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Regulation of Alpha- and Beta-actin Isoforms in the Contracting A7r5 Smooth Muscle Cell

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By

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Abstract

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By Dawn Leah Brown-Turner

Two isoforms of actin have been found to be present in A7r5 smooth muscle cells, α- and β-actin. This body of work sets out to examine the different regulatory factors of the two actin isoforms during smooth muscle contraction. The response of the actin isoforms to phorbol 12, 13-dibutyrate (PDBu) is markedly different. α-Actin remodels to podosomes around the periphery of the cell, while β-actin merely shortens. One protein involved in smooth muscle contraction induced by PDBu is protein kinase C (PKC). Two inhibitors of PKC, staurosporine and bisindolymaleimide, were used prior to PDBu stimulation and after PDBu stimulation to observe the effects of PKC on α- and β-actin stress fiber structure and remodeling. α-Actin showed a decrease in podosome formation when the inhibitors were added prior to or after PDBu stimulation. β-actin demonstrated a loss in stress fiber structure in response to PKC inhibitors. PKC appears to regulate α- and β-actin differently and could play a role in the differential remodeling seen by these two actin isoforms. Myosin light chain kinase (MLCK) is another enzyme involved in smooth muscle contraction. Even though it does not play a major role in PDBu induced contraction of smooth muscle, it has been found to interact with actin at two different actin binding sites, allowing crosslinking of actin filaments. Inhibition of the kinase domain of MLCK does not seem to have an effect on the crosslinking abilities of MLCK. These two key enzymes of smooth muscle contraction appear to not only be able to

initiate smooth muscle contraction, but also regulate reorganization of the two actin isoforms during smooth muscle contraction.

Dedication

I would like to dedicate this work to my family for without their support it would not be possible. My parents have always encouraged me to pursue my dreams. My brother has always been there and been supportive. My husband has been patient with me. We met during my second year as a graduate student, and he has been there through my comprehensive exams, my trip to Japan, and the long process of writing this dissertation. Most of all, I would like to dedicate this to my daughter, Alexis Grace. She unknowingly motivated me to get this finished and get my degree. To all of them, I say thank you, and I love you.

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Most of all, thanks to my committee members for mentoring me through my time here at Marshall. Dr. Green was always available to help and allowed me to borrow Larry the Loop. Dr. Harris helped me to get started in the lab and has always been encouraging to me. Dr. Mangiarua is always there to talk to whether about research or about anything. Dr. McCumbee was always a good neighbor who was willing to help whenever needed.

Dr. Wright I feel deserves his own paragraph. Without him, this would not be possible. I just hope these words make it into print, if not he made this impossible. Though he has a rough exterior and doesn't always say things in a way that may seem supportive, if you know him well enough, you know he wants nothing more for you than to succeed in life both professionally and personally. I owe it all to him.

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Chapter II. Differential Actin Isoform Reorganization in the Contracting A7r5 Cell

Chapter III. Role of the Kinase Domain of Myosin Light Chain Kinase in Actin Organization in the Contracting A7r5 Cell

List of Symbols/Abbreviations

α-actin	alpha-actin
ADP	adenosine diphosphate
ANOVA	analysis of variance
ATP	adenosine triphosphate
Asp	aspartate
β-actin	beta-actin
C-terminal	carboxy-terminal
Ca^{2+}	calcium
$[Ca^{2+}]_i$	intracellular concentration of calcium
CaD	caldesmon
CaM, CM	calmodulin
CO_2	carbon dioxide
DAG	diacylglycerol
DMEM	Dulbecco's modified Eagles medium
EDTA	ethylenediaminetetraacetic acid
F-actin	filamentous actin
FITC	fluorescein isothiocyanate
γ-actin	gamma-actin
G-actin	globular or monomeric actin
GFP	green fluorescent protein
Glu	glutamate
h	hour(s)

K^+	potassium
$[K^+]_o$	extracellular concentration of potassium
MAP	mitogen-activated protein
Mg^{2+}	magnesium
min	minute(s)
MLCK	myosin light chain kinase
MLCP	myosin light chain phosphatase
N-terminal	amino-terminal
NM	non-muscle
PBS	phosphate buffered saline
PBS-T	phosphate buffered saline containing 0.5% Tween-20
PDBu	phorbol 12, 13-dibutyrate
P_i	inorganic phosphate
PKC	protein kinase C
PVDF	polyvinylidene fluoride
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SE	standard error
siRNA	small, interfering ribonucleic acid
SM	smooth muscle

Chapter I

General Introduction

Dissertation Organization

This dissertation is divided into four chapters. The first chapter is an overview of the organization of the dissertation followed by a literature review of important topics that will be discussed in the following chapters. Chapter two is a manuscript that was published in the *Canadian Journal of Physiology and Pharmacology*. In this manuscript I investigated the regulatory role of protein kinase C (PKC) on α- and β-actin prior to and following initiation of contraction by phorbol 12, 13-dibutyrate (PDBu). In the third chapter, I investigated whether the kinase domain of myosin light chain kinase (MLCK) plays a role in the crosslinking of actin filaments. Chapter four is an overview of the results found in the experiments conducted within this body of work, as well as a look into what future endeavors could be examined.

Actin Isoforms

Actin is an abundant protein in all eukaryotic cell types. It is a cytoskeletal protein that plays a role in numerous functions including maintaining cell shape, cell division, endocytosis, exocytosis, secretion, signal transduction, and regulation of enzyme activities (Pollard and Cooper, 1976; Welch *et al.*, 1998). Of interest to the current work is the role actin plays in vascular smooth muscle contraction.

There are six actin isoforms known to be expressed in mammalian cells. Each of these six isoforms is encoded by an individual gene (Vandekerckhove and Weber, 1978a). These six isoforms can be classified into three groups based on isoelectric

focusing; α-, β-, and γ-actins (Garrels and Gibson, 1976; Storti *et al.*, 1976; Wahlen *et al.*, 1976; Rubenstein and Spudich, 1977). The three classes of isoforms are highly conserved with less than 10% difference between the amino acid sequences (Erba *et al.*, 1988; Gunning *et al.*, 1983; Hamada *et al.*, 1982; Ng *et al.*, 1985; Vandekerckhove and Weber, 1978a). Sequence analysis conducted by Vandekerckhove and Weber (1978b) indicated that most of the variability in the amino acid sequences arises within the first several amino acids of the amino-terminal region. α-Actin contains four charged amino acid residues within the first several amino acids of the amino-terminal region. By comparison, β-actin has three acidic residues, Asp-Asp-Asp, in the amino-terminal region. Like β-actin, γ-actin has three acidic residues as well in the amino-terminal region, but they are Glu-Glu-Glu. While there are other differences in the amino acid sequence spread throughout the molecule of the various isoforms of actin, it is the differences in the amino-terminal region that are likely to determine the specific functions and/or interactions of the isoforms, including actin-actin, acto-myosin, and actin-actin-binding protein interactions (Herman, 1993).

Another way to classify the actin isoforms is based on whether they are contractile or cytoplasmic actins. Contractile actins, or muscle actins, play a role in muscle contraction. All α-actin isoforms are contractile proteins. Cytoplasmic actins are also known as non-muscle actins, meaning they are not thought to play a role in muscle contraction, but rather play a structural support role in the cell. β-actin is a cytoplasmic actin. γ-actins have isoforms that can fit into either of these groups (Mossakowska and Strzelecka-Golaszewska, 1985).

Smooth muscle has been found to express four actin isoforms. Two of the isoforms are smooth muscle (SM) specific α-SM and γ-SM actins, while the other two are non-muscle (NM) actins, β-NM and γ-NM actins (Drew and Murphy, 1997). Of the two smooth muscle specific actins, α-SM actin is the isoform found predominantly in vascular smooth muscle, comprising up to 70% of the total actin (Fatigati and Murphy, 1984; Small, 1995). Of the two cytoplasmic actins, β-actin is more abundant in vascular smooth muscle (Franke *et al.*, 1980; Owens *et al.*, 1986; Skalli *et al.*, 1987). All four isoforms, however, can be found in vascular smooth muscle during fetal development with β-actin being the predominant isoform (Franke *et al.*, 1980; Owens *et al.*, 1986; Skalli *et al.*, 1987). The switch from predominantly β-actin to α-actin marks the differentiation of smooth muscle cells (Owens and Thompson, 1986; Skalli *et al.*, 1987). Cells that have undergone differentiation are generally non-dividing cells that have the ability to contract (Murphy, 1992). An interesting phenomenon that has been documented is the switching from α-actin as the predominant isoform back to β-actin as the predominant isoform after differentiation of the smooth muscle cells. This phenomenon is known as dedifferentiation and results in loss of contractility. Some instances where this has been found include during isolation of cells for primary culture (Chamley-Campbell *et al.*, 1979; Chamley-Campbell *et al.*, 1981; Yau-Young *et al.*, 1981), during cell proliferation and migration (Barja *et al.*, 1986), and during conditions like intimal thickening of rat aorta after endothelial injury or human atheromatous plaque development (Gabbiani *et al.*, 1984).

With the presence of multiple actin isoforms within a given cell type, a common question arises as to why a cell would have multiple forms of a protein that is so similar

in amino acid sequence and thus structure. The two common topics that attempt to answer this question revolve around the location of the isoforms in the cell and the function of the isoforms in the cell.

Many studies have looked at the possibility of compartmentalization of the various actin isoforms within a given cell type. As mentioned previously, the actin isoforms are often referred to as contractile or cytoplasmic actins. Results from some of these studies indicate that the actin is compartmentalized in such a way as to yield contractile domains and cytoplasmic domains. According to work conducted by Small *et al.* (1986) and Draeger and co-workers (1990), the contractile domains are distinguished by the presence of actin and myosin, while the cytoskeletal domains are distinguished by actin, intermediate filaments, and dense bodies. Another study by Lehman (1991) also indicated two distinct populations of actin filaments that were isolated from smooth muscle cells using two distinct antibodies. The use of an anti-filamin antibody revealed actin filaments associated with tropomyosin, filamin, and calponin. The presence of filamin, an intermediate filament protein, suggests that this set of actin filaments would be part of the cytoskeletal domain. The use of an anti-caldesmon antibody resulted in actin filaments associated with tropomyosin and caldesmon. Caldesmon is known to modulate contractile activity, suggesting that this set of actin filaments would be part of the contractile domain.

In some instances, spatial separation of the actin isoforms in smooth muscle has been found. DeNorfrio *et al*. (1989) and Herman (1993) have both found α-actin to be present in stress fibers, while NM-actins are localized to regions of advancing cortical cytoplasm within vascular smooth muscle cells in culture. Similarly, North *et al.* (1994)

found that in chicken gizzard smooth muscle cells, the two actin isoforms, β-NM-actin and γ-SM-actin in this muscle type, are arranged differently within the cytoplasm. β-Actin was found to be arranged in a more longitudinal direction and localized to dense bodies, dense plaques, and in channels with intermediate filaments. Again, β-actin was found to be closely associated with filamin, except at dense bodies, suggesting a cytoskeletal role for β-actin. A number of studies have indicated that γ-actin, on the other hand, is arranged more obliquely within the cytoplasm of the cells (Fay and Delise, 1973; Small, 1974; Fisher and Bagby, 1977; Small *et al.*, 1990). North *et al.* (1994) suggested a model where γ-actin is not present in dense bodies, and therefore, it is not a component of the cytoskeletal domain. However, they do not exclude the possibility that the γ-actin, associated with myosin into contractile domains and running obliquely across the cell, does not at some point cross through the cytoskeletal domains.

