c). MYPT1 remains at the plasmamembrane while PP1c returns to a diffuse distribution throughout the cell. Dissociated from MYPT1, PP1c would have a reduced ability to bind to and dephosphorylate MLC₂₀.

The reported differences in PKC α – CPI-17 spatial-temporal localization in SM tissues and isolated cells in this paper, along with numerous reports by others on these and other proteins (RhoA, ROCK, other PKC isoforms, calponin, vinculin, talin, paxillin, Fak) that

have been reported to translocate to and from the plasmamembrane in isolated and/or cultured cells (and undoubtedly many other proteins that have not been reported on) provides a means to better understand spatial-temporal regulation of cell function. What keeps PKC α localized at the plasmalemma, why it doesn't remain localized there in freshly isolated SMCs, and what allows/causes it to translocate back to the plasmalemma with activation of freshly isolated SMCs? If CPI-17 translocates to the plasmalemma to be activated by PKC α with tissue activation, but does not return to the cytosol in a physiologically relevant timeframe, how does it inhibit MLCP? Does MLCP also translocate to the plasmalemma in intact tissues following SM activation as it does in freshly isolated SMCs (Shin et al., 2002) and is this spatial-temporal separation of MLCP from myosin in the cytoplasm what allows MLC₂₀ phosphorylation levels to be maintained? Or does the PKC α – CPI-17 pathway only regulate MLC₂₀ phosphorylation levels of myosin spatially located near the plasmalemma? Using SM tissue and freshly isolated SMC experiments in parallel to address these questions may provide answers to these and many related questions as well as increasing our understanding of pathways involved in Ca²⁺ sensitization and which of these mechanisms are involved in SM tissue and organ function? In addition to the above questions related specifically to the PKC α -CPI-17- MLCP pathway are questions about what other proteins might also be translocating, and where, when and how is this happening. The very consistent alternating distribution of adherens junctions and caveolae at the plasma membrane (fig 1) brings the cytoskeletal organization for transmitting the actomyosin force generation to the membrane (and adjacent cells when in intact tissues) in close association with high density clustering of multiple receptors, channels, and signaling molecules. Significant changes in these localized domains, but not global changes throughout the cell, may be the key to regulation of many of these cellular processes. Are these two closely associated but distinct domains truly distinct and anatomically “fixed” in relative location to each other, or is significant regulation determined by their “co-mingling” with each other? New imaging techniques with improved resolution that can be used in living tissue should allow answers to these questions and many others that we still do not know.

Near the beginning of this article I suggested that understanding the functional differences between tonic and phasic smooth muscle is of physiological, clinical and academic relevance. I will also propose that there is physiological, clinical and academic relevance to if, when, and how protein translocation occurs in tonic and phasic SM tissues. Physiologically, it is possible that differences in if, when and how proteins translocate in SM tissues can explain tonic vs. phasic contraction specifically, and SM contraction generically. Maintained force despite decreases in [Ca²⁺]_i and myosin light chain 20 (MLC₂₀) phosphorylation to intermediate levels could result from spatial-temporal differences in any or all of the proteins involved in force generation, transmission and regulation. Clinically, cardiovascular disease remains the number one cause of morbidity and mortality in this and many other countries, and this does not take into account all the devastating consequences due to pathologies in the numerous other SM organ systems. Knowing how SM tissues function in any or all of these organ systems in physiological conditions is the first step in understanding how this differs from pathological conditions. And finally, academically (in addition to physiologically and clinically), if any of the translocation mechanisms identified

in freshly isolated or cultured SMCs is an artifact of SMC isolation and culturing – wouldn't we all want to know this? But either way - physiologically relevant or an artifact - protein translocation in isolated and or cultured SMCs gives us an ideal experimental system to study the cellular mechanism(s) of protein translocation and ultimately to understand its significance in SMC function.

In this article I have reviewed (and presented) data for the redistribution of vinculin (in approximately 50% of the cells) and PKC α in freshly isolated SMCs relative to that observed in intact SM tissues. In the former case (vinculin) this redistribution from the plasmalemma to the cytosol does not return to its primarily peripheral cellular distribution with isolated SMC activation, while in the latter case (PKC α) cell stimulation (PDBu) returns the PKC α to its primarily peripheral cellular distribution. The reason for this redistribution of these proteins from the plasma membrane to the cytosol in isolated SMCs is not known, but is hypothesized to be a result of the proteolysis and or loss of extracellular matrix proteins in the process of cell isolation. In this altered state, freshly isolated SMCs can be used to study how SMC function is altered by this new spatial-temporal distribution of these proteins. In the case of PKC α , studies can also be done examining how and when PKC α translocates back to the membrane with activation. While it remains unclear if there is a physiological relevance for these protein redistributions in SMCs in intact SM tissues, it seems likely that these changes do occur in development, growth, and pathological situations. Using both freshly isolated SMC and intact SM tissue studies in parallel will greatly enhance our understanding of basic cellular mechanisms and regulation of SMC function in tissues in physiological as well as pathological conditions.

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Fig 1.
Fluorescent photomicrograph of non-stimulated transverse section of dog intestine immunoreacted for vinculin (green), caveolin (red) and filamentous actin (phalloidin – blue). The filamentous actin appears uniformly distributed throughout the SMCs while vinculin (green, adherens junction) and caveolin (red, caveolae) are localized primarily at the plasmalemma in an alternating punctate pattern. Note that the adherens junctions and caveolae align at the membranes of adjacent cells.