In other instances, actin isoforms have not been found to be compartmentalized within cells, and in some cases, have been found to copolymerize with each other. Experiments looking at the kinetics of polymerization of the various actin isoforms have found that under physiological conditions there is little or no difference between the polymerization of the various actin isoforms (Gordon *et al.*, 1977; Mossakowska and Strzelecka-Golaszewska, 1985; Umemoto and Sellers, 1990). Drew and co-workers (Drew *et al.*, 1991; Drew and Murphy, 1997) found that thin filaments in adult swine stomach smooth muscle contained both smooth muscle actins and cytoplasmic actins randomly distributed along the filaments. They could not rule out, however, that individual filaments may have specific accessory proteins associated with them that may dictate further the functioning of these filaments within the cell either as cytoskeletal or

contractile filaments (Langanger *et al*., 1986). Song *et al*. (2000) also showed that smooth muscle and nonmuscle actin isoforms are not separated into two populations of thin filaments, but rather are copolymerized into the same filaments in smooth muscle cells cultured from rabbit aorta. In this cell type, the two actin isoforms are α-SM-actin and β-NM-actin. They did find, however, that the α-actin tended to make up the portion of the actin filament found in the central region of the cell, while the β-actin tended to form the portion of the same fiber found at the cell periphery. Either way, the localization of the actin isoforms often times is similar to the expression of the actin isoforms: tissue specific.

It is fairly obvious that if actin isoforms are separated into different compartments or domains that they may perform different functions. For example, the work by North *et al.* (1994), showed smooth muscle actins were arranged obliquely and associated with myosin, and nonmuscle actins are arranged longitudinally and associated with filamin. This clearly demonstrates that the actin associated with myosin is the actin involved in the contraction of the cells, while suggesting the nonmuscle actin plays a structural role typical of what would be seen of the cytoskeleton in nonmuscle cell types. However, Khaitlina (2001) has cautioned that sorting of actin isoforms into different compartments within the cell does not have to mean that they perform different functions but that they could be performing the same functions in different regions of the cell.

On the other hand, Murphy (1992) suggested two criteria in determining if there are functionally different roles for smooth muscle versus nonmuscle actins contained within the same cell type. The first criteria is the presence of two distinct populations of thin filaments characterized by the actin isoform composition of the filaments and

possibly by the types of actin binding proteins associated with those filaments. The second criteria is the separation of these two populations of actin filaments on the basis that only the smooth muscle actins are associated with myosin. Throughout the remainder of the dissertation, I try to satisfy these criteria and demonstrate that the actin isoforms within A7r5 smooth muscle cells are two distinct populations with different functions as well as different regulatory factors.

A7r5 Cell Line

The model utilized in this body of work is the A7r5 smooth muscle cell line. This smooth muscle cell line was derived from embryonic rat aorta (Kimes and Brandt, 1976) between days 14-17 gestation (Furulli *et al.*, 1998). These cells do, however, exhibit a phenotype much like that of adult smooth muscle cells. Furulli *et al.* (1998) examined the expression of numerous markers specific to differentiated smooth muscle cells within the A7r5 cell line. Among the markers they chose to study were smooth muscle myosin heavy chain (one of the most specific markers for smooth muscle cells), smooth muscle calponin, SM22, and smooth muscle α-actin. They found the A7r5 cell line expressed transcripts for all of these smooth muscle cell markers. The A7r5 cell line retains these marker proteins even after being maintained in cell culture conditions for prolonged periods of time (Gimona, *et al*., 2003). This suggests the A7r5 cell line is a good model for study of smooth muscle.

As mentioned previously, smooth muscle cells can have a range of phenotypes, which can be determined partly by the actin isoform which is dominant. When cells function primarily in contraction, there is a predominance of α-actin (Campbell and

Campbell, 1993; Mosse, *et al.*, 1985; Manderson, *et al.*, 1989). As was mentioned in the previous paragraph, smooth muscle α-actin is present within the A7r5 cells and is the predominant actin isoform in this cell line.

Another important feature of the A7r5 cell line as a model for this work is its ability to contract in response to various stimuli. The work presented here will look at contraction of the A7r5 cells in response to phorbol 12, 13-dibutyrate, which will be discussed in more detail in a later section. However, it has been found that cells of the A7r5 cell line are also able to contract in response to other stimuli including vasopressin, phenylephrin, and elevated potassium levels (Gimona *et al*., 2003).

A final feature about the A7r5 cell line that has made it an ideal model in this body of work is its ability to be transfected. Furulli *et al*. (1998) examined four rat smooth muscle cell lines: A7r5, adult and pup aortic, and PAC1. Among these four cell lines, A7r5 was found to be the most highly transfected cell line of the four studied in terms of the uptake of exogenous plasmid DNA. As will be seen in later chapters, the transfection of the A7r5 cell line with plasmid containing β-actin DNA was a common technique used to study the regulation and function of β-actin during phorbol contraction. All of these points taken together indicate the A7r5 cell line is a good model for smooth muscle research.

Smooth Muscle Contraction

The first step in smooth muscle contraction requires a stimulus to initiate contraction. Such stimuli fall into one of two categories: electromechanical coupling or pharmacomechanical coupling. In arterial smooth muscle, electromechanical coupling

occurs through a change in the membrane potential of the cell either initiated by a contractile agonist or by an increase in the extracellular concentration of potassium ($[K^+]_o$) (Rembold, 1996). Action potentials or an increase in $[K^+]_o$ activate L-type calcium channels allowing calcium to enter the cell, causing the intracellular calcium concentration ($[Ca^{2+}]_i$) to rise (Hermsmeyer *et al.*, 1988). Agonists such as norepinephrine (Nelson *et al.*, 1988; Haeusler and De Peyer, 1989; Neild and Koteca, 1987), histamine (Droogmans *et al.*, 1977; Casteels and Suzuki, 1980; Keef and Bowen, 1989; Keef and Ross, 1986), and endothelin (McPherson and Angus, 1991) also can cause depolarization of the smooth muscle membrane primarily through L-type calcium channels or other voltage-gated calcium channels. In each of these instances, an increase in $[Ca^{2+}]_i$ occurs, which activates the cellular contractile machinery.

Pharmacomechanical coupling in arterial smooth muscle occurs when an agonist causes the release of calcium from intracellular stores, when agonists activate either voltage-dependent or voltage-independent calcium channels in the plasma membrane to allow calcium to enter the cell (at a degree higher than expected with electromechanical coupling), or when an agonist increases $[Ca^{2+}]_i$ sensitivity (Rembold, 1996). In any of these instances, an increase in $[Ca^{2+}]_i$ or a change in the response to a given level of $[Ca^{2+}]_i$ may occur without a change in the membrane polarity of the cell (Rembold, 1996).

Once a stimulus has caused the $[Ca^{2+}]_i$ to increase, the calcium binds to calmodulin (Kretsinger and Nockolds, 1973). Calcium induces a conformational change in calmodulin (Seamon, 1980) that allows it to interact with myosin light chain kinase (MLCK) (Kamm and Stull, 1985; Walsh, 1994; Somlyo and Somlyo, 1994). The

interaction of calcium-calmodulin with MLCK activates MLCK, allowing it to phosphorylate the regulatory light chains of myosin at serine 19 (Adelstein and Conti, 1975; Gallagher *et al.*, 1997). This step activates the actin-dependent ATPase activity of myosin needed during cross-bridge cycling (Erdodi *et al.*, 1996). The phosphorylation of the regulatory light chains of myosin is the primary regulatory event leading to the initiation of smooth muscle contraction (Ogut and Brozovich, 2003; Kamm and Stull, 1985).

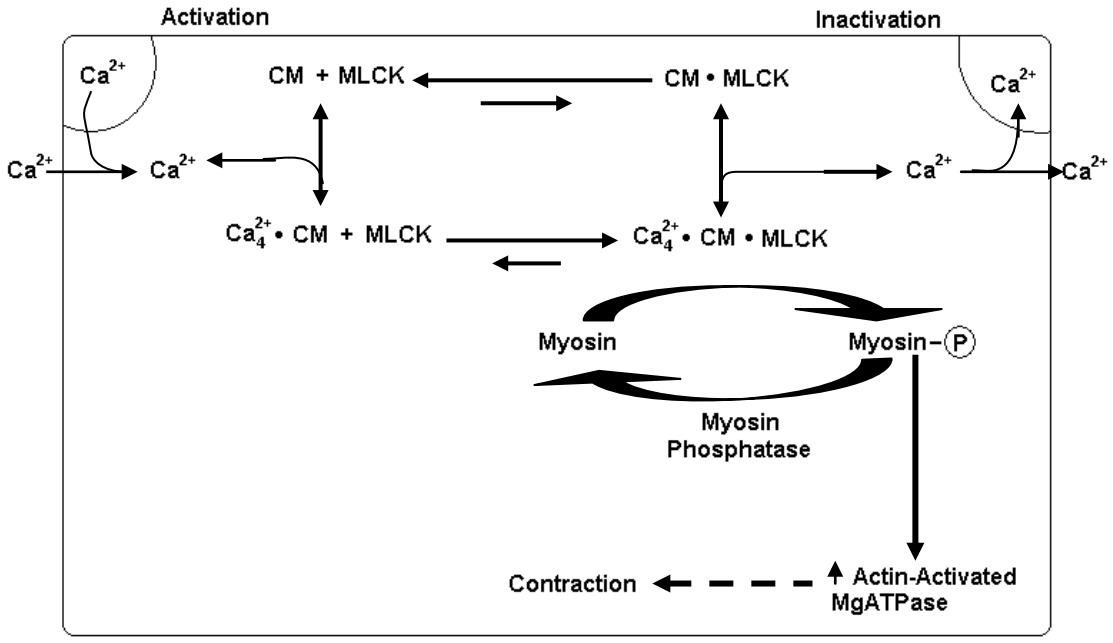

Figure 1. Schematic of the biochemical events of smooth muscle contraction.

After phosphorylation of the regulatory light chains occurs, actin and myosin can interact and begin cross-bridge cycling to develop tension and shorten the muscle cell. In smooth muscle, it is thought that the phosphorylation of myosin allows the actin to bind

to myosin, suggesting that this phosphorylation event is a regulatory step that occurs prior to actin binding to myosin (Hartshorne and Gorecka, 1980). Once the actin has bound to myosin, an inorganic phosphate (P_i) that is bound to the myosin from the previous cross-bridge cycle is released, leaving an ADP bound to the actinomyosin complex. The ADP is then released from the complex as well causing the myosin to pull the actin to generate force and shortening. Once the ADP and P_i are released, an ATP molecule can bind to the myosin of the actinomyosin complex. Once the ATP has bound, the actin is released from the myosin. The ATPase of the myosin then hydrolyses the ATP into ADP and P_i to initiate the cycle again.