Fig 2.

Vinculin distribution in relaxed (left) and stimulated (right) SMCs. Freshly isolated rabbit stomach single SMCs were attached to cover glasses, incubated at 37°C in PSS without Ca^{2+} (left) or following incubation with 10 μM phorbol 12, 13-dibutyrate (PDBu; right) for 15 minutes before fixation and immunoreacting for vinculin (green) and counterstained with phalloidin (filamentous actin; red) and DAPI (nuclei; blue). Large main panels on left and right are central z-stack longitudinal optical sections including multiple SMCs showing the vinculin (green) distribution as either randomly distributed throughout the cytoplasm, or primarily at the plasmalemma. Smaller fields above and to the right of each main panel shows a transverse optical section of the cells at the point where the line in the main panel bisects a SMC. Cells in the main panel that appear to have a random vinculin distribution throughout the cytoplasm show uniform vinculin in the transverse rendering, while cells that show vinculin at the plasmalemma in the main panel show a ring of vinculin surrounding the F-actin cytoplasmic core in the traverse rendering. Note that vinculin immunofluorescence distribution in freshly isolated SMCs is independent of relaxation in PSS without Ca^{2+} or with PDBu activation.

Figure 3.

Low magnification montage of transverse double labeled immunofluorescence sections of dog tracheal SM strip showing entire transverse section. Panel A is caveolin immunoreactivity of the entire cross-sectional area of the muscle strip following papain digestion showing caveolin's immunoreactivity is not lost with papain digestion. Panel B is the fibronectin immunoreactivity of this same transverse section showing only a small amount of immunoreactivity remaining near the center of the muscle strip. Quantitative image analysis allows immunofluorescent cross-sectional areas to be measured and

compared to the percent area of fibronectin lost. The percentage decrease of original force generation following papain digestion for this tracheal strip was 86.0 % and the percentage area of the strip with fibronectin immunofluorescence absent is 85.5%. Scale bar equals 100 μm . Peripheral fibronectin distribution in control tissues can be seen in (Eddinger et al., 2005, 2007).

Figure 4.

Transverse sections of dog colon longitudinal SM immunoreacted for caveolin in intact tissue (panel A) or following papain digestion of the tissue (panel B). In intact tissue, caveolin immunoreactivity is peripherally located in a punctate pattern (panel A). Following enzymatic digestion caveolin immunoreactivity remains peripherally located but is more diffuse such that the punctate pattern is not as prevalent as in panel A.

Figure 5.

Serial transverse sections of dog colon longitudinal smooth muscle (left panel set) and dog stomach body smooth muscle (right panel set) immunoreacted for vinculin (panels a), fibronectin (panels c), and talin (panels e) following papain digestion of the tissue. Caveolin immunoreactivity for these same fields (a, c, and e) are shown in panels b, d, and f respectively. Vinculin and talin immunoreactivity is not diminished and remains unaltered at the plasmalemma following papain digestion while the fibronectin immunoreactivity is significantly reduced by the enzymatic digestion (panels c, absence of signal). Caveolin immunoreactivity remains peripherally located following digestion. Distribution of these proteins in control tissues can be observed in (Eddinger et al., 2005, 2007).

Fig 6.

PKCα distribution in relaxed (left) and stimulated (right) SMCs. Freshly isolated rabbit stomach single SMCs were attached to cover glasses, incubated at 37°C in PSS without Ca²⁺ (left) or following incubation with 10 μM phorbol 12, 13-dibutyrate (PDBu; right) for 15 minutes before fixation and immunoreacting for PKCα (green) and counterstained with DAPI (nuclei; blue) and phalloidin (stimulated cells only, filamentous actin; red). Large main panels on left and right are central z-stack longitudinal optical sections including multiple SMCs showing the PKCα (green) distribution as randomly distributed throughout the cytoplasm when cells are not stimulated (left) , or primarily at the plasmalemma following stimulation (right) . Smaller fields above and to the right of each main panel shows a transverse optical section of the cells at the point where the line in the main panel bisects a SMC. Cells in the main panel that appear to have a random PKCα distribution throughout the cytoplasm show uniform PKCα in the transverse rendering, while cells that show PKCα at the plasmalemma in the main panel show a ring of PKCα surrounding the cytoplasmic core. Note that PKCα immunofluorescence distribution in freshly isolated SMCs is randomly distributed throughout the cytoplasm when the SMCs are relaxed in PSS without Ca²⁺ but become localized at the periphery following stimulation with PDBu.

FIGURE 7.

Immunohistochemical staining results from transverse-sectioned unstimulated (left three columns) and stimulated (right column) swine antrum tissue. Left three panels in the same row are from the same tissue section. Antibodies for respective proteins are labeled in corresponding panels. Co-localization of proteins appears as regions emitting a yellow or orange hue. Note that vinculin and PKC α do not appear to co-localize with or without stimulation while caveolin and PKC α appear to co-localize with and without stimulation. Nuclei were DAPI stained (blue). White bars = 10 μ m.

Figure 8.

Pig antrum activated with PDBu and reacted with PKCα (left column, green) and CPI-17 (middle column, red) and merged with nuclei (right column, PKCα-green; CPI-17 – red; DAPI – blue). Co-localization of PKCα and CPI-17 results in yellow/orange fluorescence.