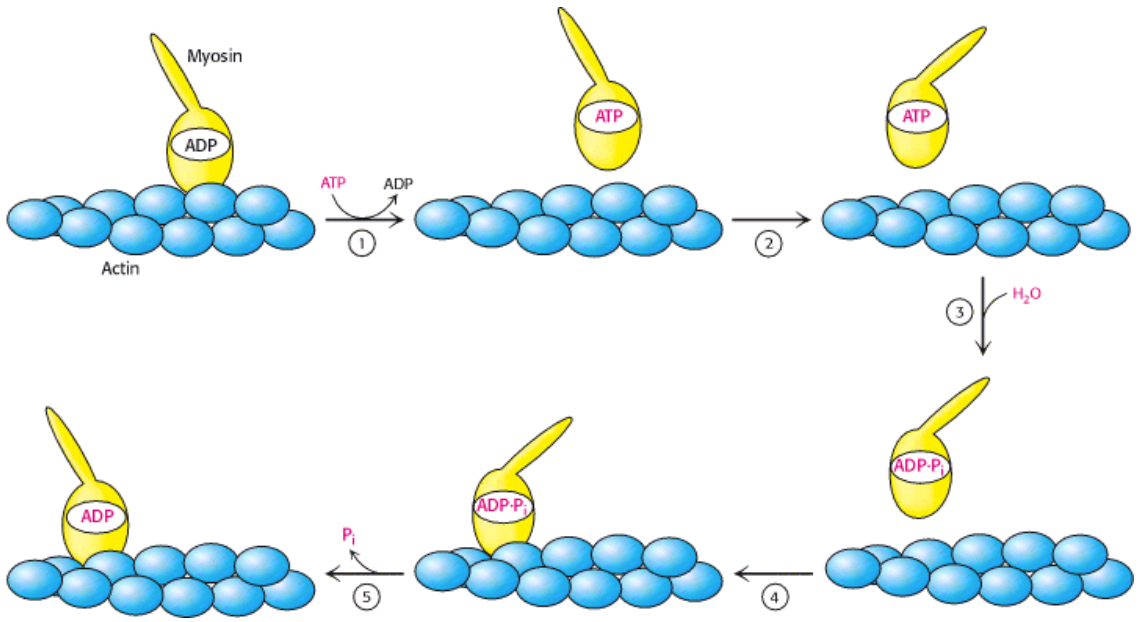

Figure 2. Cross-bridge cycling of actin and myosin during muscle contraction. J.M. Berg, J.L. Tymoczko, and L. Stryer. (2002). *Biochemistry*. New York: W. H. Freeman and Company.

If phosphorylation of the regulatory light chains of myosin is a regulatory step to allow cross-bridge attachment and cycling, then there must be a way to reverse this step to cease cross-bridge cycling. The opposing enzyme to MLCK is myosin light chain

phosphatase (MLCP), which removes the phosphate group from serine 19 of the regulatory light chains of myosin (Alessi *et al.,* 1992; Shimizu *et al.,* 1994; Shirazi *et al.,* 1994). It is the balance of activities of MLCK and MLCP that determines to what degree the regulatory light chains of myosin are phosphorylated (Erdodi *et al.,* 1996).

In smooth muscle, however, contraction can be initiated and/or maintained without phosphorylation of the regulatory light chains of myosin (Chatterjee *et al.,* 1987; Somylo *et al.,* 1988; Adam *et al.,* 1989; Katsuyama *et al.,* 1992). Often times the relationship between the amount of force developed and the degree to which the regulatory light chains of myosin are phosphorylated depends on what initiates the contraction or the length of time the muscle is contracted (Haeberle *et al.,* 1985; Rembold and Murphy, 1986; Jiang and Morgan, 1989; Laporte *et al.,* 1994). One common theory, known as the latch state, tries to explain how smooth muscle can remain tonically contracted with the use of very little energy (Dillon and Murphy, 1982). This theory was brought about by observations that even under circumstances where $[Ca^{2+}]_i$ is low and the level of myosin light chain phosphorylation is low, there can still be a substantial amount of force maintained even with a reduction in cross-bridge cycling and ATP hydrolysis (Siegman *et al.,* 1984; Rembold and Murphy, 1986). In the context of this paper, the idea that there are other means to initiate contraction without phosphorylation of the regulatory light chains is of great importance and will be dealt with in more detail in later sections.

Phorbol Esters and PKC Isozymes

In the experiments we conducted, the A7r5 smooth muscle cell was contracted with phorbol esters, specifically phorbol 12, 13-dibutyrate. Phorbol esters are derived from the plant *Croton tiglium,* as well as from other plants belonging to the family Euphorbiaceae (Hecker, 1968). Phorbol esters were originally utilized as tumor promoters (Boutwell, 1974), but since then they have been found to have numerous other roles, including the initiation of slow, sustained contractions in vascular smooth muscle strips (Danthuluri and Deth, 1984; Rasmussen *et al.*, 1984; Jiang and Morgan, 1987, 1989; Singer and Baker, 1987).

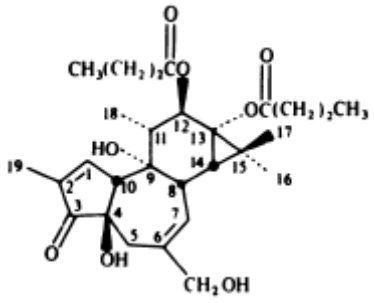

Figure 3. Structure of phorbol 12, 13-dibutyrate.

The mechanism by which phorbol esters work in the cell is by mimicking the effects of diacylglycerol (DAG) (Kazanietz, 2005). DAG is a lipid second messenger known to bind to protein kinase C (PKC) (Ron and Kazanietz, 1999). DAG is typically generated in the cell through the interaction of a hormone or other extracellular effector that binds to a receptor on the extracellular surface of the plasma membrane that is then linked to the enzyme phospholipase C via a G-protein mediated process (Berridge, 1984;

Majerus *et al.*, 1986). Once phospholipase C is activated it cleaves phosphatidylinositol 4,5-bisphosphate to produce DAG and inositol 1,4,5-triphosphate (Blumberg, 1988). DAG can then interact with PKC thereby activating it, as will be described in further detail later. Phorbol esters mimick DAG by binding to the same site on PKC as DAG.

PKC was originally discovered in 1977 by Nishizuka and co-workers (Takai *et al.*, 1977). Ten isozymes of PKC have been found; each of which is a product of a separate gene with exclusion of PKCβI and PKCβII, which are formed by alternative splicing (for reviews see Mellor and Parker, 1998; Newton 1995, 1997). These 10 isozymes are divided into three subclasses based on the properties that regulate them. The "conventional" or "classic" PKCs include the isozymes PKC α, βI, βII, and γ. This subclass of isozymes is activated by calcium and/or DAG and phorbol esters. The second subclass of isozymes is known as the "novel" PKCs. This subclass consists of the isozymes PKC δ, ε, θ, and η. The novel PKCs are activated by DAG and phorbol esters but not calcium. The last subclass is known as the "atypical" subclass of PKCs that includes PKCζ and PKCι. This subclass of PKCs is not activated by either calcium or DAG and phorbol esters. The isozymes of PKC that have been found in A7r5 cells include PKCα (Kaplan-Albuquerque and Di Salvo, 1998; Fan and Byron, 2000), PKCβ, and PKCγ (Fan and Byron, 2000). Evidence indicates that PKCα is the primary isozyme involved in contraction in response to phorbol esters (Hai *et al.*, 2002), which will be discussed further in the next section.

Ron and Kazanietz (1999) reviewed the structure of the PKC isozymes. The structure of the PKC isozymes consists of a single amino acid chain with two distinct

domains joined by a hinge region. On the carboxy-terminal end is the kinase domain. This domain consists of regions that interact with ATP and the specific substrates of the isozyme. On the amino-terminal end of the protein is the regulatory domain. It is this domain that varies most between the isozymes. The regulatory domain consists of binding sites for DAG/phorbol esters and calcium, as well as regions that participate in protein-protein interactions that help to regulate activation of PKC and its localization within the cell.

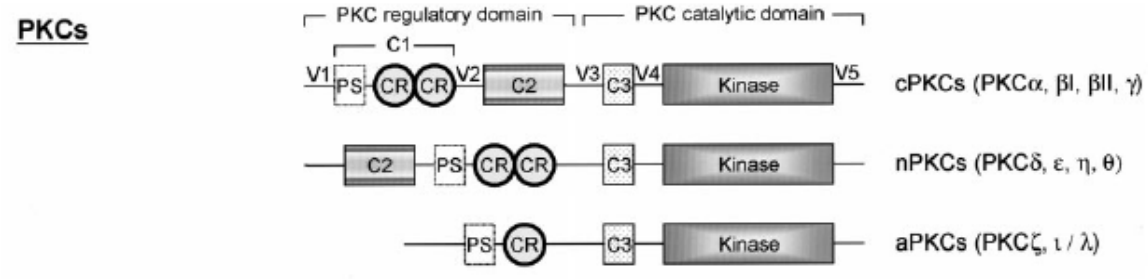

Figure 4. Structure of the subclasses of PKCs. Modified from Ron and Kazanietz, 1999.

The C1 region of the regulatory domain is of particular interest to the topic of phorbol esters binding to and activating PKC isozymes. The C1 region is a highly conserved region among the PKC isozymes. This region consists of a pseudosubstrate domain (autoinhibitory domain) and cysteine-rich domain(s). The pseudosubstrate domain acts to keep the enzyme inactive by binding to the substrate binding site in the catalytic domain when DAG/phorbol esters and/or calcium are not bound to the enzyme (Orr *et al.*, 1992). The pseudosubstrate domain can bind to the substrate binding domain, because it resembles the substrates that would be able to bind to this site with exclusion of a nonphosphorylated amino acid residue in place of the serine or threonine residue that

the enzyme would typically phosphorylate (House and Kemp, 1987). The cysteine-rich domains within the C1 region are the sites where DAG or phorbol esters bind in the conventional and novel PKCs (Burns and Bell, 1991; Kazanietz *et al.*, 1995a; Kazanietz *et al.*, 1995b; Ono *et al.*, 1989).

There are three other highly conserved regions within the PKC isozymes. One of these three is also in the regulatory region and is known as the C2 domain. The C2 domain is responsible for the binding of calcium in conventional PKC isozymes. There is a C2-like domain within the regulatory region of novel PKC isozymes as well (Sossin and Schwartz, 1993), but it does not play a role in calcium binding to these isozymes. The other two highly conserved regions are within the catalytic domain of the isozymes and are referred to as the C3 and C4 domains. The C3 domain contains the site for the binding of ATP, while the C4 domain is the site for recognition and binding of the substrate. It is the C4 domain to which the pseudosubstrate domain of the C1 region binds to when the PKC isozyme is in its inactive form.

Phorbol esters have been found to bind to and activate conventional and novel PKC isozymes. The manner in which they bind involves the presence of a phospholipid cofactor, generally phosphotidylserine (Konig *et al.*, 1985). It is important for the PKC isozymes to be associated with phospholipids to regulate the translocation of PKC after treatment with phorbol esters (Kazanietz, et al., 2000). Once the phorbol ester and cofactor are bound, a conformational change occurs within the PKC isozyme resulting in the removal of the pseudosubstrate domain of the C1 region from the binding site in the C4 domain, which causes the activation of the PKC isozyme (Ron and Kazanietz, 1999). It has been found that even under conditions where calcium and phosholipid cofactors are

limited, phorbol esters still have the ability to activate PKC isozymes (Castagna *et al.*, 1982).

Initiation of Contraction by PKC

There is much debate on what role calcium plays in vascular smooth muscle contraction in response to phorbol. Several investigators have found that treatment with phorbol esters do cause an increase in $[Ca^{2+}]_i$ leading to contraction of the smooth muscle by means of MLCK, as discussed in the earlier section on smooth muscle contraction. Others have found that phorbol esters can initiate contraction without an increase in $[Ca^{2+}]_i$ or phosphorylation of myosin light chain (Sybertz *et al.*, 1986; Jiang and Morgan, 1987, 1989; Singer 1990). If myosin light chain is not phosphorylated, then phorbol esters could initiate contraction by PKC phosphorylating other proteins that will result in contraction (Walsh *et al.*, 1994) or by increasing the sensitivity of the contractile response to calcium so that contraction can occur at resting levels of intracellular calcium (Jiang and Morgan, 1987).

Many studies have been conducted examining the role of calcium in contraction of A7r5 cells in response to phorbol esters. There is conflicting evidence as to whether there is an increase in intracellular calcium in the A7r5 cell line in response to phorbol esters. In a study using A7r5 cells, Nakajima *et al.* (1993) proposed that the increase in $[Ca^{2+}]_i$ they found in response to phorbol esters appears to be caused by PKC modulating calcium currents through voltage-dependent calcium channels (Fish *et al.*, 1988). Sperti and Collucci (1987) also reported an increase in the intracellular calcium in A7r5 cells in response to treatment with phorbol diesters, which is blocked by dihydropyridines

suggesting influx through voltage-dependent calcium channels. Rembold and Murphy (1988) agreed with Sperti and Colluci with the added information that the effects of PDB on intracellular calcium could be dependent on membrane potential of the cells. On the other hand, Galizzi *et al.* (1987) found that treatment with phorbol esters of A7r5 smooth muscle cells causes inhibition of calcium channel activity, suggesting calcium does not play a role. Vigne *et al.* (1988) has shown that phorbol esters activate sodium-calcium exchangers in A7r5 cells, which would cause a decrease in the intracellular levels of calcium. Our laboratory (Li *et al.* 2001), has found that calcium appears to be necessary, at least at low levels, to obtain the characteristic contraction and remodeling of α-actin in response to PDBu.

In regards to increased sensitivity of the contractile response to calcium, Jiang and Morgan (1987) showed results indicating that at resting levels of intracellular calcium, phorbol esters can initiate contraction in rat and ferret aorta. These results confirm *in vitro* studies indicating that phorbol esters and diacylglycerol increase the affinity of PKC for calcium from the 1×10^{-6} M range to the 1×10^{-7} M range, which falls within the range of resting intracellular calcium concentrations (Nishizuka, 1986; Yamanishi *et al.*, 1983).

Addressing the last issue mentioned previously concerning other possible substrates for PKC that could induce contraction in response to phorbol esters, there are several that have been mentioned in the literature ranging from proteins previously mentioned to be involved in smooth muscle contraction, such as MLCK, myosin light chain, and myosin phosphatase, to other proteins that interact with the cytoskeleton, such as caldesmon and calponin. While MLCK has been found to be a substrate for PKC *in*

vitro (Ikebe *et al.*, 1985; Nishikawa *et al.*, 1985), it has been documented that phosphorylation of MLCK by PKC does not have an effect on contractility (Stull *et al.*, 1990).

Myosin light chain has also been found to be an *in vitro* substrate of PKC (Bengur *et al.*, 1987; Ikebe *et al.*, 1987). These *in vitro* studies indicated that PKC phosphorylates myosin light chain at sites different from MLCK. PKC phosphorylates myosin light chain at serine 1, serine 2, and threonine 9, while MLCK phosphorylates myosin light chain at serine 18 and serine 19. Phosphorylation at PKC sites has been found to inhibit actin-activated myosin Mg^{2+} ATPase activity and contraction (Bengur *et al.*, 1987; Sutton and Haeberle, 1990). There have been studies indicating that while myosin light chain is phosphorylated at specific sites related to PKC, there is also a degree of phosphorylation of myosin light chain that corresponds to the sites typically phosphorylated by MLCK (Singer *et al.*, 1989; Singer, 1990; Barany *et al.*, 1992). It has been suggested that the phosphorylation at the characteristic MLCK sites could be a result of inhibition of myosin phosphatase (Masuo *et al.*, 1994). The means by which this inhibition occurs is by the phosphorylation of CPI-17 by PKC (Kitazawa *et al.*, 1999).

There are two other possible substrates for PKC to interact with in the cytoskeleton. One of these is caldesmon. Caldesmon is a protein that can cross-link actin and myosin filaments (Ikebe and Reardon, 1988). Caldesmon functions to inhibit actin-activated myosin Mg^{2+}-ATPase activity (Ngai and Walsh, 1984). Phosphorylation of caldesmon by PKC results in the release of actin thereby releasing the inhibition of the actomyosin ATPase (Andrea and Walsh, 1992), which theoretically could lead to contraction. It is thought that this interaction between caldesmon and PKC is not direct,

but rather is a result of PKC acting on caldesmon via a cascade involving Ras, Raf, mitogen-activated protein (MAP) kinase kinase, and MAP kinase (Adam and Hathaway, 1993).

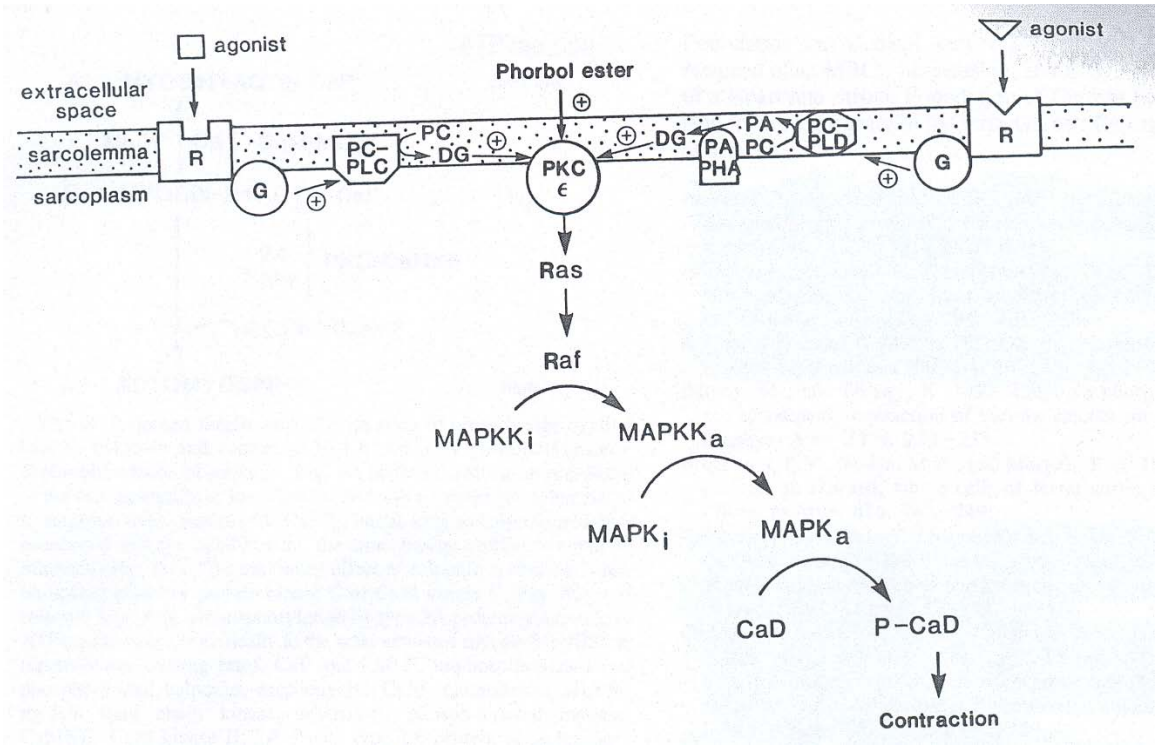

Figure 5. Signal transduction pathway linking PKC to caldesmon (CaD) to initiate contraction. (Walsh *et al*., 1994)

Calponin is the other possible PKC substrate found to interact with the cytoskeleton (Winder and Walsh, 1993). Calponin also inhibits actin-activated myosin Mg^{2+}-ATPase activity by binding to actin (Winder and Walsh, 1990). PKC directly phoshporylates calponin at serine 175 to release actin and the inhibition of the actomyosin ATPase (Winder and Walsh, 1993, 1990), which has the potential to lead to smooth muscle contraction.

Podosomes

Podosomes were first reported in the mid-1980's as structures that formed where cells made contact when grown on an artificial surface (Marx, 2006). To date, no podosomes have been reported to be found *in vivo* (Marx, 2006). Podosomes were first described in cells that had been transformed by Rous sarcoma virus (David-Pfeuty and Singer, 1980) and in cells derived from monocytes, such as osteoclasts (Marchisio *et al.*, 1984) and macrophages (Marchisio *et al.*, 1987). Podosomes are generally 1-2 μm in diameter (Marx, 2006) and are finger-like projections that extend up through the cell from the ventral membrane, where the cell is in contact with the substrate, toward the dorsal surface of the cell (Linder and Aepfelbacher, 2003). The core of the podosome contains columns of actin filaments with proteins associated with actin polymerization, such as N-WASp (Mizutani *et al.*, 2002), Arp2/3 (Linder *et al.*, 2000; Burns *et al.*, 2001; Kaverina *et al.*, 2003), and cortactin (Schuuring *et al.*, 1993, Ochoa *et al.*, 2000; Pfaff and Jurdic, 2001; Mizutani *et al.*, 2002; Destaing *et al.*, 2003). Surrounding the actin core is a ring of proteins typically associated with focal adhesions, such as α-actinin and vinculin (Marchisio *et al.*, 1984; Tarone *et al.*, 1985; Marchisio *et al.*, 1988; Sobue *et al.*, 1989; Babb *et al.*, 1997; Fultz *et al.*, 2000; Hai *et al.*, 2002; Destaing *et al.*, 2003). The actin core does not appear to be associated with the rest of the actin cytoskeleton (Gimona and Buccione, 2006).

The function of podosomes is still a matter of debate. It is generally thought that because they adhere to artificial substrates, podosomes play a role in cell adhesion and cell motility (Marx 2006). In other instances, podosomes have been found to play a role in remodeling of the cytoskeleton and matrix by controlling the localized turnover of

cytoskeleton attachments and degradation of the extracellular matrix (Gimona and Buccione, 2006).

Fultz *et al.* (2000) was the first to report the presence of podosomes in A7r5 cells as a response to phorbol esters. However, he did not describe them as podosomes but as peripheral bodies. It was Hai (2002) that first described these peripheral bodies as podosomes. Fultz *et al.* (2000) found that cells stained with phalloidin and stimulated to contract with PDBu developed discrete peripheral structures that were confirmed to result from the remodeling of α-actin. The α-actin was found to disassemble from stress fibers and reassemble into these peripheral structures resulting in the loss of most of the α-actin stress fibers. A protein found to be associated with the actin core was α-actinin. Another feature of the peripheral bodies was found to be that they extended vertically up from the base of the cell.

Hai *et al.* (2002) confirmed the fact that the peripheral bodies arose from the base of the PDBu treated cells, and they connected this to the defining feature of podosomes (Nitsch *et al.,* 1989; Wakino *et al.,* 2001). They also found that vinculin, which is a marker for focal adhesions, colocalized to the peripheral structures, suggesting that these structures may arise from focal adhesions. As mentioned previously, podosomes have been found to have both α-actinin (Fultz *et al.,* 2000) and vinculin associated with the actin core. The observations published in the work by Fultz *et al.* (2000) and the observations made by Hai *et al.* (2002) led Hai to label the peripheral bodies as podosomes.

Numerous others have begun to study podosomes in A7r5 cells as well. Kaverina *et al.* (2003) reported that the initiation and maintenance of podosomes in A7r5 cells requires Arp2/3-dependent actin polymerization, which is consistent with findings about podosomes in other cell types. They also found that the site of podosome formation appears to be at the junction of actin stress fibers with focal adhesions. Work done by Burgstaller and Gimona (2005) further indicated that podosomes in A7r5 cells are sites of substrate degradation. Work continues in this area to define the structure and function of these structures.

Myosin Light Chain Kinase

MLCK has already been mentioned as an enzyme involved in the regulation of smooth muscle contraction by phosphorylating the regulatory light chain of myosin thereby activating myosin ATPase (Aksoy *et al*., 1976; Chacko *et al*., 1977; DiSalvo *et al*., 1978; Gorecka *et al*., 1976; Ikebe, *et al*., 1977; Sobieszek 1977). This section is going to focus on the structure of the enzyme, and its role in binding to actin.

There are two genes responsible for expressing MLCK in vertebrates, but only one gene is expressed in smooth muscle (Stull *et al*., 1986; Gallagher *et al*., 1997). The other MLCK gene has been found to be expressed in striated muscle. MLCK is between 130-150 kDa depending on what species it is isolated from (Stull *et al*., 1998). The catalytic site is found near the central region of the enzyme and is similar to other serine/threonine protein kinases (Olson *et al*., 1990; Stull *et al*., 1986; Gallagher *et al*., 1997). The C-terminal region is known as the telokin domain and is thought to be a site for myosin binding (Ito *et al*., 1989). There are also two sites to which calmodulin binds

to MLCK often referred to as the regulatory sites. One is found in the N-terminal region of the enzyme between amino acids 26-41, and the other is found in the C-terminal region between amino acids 787-815 (Olson *et al*., 1990; Ye *et al*., 1997; Gao *et al*., 2001). The calmodulin binding site in the C-terminal region acts to regulate the kinase activity of MLCK (Olson *et al*., 1990). The calmodulin binding site in the N-terminal region acts to regulate the actin binding activity of MLCK (Ye *et al*., 1997). MLCK also has two actin binding domains within the N-terminal region of the enzyme (Ye *et al*., 1997). One of the actin binding domains is within amino acids 1-41, while the other is within amino acids 138-218 (Ye *et al*., 1997; Gao *et al*., 2001). Notice that one of the actin binding sites overlaps with one of the calmodulin binding sites. This particular actin binding site is referred to as a Ca^{2+}/CaM sensitive binding site. Binding of Ca^{2+}/CaM to this site causes MLCK to release actin from this binding site (Ye *et al*., 1997). The other actin binding site is referred to as a Ca^{2+}/CaM insensitive binding site (Ye *et al*., 1997).

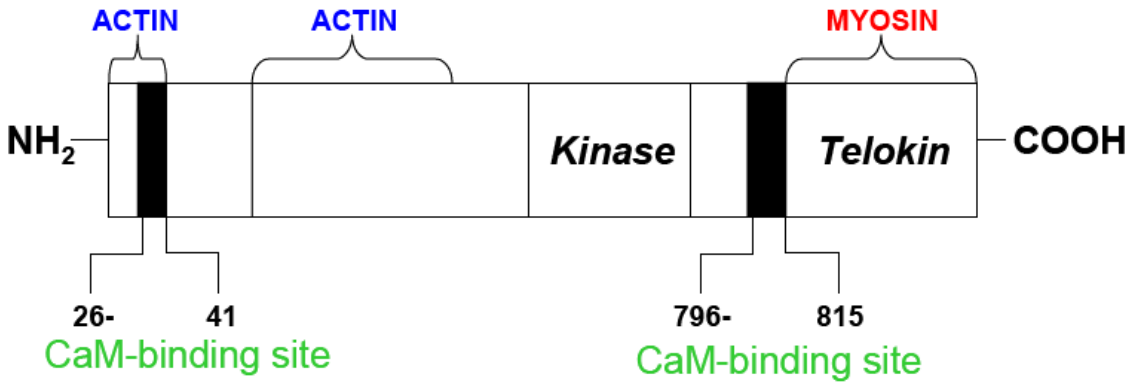

Figure 6. Schematic structure of MLCK. Redrawn from *Molecular Mechanisms of Smooth Muscle Contraction*, Chapter 2, Hayakawa *et al*., 1999. The red, blue, and green color regions indicate the location of the binding of listed molecules to MLCK.

MLCK has been found to bundle actin filaments by cross-linking actin filaments between the Ca^{2+}/CaM sensitive and insensitive actin binding sites found within the N-terminal region of MLCK (Hayakawa *et al*., 1994, 1999; Gao *et al*., 2001). When MLCK bundles actin filaments, it also causes an inhibitory effect on the interaction of actin and myosin. This inhibition is relieved in the presence of Ca^{2+}/CaM (Kohama *et al*., 1992). Hayakawa *et al*. (1999) concluded the bundling activity of MLCK with actin is too low to possess any physiological relevance without the interaction of an additional protein yet to be identified.

Summary

Taken together, the literature indicates that smooth muscle contraction has a unique way of developing force that cannot be solely explained on the basis of the classical actin/myosin sliding filament theory, even though some of the concepts may apply to smooth muscle. Our laboratory and others have proposed that in smooth muscle the actin cytoskeleton undergoes dynamic remodeling during force development. This remodeling is important to maintain actin and myosin at optimal opposition to enhance force development and to allow for slow, sustained force development, a unique characteristic of smooth muscle contraction.

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Chapter II

Differential Actin Isoform Reorganization in the Contracting A7r5 Cell

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Abstract

In the present study, we investigated the reorganization of α- and β-actin in the contracting A7r5 smooth muscle cell. The remodeling of these actin variants was markedly different in response to increasing concentrations of phorbol 12, 13-dibutyrate (PDBu). At the lowest concentrations ($\leq 10^{-7}$ mol/L), cells showed an ~70% loss in α-actin stress fibers with robust transport of this isoform to podosomes. By comparison, β-actin remained in stress fibers in cells stimulated at low concentrations ($\leq 10^{-7}$ mol/L) of PDBu. However, at high concentrations ($\geq 10^{-6}$ mol/L) ~50% of cells showed transport of β-actin to podosomes. Consistent with these findings, staining with phalloidin indicated a significant decrease in the whole-cell content of F-actin with PDBu treatment. However, staining with DNase I indicated no change in the cellular content of G-actin, suggesting reduced access of phalloidin to tightly packed actin in the podosome core. Inhibition of protein kinase C (staurosporine, bisindolymaleimide) blocked PDBu-induced (5×10^{-8} mol/L) loss in α-actin stress fibers or reversed podosome formation with re-establishment of α-actin stress fibers. By comparison, these inhibitors caused partial loss of β-actin stress fibers. The results support our earlier conclusion of independent remodeling of α- and β-actin cytoskeletal structure and suggest that the regulation of these structures is different.

Introduction

Several isoforms of actin have been isolated from eukaryotic cells that, based on their motility in isoelectric focusing, have been classified as α-, β-, and γ-actins. Sequence analysis has indicated that actin structure is highly conserved with only minor heterogeneity (<10%) among isoforms (Khaitlina 2001). Nevertheless, there is clear evidence for tissue specificity of actin isoform expression. Four isoforms of actin have been identified in mammalian and avian smooth muscle: α- and γ-smooth muscle and β- and γ-nonmuscle or cytoplasmic actin. The β-actin isoform has been found in all smooth muscle tissue examined thus far, whereas, the expression of the remaining isoforms is tissue-specific (Hartshorne 1987). The γ-actin isoform is found in visceral smooth muscle (Hartshorne 1987; North et al. 1994; Toullec et al. 1991), whereas α-actin is the predominant muscle actin isoform in vascular smooth muscle (Fatigati and Murphy 1984; Hartshorne 1987). The expression of multiple actin species within a single cell type suggests that the isoforms serve different functions. Consistent with this idea, actin isoform compartmentalization has been reported in a variety of cell types (Khaitlina 2001). Based on immunohistochemistry and the distribution of actin-associated proteins, it was proposed early on that smooth muscle cell actins could be separated into contractile and cytoplasmic domains containing smooth muscle-specific and nonmuscle isoforms, respectively (DeNofrio et al. 1989; Herman 1993; North et al. 1994; Small 1995). However, other investigators have challenged the spatial separation of actin isoforms in smooth muscle. Drew and Murphy (1997) reported that actin filaments isolated from swine stomach fundus consisted of copolymerized muscle and nonmuscle actins at about the same composition ratio of tissue homogenates. Stromer et al. (2002)

showed localization of β-actin, α-, and (or) α/γ-actin and α-actinin at discrete cellular and membrane foci, confirming that both muscle and nonmuscle filaments are associated with cell dense bodies and plaques in several types of smooth muscle. Based on their observation of low level staining for β-actin throughout filament structure, however, they concluded that there was extensive overlap of muscle-specific and nonmuscle isoforms, arguing against spatial separation into contractile and cytoplasmic domains. By comparison, Song et al. (2000) showed a distinctly herterogenous distribution of actin isoforms in cultured smooth muscle cells. They noted a predominate localization of β-actin at the cell periphery associated with vinculin plaques, striated myosin, and α-actinin aggregates, whereas α-actin was observed in the central region of the cell associated with continuous myosin and punctate α-actinin staining. However, they further showed an apparent transition in composition of individual stress fibers from β-actin to α-actin from periphery to cell center, indicating incomplete separation into discrete domains.

Taken together, these studies suggest at least partial segregation of smooth muscle-specific and nonmuscle actin isoforms. This, in turn, suggests that, despite the high level of homology, mechanisms are available for sorting actin isoforms. Consistent with this conclusion, Mounier et al. (1997) have reported direct evidence of differentiated sorting of labeled actin isoforms introduced into smooth muscle and nonmuscle cells. In addition to evidence of spatial segregation, several studies have suggested that α- and β-actin organization may be governed by different mechanisms and could serve different functional roles in the contractile and motility properties of fibroblasts and smooth muscle cells. For example, experimental manipulation of α- and β-actin structure and expression levels has been shown to selectively alter contraction and cell motility,

respectively (Hinz et al. 2001; Ronnov-Jessen and Petersen 1996; Schedlich et al. 1997). Recent evidence has further indicated that the introduction of the NH_2-terminal sequence of α-actin into myofibroblasts inhibits contractile activity, whereas the NH_2-terminal peptide of β-actin had no effect (Hinz et al. 2002).

Our laboratory has reported that the reorganization of α- and β-stress fibers is different during phorbol ester-induced contraction of A7r5 cells (Fultz et al. 2000; Li et al. 2001*a*). During the interval of cell contraction, β-actin fibers remained stable, whereas α-actin reformed into intensely fluorescing peripheral bodies that have been subsequently identified as podosomes (Hai et al. 2002), suggesting the isoforms were subject to different regulatory control. Gimona et al. (2003) have recently reported that actin stress fibers decorated with h1-calponin remained stable, whereas those associated with SM22 were transported to podosomes in PDBu-treated A7r5 cells, suggesting 2 functionally distinct actin filament populations. However, in contrast to our earlier observations, they found that β-actin as well as α-actin was localized at podosomes. One possible explanation of results was the difference in concentrations of PDBu used to stimulate cells in the 2 studies. In the present study we examined the remodeling of α- and β-actin in response to a range of PDBu concentrations as well as comparing the effects of contractile antagonists on their organization during PDBu stimulation. The results support earlier conclusions of spatial and regulatory segregation of α- and β-actin in the A7r5 cell.

Materials and Methods

Cell Culture

A7r5 smooth muscle cells, derived from embryonic rat aorta and shown to maintain the ability to contract to phorbol esters (Fultz et al. 2000; Nakajima et al. 1993), were obtained from American Type Culture Collection (Manasass, Va.). Cells were plated on 75-cm^2 flasks and grown to approximately 85% confluence at 37^oC in a humidified atmosphere of 5% CO_2 in air. The cells were maintained in Dulbecco's modified Eagles medium (DMEM) supplemented with 10% fetal bovine serum, 100 U/mL penicillin G, and 100 μg/mL streptomycin. Media was changed every other day and cells were passaged at least once a week. Passaging was accomplished by addition of trypsin-EDTA solution in PBS and collection of cells by centrifugation.

Confocal Microscopy

In experiments employing immunostaining, cells were seeded onto glass coverslips, placed in 6-well culture plates and returned to the incubator for a minimum of 24 h to allow for attachment and spreading. After experimental treatment, cells were fixed and permeabilized by addition of ice cold acetone for 1 min. The cells were washed several times with PBS containing 0.5% Tween-20 (PBS-T), pH 7.5, followed by a 30-min incubation in blocking solution containing 5% nonfat dry milk in PBS. α-Actin staining was accomplished by incubation of cells in a 1:500 dilution of monoclonal-α-smooth muscle actin, clone 1A4 FITC-labeled antibody (Sigma Chemical Co., St. Louis, Mo.) for 60 min at room temperature. β-Actin was imaged using a 1:500 dilution of monoclonal anti-β-actin, clone AC-15 primary antibody (Sigma) followed by incubation

with an Alexa 488 labeled secondary antibody (Molecular Probes, Eugene, Ore.). Cells were imaged by mounting on a Nikon Diaphot Microscope and confocal microscopy performed with a Bio-Rad Model 1024 scanning system with a Krypton/Argon laser. Micrographs were constructed by projection of Z-plane acquisitions and analyzed by Lasersharp and Confocal Assistant Software (BioRad, Hercules, Calif.). The ratio of cellular filamentous (F-actin) to monomeric (G-actin) actin was estimated using dual staining with phalloidin and DNase I by a modification of the method of Knowles and McCulloch (1992). Cells were fixed with ice-cold acetone for 1 min, rinsed with PBS (3X) and then incubated with Alexa Fluor-DNase I and Alexa Fluor-phalloidin (Molecular Probes, Eugene, Ore.) for 1 h. Cells were thoroughly rinsed with PBS and embedded in Gel-Mount media (Fischer, Chicago, Ill.) on glass slides. Cells were then visualized using a Bio-Rad 1024 scanning confocal microscope with laser settings kept constant for all experimental groups. Nuclear fluorescence was eliminated using Paint Shop Pro. Whole-cell pixel counts were obtained by boxing cells using the rectangular tool with ImageJ program (NIH, Bethesda, Md.). F-actin to G-actin ratios were calculated by dividing pixel counts obtained for phalloidin and DNase I staining, respectively, for each cell. Because 20% to 30% of cells do not respond to PDBu, cells showing robust podosome formation were selected for evaluation.

Cell treatments and analysis of actin remodeling

PKC inhibitors staurosporine and bisindolymaleimide were added at a final concentration of 10x the reported IC_{50} or K_i value. The effect of the compounds were evaluated in their ability to alter α-actin and β-actin structure both during PDBu-

stimulated (5×10^{-8} mol/L) reorganization and after PBDu-induced reorganization was established. For the former experiments, an inhibitor was added 30 min before PDBu with the cells fixed for imaging 30 min after PDBu stimulation. For the latter, the inhibitor was added 30 min following PDBu with cells fixed for imaging at 30 min after the introduction of inhibitor. Vehicle-control cells were fixed at identical intervals as the inhibitor-treated cells.

Based on the work of Chrzanowska-Wodnicka and Burridge (1996) showing that internal strain on the cytoskeleton was required for formation of stress fibers, we assumed that contractile antagonists would result in significant loss of tension bearing structures. Consequently, in addition to qualitative assessment of actin stress fibers (fiber breakage, compression), an attempt was made to obtain quantitative information concerning loss of structure. Surprisingly, the contractile antagonists did not result in loss of α-actin stress fibers. Rather, the protein kinase inhibitors blocked α-actin stress fiber remodeling to podosomes when added before PDBu and caused a reversal of podosome formation with reformation of stress fibers when added after PDBu. Therefore, counts of cells exhibiting podosome formation were used as a measure of inhibitor effect on α-actin remodeling. Cell counts were performed in 3 separate experiments with a total of approximately 300 cells counted per experiment. Cells were scored by 2 viewers and the counts were averaged.

In contrast to α-actin, inhibitors caused noticeable loss in β-actin structure. To estimate losses in β-actin stress fibers, confocal images of treated and control cells were obtained at fixed confocal settings and analyzed using Image J software (NIH). Using the line scan tool, the cell was bisected in the long axis at mid-cell and the resulting plot

was imported into Microsoft Office Excel (Fig. 1). The point of lowest intensity was selected as baseline and the graph was then analyzed using Peakfit 4.0 software to determine the area under the curve (I = total intensity). This value was then divided by line pixel number (p) to normalize for line length. The final value, β-actin filament intensity = I/p was calculated using Excel.

Western blot analysis

A7r5 cells were plated on 150 mm x 25 mm tissue culture dishes and grown to approximately 85% confluence as described in the cell culture section. Cells were treated with vehicle (Control) or 10^{-7} mol/L PDBu for 30 min to allow cells to contract. After contraction, control and PDBu treated cells were collected by addition of trypsin-EDTA and centrifugation. Cells were resuspended in 300 μL RIPA lysis buffer containing Complete protease inhibitor cocktail tablet (Roche Diagnostics, Indianapolis, Ind.) for 30 min on ice. To separate G-actin from F-actin, the cell lysates were centrifuged at 100,000g for 1 h at 4^{o}C. Upon completion of centrifugation, the supernatant was removed for analysis of G-actin. An amount of lysis buffer equal to the supernatant was added to the pellet and allowed to soften on ice for 1 h. The pellet was then resuspended and sonicated for analysis of F-actin. Concentration of total protein per sample tube was determined using BCA (Pierce) protein assay. Samples were denatured in SDS sample buffer and subjected to 12.5% SDS-PAGE. Protein was transferred to PVDF membrane (Pierce) using a Fischer Biotech semi-dry blotting unit. Blots were blocked for 1 h with 5% nonfat dry milk in PBS followed by washing 3 times for 5 min each. The blot was then probed with a 1:500 dilution of monoclonal-anti-actin, α-smooth muscle primary

antibody (Sigma, St. Louis, Mo.) for 1 h followed by washing. The blot was then incubated for 1 h with anti-mouse Ig, horseradish peroxidase-linked secondary antibody (Amersham Biosciences, Piscataway, N.J.) followed by washing. Horseradish peroxidase activity was detected using the ECL Western Blotting Detection system (Amersham Bioscience).

Data analysis

Analysis of differences between treatment means was performed by 1-way ANOVA followed by Student's unpaired *t* test (Sigma Stat, SPSS, Chicago, Ill.). Differences between means were considered significant if $p \leq 0.05$. Averaged results are presented as mean ± SE throughout the text.

Results

Actin concentration response to PDBu

There was a marked dichotomy in the concentration-response of PDBu-stimulated α- and β-actin reorganization into podosomes (Table 1; Fig. 2). A total of 40% to 70% of cells stained for α-actin showed robust podosome formation with an approximate 70% loss in stress fibers (Table 1) at the lowest PDBu concentrations (10^{-8} mol/L and 10^{-7} mol/L). In contrast, few cells imaged for β-actin showed evidence for podosome formation (Fig. 2) and loss of stress fibers (Table 1) at lower PDBu concentrations. However, approximately 50% of these cells exhibited formation of these structures at PDBu concentrations $\geq 10^{-6}$ mol/L (Fig. 2).

Dual staining of control cells with phalloidin and DNase I indicated F-actin incorporation into stress fibers (Fig. 3A) with G-actin diffusely distributed within the cell body (Fig. 3B). Contraction of the cell with 10^{-7} mol/L PDBu resulted in an apparent loss in stress fibers with translocation of F-actin to podosomes (Fig. 3C), a pattern of reorganization that appeared to be intensified in cells treated with 10^{-5} mol/L PDBu (Fig. 3E). Interestingly, there was a clear redistribution of G-actin to the cell periphery with PDBu treatment (Fig. 3D, 3F). Particularly in cells contracted with 10^{-5} mol/L PDBu, G-actin appeared to diffusely distribute about the F-actin-containing core of podosomes. As expected from visual evaluation of images, simultaneous staining with phalloidin and DNase I indicated a significant reduction in the ratio of F-actin to G-actin in PDBu-treated cells (Table 2). However, this change was due to a decrease in F-actin

fluorescence with no evidence of significant alteration in the cellular content of G-actin. This suggests that PDBu stimulation did not result in a net depolymerization of actin but that the balance of actin from stress fibers was incorporated as tightly packed filaments in the core of podosomes. Consistent with this idea, Western blot analysis indicated no change in the content of F- and G-α-actin in PDBu-treated compared with control cells (Fig. 4).

Effects of protein kinase inhibitors on actin structure

Figure 5 shows the distribution of α- and β-actin in unstimulated and PDBu-treated (5×10^{-8} mol/L) A7r5 control cells. In the resting cell, both isoforms are incorporated into stress fibers arranged in parallel. During PDBu-induced contraction, the 2 actins undergo distinct modes of reorganization. As the cell contracts, α-actin structure exhibits a loss (~70%) in stress fibers (Table 1) with the formation of podosomes. By comparison, β-actin stress fibers shorten in the interval following PDBu (Fig. 5) without loss in structure (Table 1). The introduction of PKC inhibitors prior to PDBu resulted in variable effects on α-actin structure (Table 3A; Fig. 6). Both staurosporine and bisindolymaleimide blocked α-actin remodeling to podosomes. However, these cells showed well developed α-actin stress fibers suggesting sufficient strain on filaments to maintain these structures and their anchorage sites at focal adhesions. Staurosporine but not bisindolymaleimide caused significant losses in β-actin stress fibers (Table 3B; Fig. 6) when added prior to PDBu. The addition of PKC inhibitors after PDBu addition and the establishment of actin reorganization resulted in the reversal of podosome formation and re-establishment of an extensive system of α-

actin stress fibers (Table 3A; Fig. 7). By comparison, inhibitor treatment of cells after PDBu stimulation resulted in highly significant losses in β-actin stress fibers (Table 3B; Fig. 7).

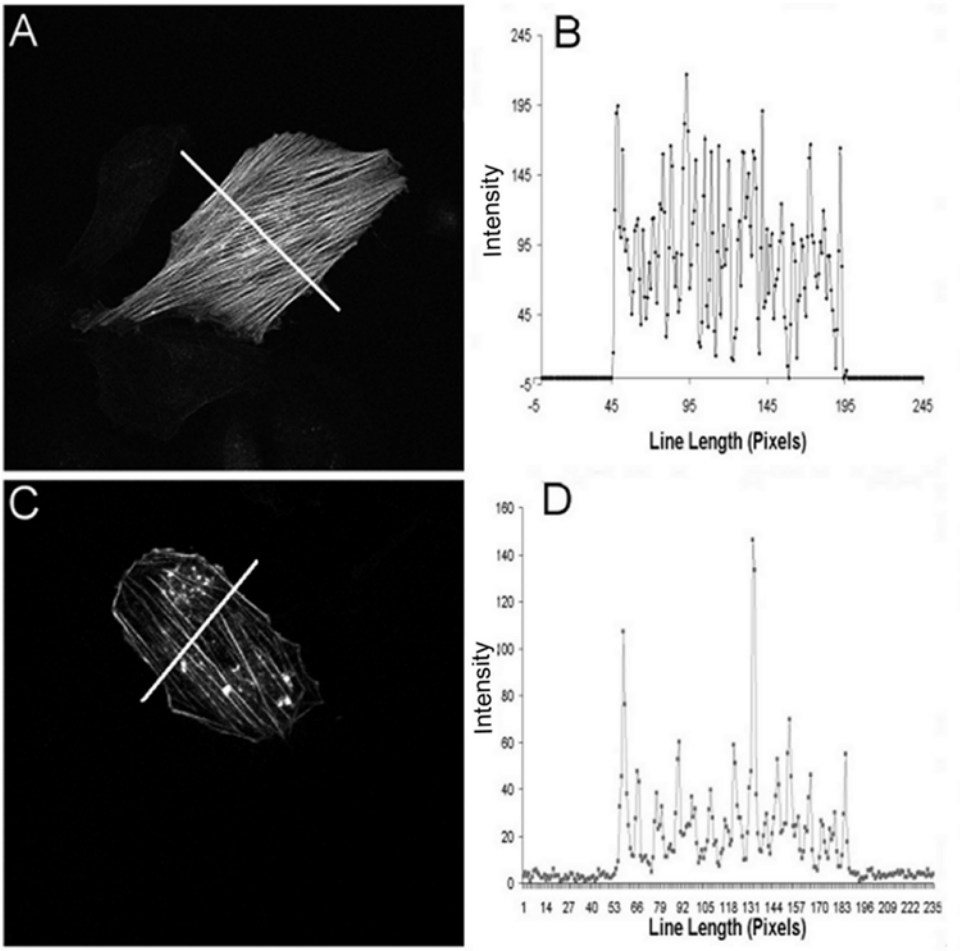

Fig. 1. Quantitative assessment of actin filament structure loss. Images of (A) control and (C) inhibitor-treated cells were obtained at fixed confocal settings and analyzed using Image J software. Each cell was bisected in the long axis by line scan and the resulting plot (B, D) imported to Microsoft Office Excel. The graph was analyzed by Peakfit 4.0 software to obtain the area under the curve (I = total intensity) that was divided by line pixel number (p) to normalize for cell width. The final number, filament intensity = I/p, was calculated in Excel.

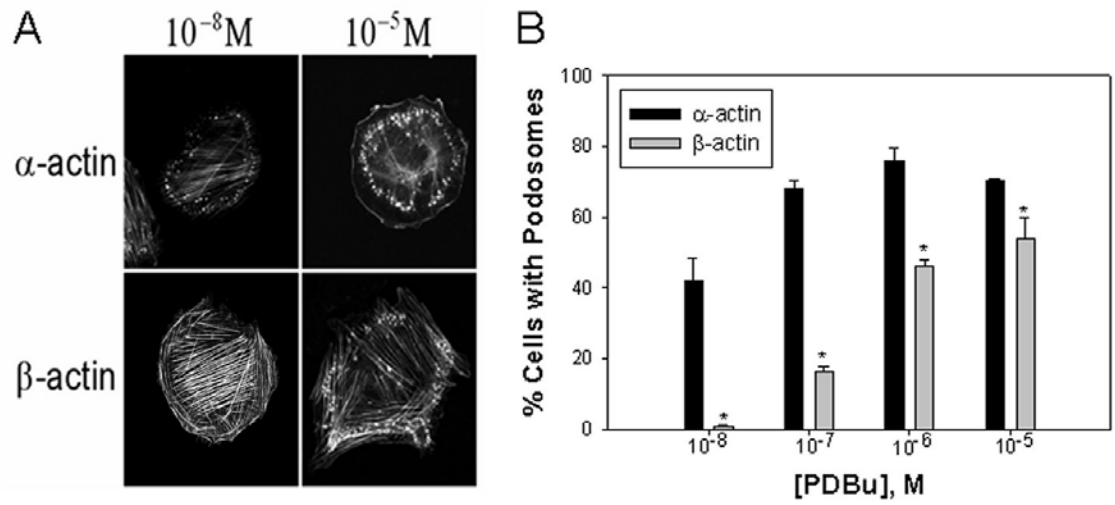

Fig. 2. The concentration-response of α- and β-actin remodeling to PDBu. (A) Images of cells showing α-actin and β-actin structure after 30 min incubation at 10^{-8} or 10^{-5} mol/L PDBu. α-Actin was imaged using a monoclonal α-smooth muscle actin, clone 14C FITC-labeled antibody. β-Actin was imaged by use of a monoclonal anti-β-actin, clone AC-15 primary antibody followed by an Alexa 488-labeled secondary antibody. (B) Graphical presentation of the percentage of cells imaged for α- or β-actin that exhibited podosome formation at increasing concentrations of PDBu. The results indicate a significant difference in the response of the 2 isoforms. α-Actin showed robust localization at podosomes even at the lowest concentration studied. By comparison, β-actin remained in stress fibers and was transported to podosomes only at high ($\geq 10^{-6}$ mol/L) concentrations of PDBu.

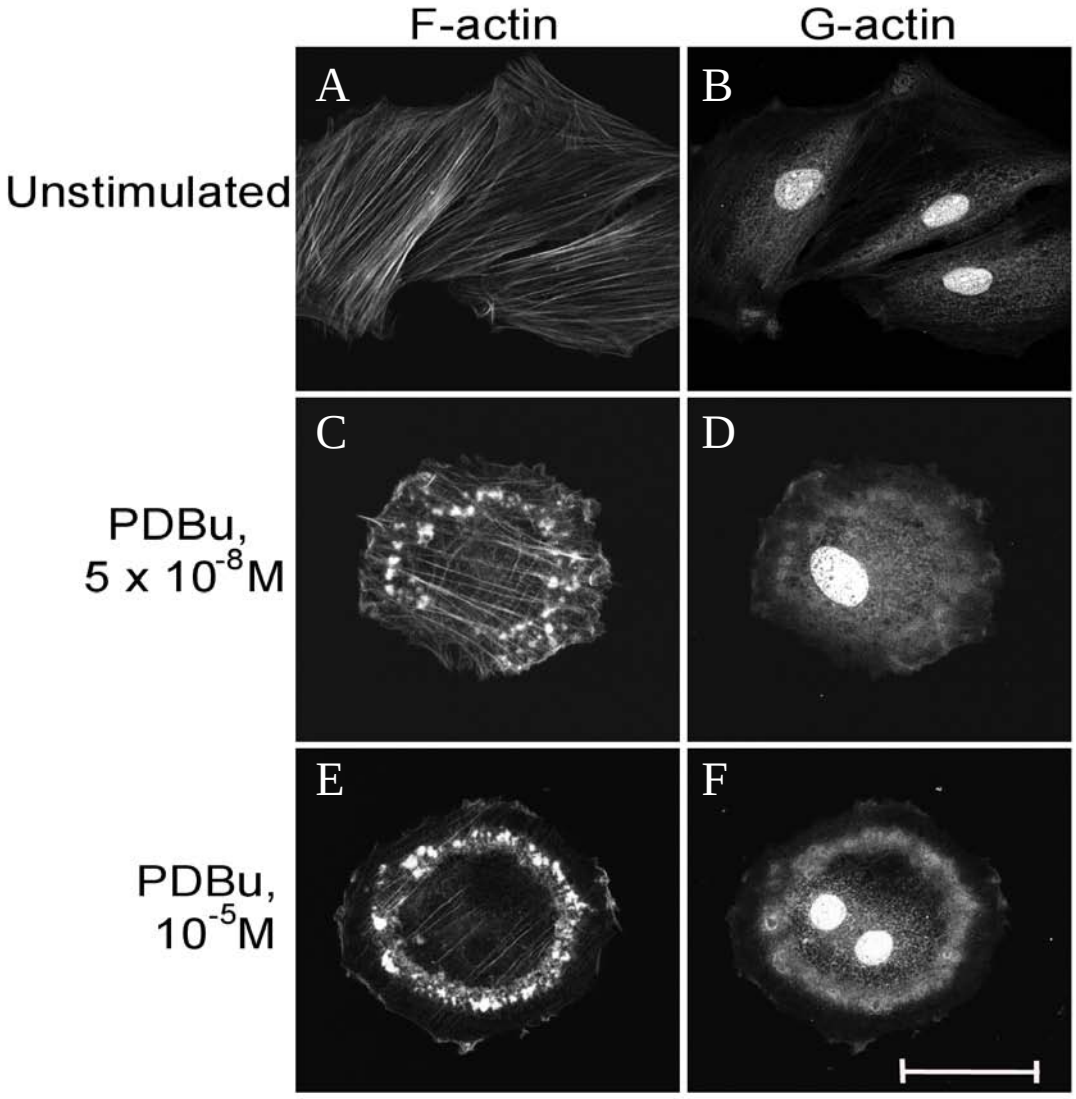

Fig. 3. Visualization of F-actin and G-actin in control (unstimulated) and PDBu-stimulated (5 x 10^{-8} mol/L) A7r5 cells. Cells were fixed with acetone and then stained with Alexa Fluor-Phalloidin and Alexa Fluor-DNase I for imaging with a Bio-Rad 1024 scanning confocal microscope. The images are typical of cells showing F-actin translocation from stress fibers to podosomes with redistribution of G-actin to the periphery about the podosomes after PDBu treatment. Bar represents 50 µm.

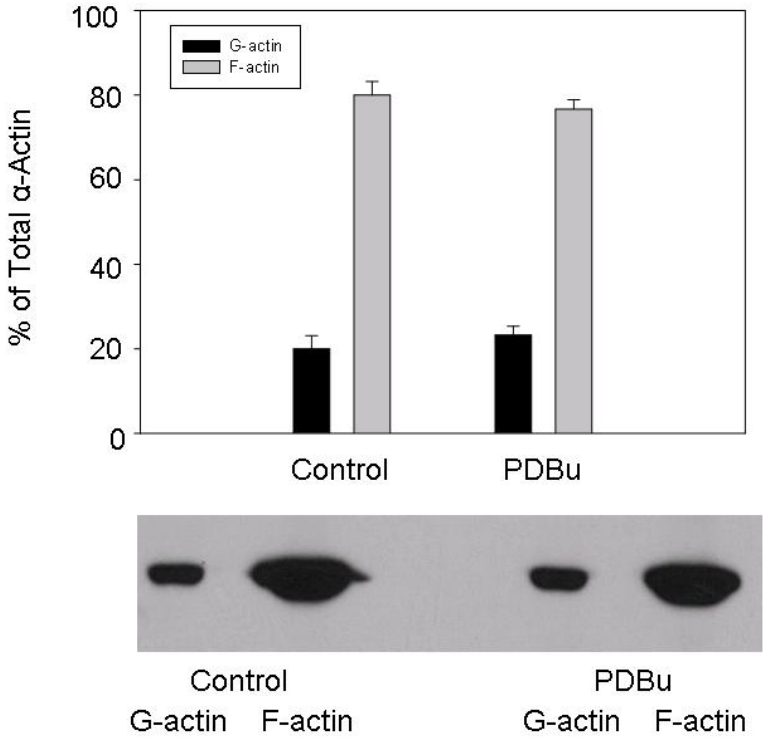

Fig. 4. Western blot analysis of monomeric (G-actin) and filamentous (F-actin) α-actin in unstimulated (control) and PDBu-treated A7r5 cells. Cells were lysed and, following centrifugation, protein from the pellet and supernatant were separated on 12.5% SDS-PAGE. Blots were probed with monoclonal anti-actin α-smooth muscle primary antibody followed by anti-mouse, horseradish peroxidase-linked secondary antibody. Values were calculated as percent of total α-actin protein. Bars indicate the average of 6 independent experiments.

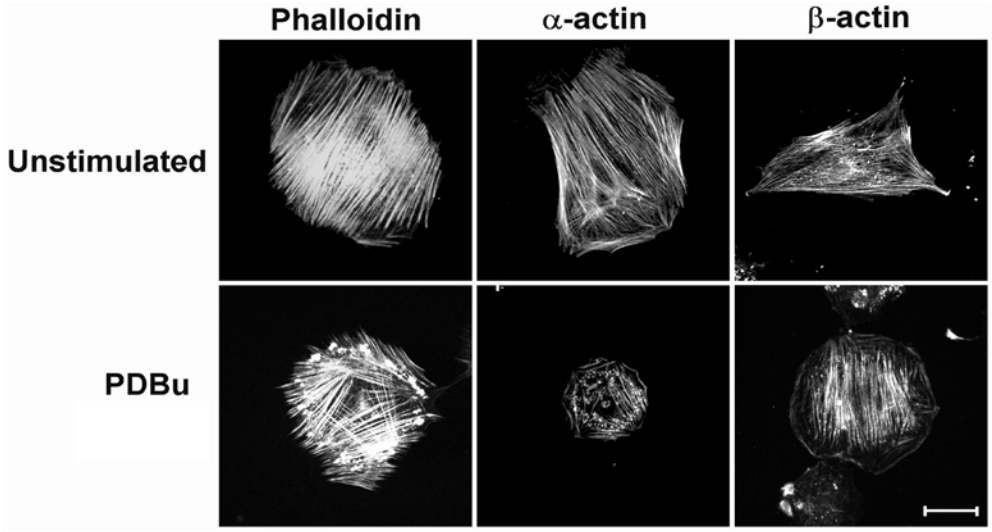

Fig. 5. The reorganization of α- and β-actin in unstimulated and PDBu (5 x 10^{-8} mol/L) stimulated control cells. Cells were stained with FITC-labeled phalloidin, monoclonal α-smooth muscle actin, clone 14A FITC-labeled antibody, or a monoclonal anti-β-actin, clone AC-15 primary antibody followed by an Alexa 488-labeled secondary antibody. The results demonstrate the loss in stress fibers and localization of α-actin at podosomes. By comparison, β-actin is retained in stress fibers that shorten. Bar represents 50 μm.

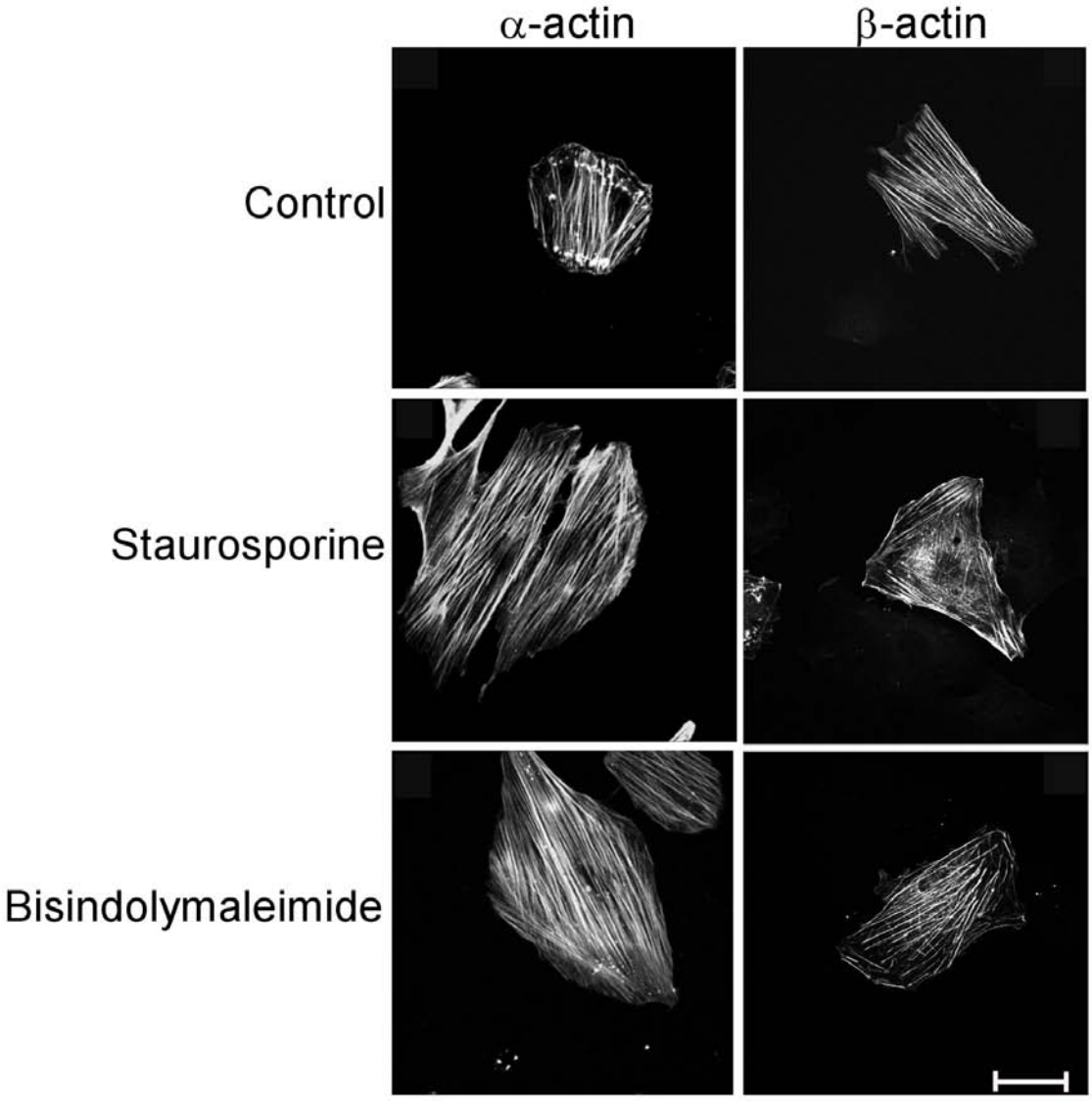
α-actin
β-actin
Control
Staurosporine
Bisindolymaleimide

Figure 6

Fig. 6. Changes in α- and β-actin structure resulting from the addition of PKC inhibitors, staurosporine and bisindolymaleimide, 30 min prior to PDBu (5×10^{-8} mol/L) stimulation. α-Actin was imaged using a monoclonal α-smooth muscle actin, clone 14A FITC-labeled antibody. β-actin was imaged using a monoclonal anti-β-actin, clone AC-15 primary antibody followed by an Alexa 488-labeled secondary antibody. Inhibitors or vehicle was added 30 min prior to PDBu and the cells were fixed for staining 30 min after PDBu. The results indicate that inhibitors blocked PDBu-induced losses in α-actin stress fibers and formation of podosomes. By comparison, staurosporine caused a significant loss in β-actin structure. Bar represents 50 μm.

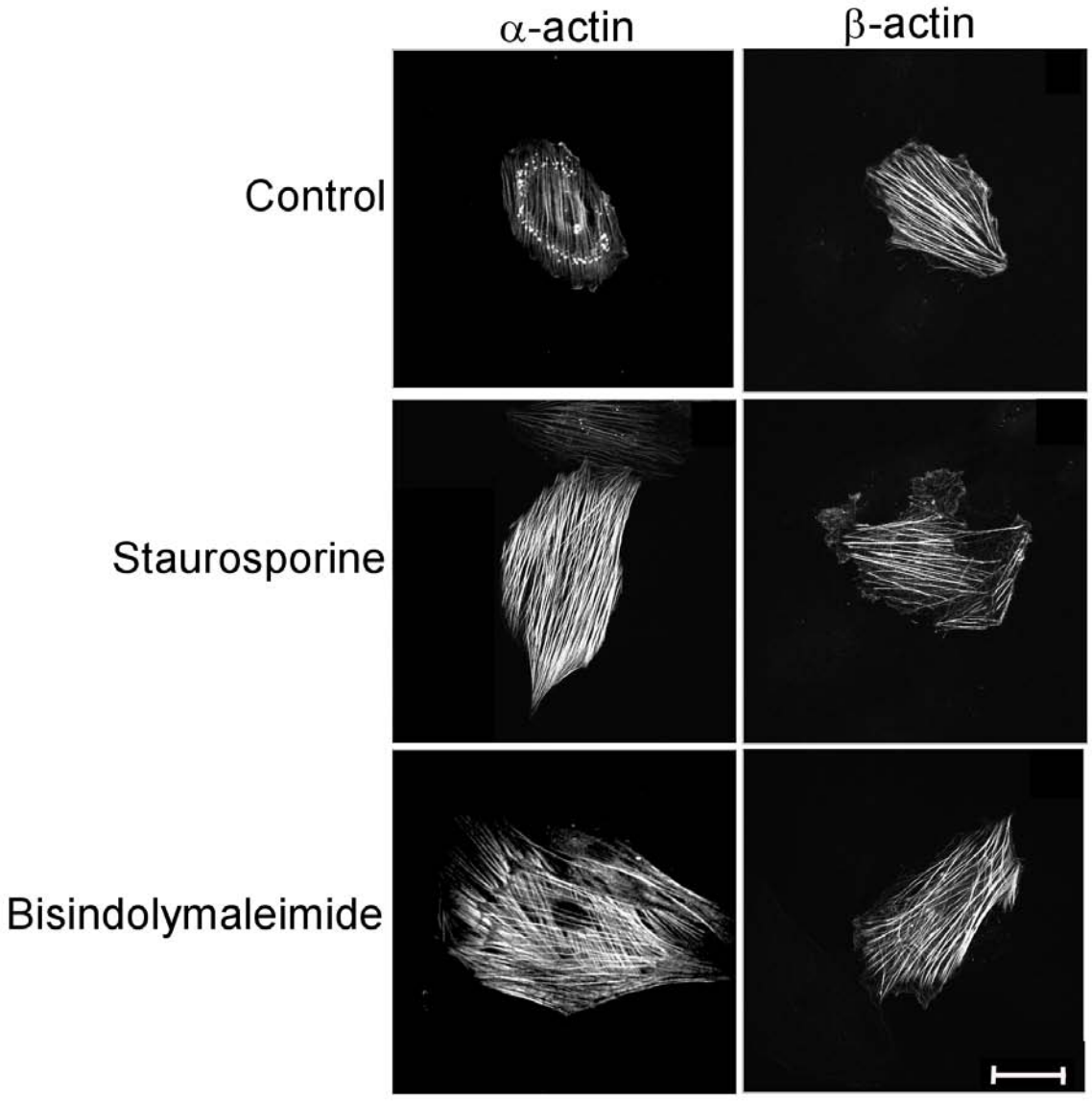
α-actin
β-actin
Control
Staurosporine
Bisindolymaleimide

Figure 7

Fig. 7. Changes in α- and β-actin structure resulting from the addition of PKC inhibitors, staurosporine and bisindolymaleimide, after stimulation of cells with 5×10^{-8} mol/L PDBu and the formation of podosomes. Inhibitors were added 30 min after PDBu and cells were fixed for staining 30 min after inhibitor addition. α-Actin was imaged using a monoclonal α-smooth muscle actin, clone 14A FITC-labeled antibody. β-Actin was imaged using a monoclonal anti-β-actin, AC-15 primary antibody followed by an Alexa 488-labeled secondary antibody. The results indicate that inhibitors reversed podosome formation with re-establishment of α-actin stress fiber structure. By comparison, both inhibitors caused significant losses in β-actin stress fiber structure. Bar represents 50 μm.

Table 1. Comparison of α-actin vs. β-actin filamentous structure in the A7r5 cell.

	α-Actin	β-Actin
Control	147.7 ± 12.1	73.0 ± 3.1
PDBu	43.8 ± 5.7*	77.5 ± 4.5
% Change	-70.4	+6.1

Note: Confocal microscopy line scan analysis of actin filamentous structure in control and PDBu-stimulated (5×10^{-8} mol/L) cells. Results are presented as fluorescence intensity/pixel number (*I/p*). A minimum of 10 cells were evaluated in each group. $*p \leq 0.05$ vs. control.

Table 2. The ratio of cellular F-actin to G-actin obtained from whole cell fluorescence intensity measurements of A7r5 cells simultaneously stained with phalloidin (F-actin) and DNase I (G-actin).

	F-Actin	G-Actin	Ratio (F/G)
Control	100.0 ± 6.5	100.0 ± 8.4	1.68 ± 0.12
PDBu (10^{-7} mol/L)	55.1 ± 4.1*	85.9 ± 11.2	1.08 ± 0.05*
PDBu (10^{-5} mol/L)	74.5 ± 5.0*	106.1 ± 9.4	1.11 ± 0.04*

Note: Prior to fixation for staining, cells were unstimulated (control) or were contracted by addition of 10^{-7} mol/L or 10^{-5} mol/L phorbol 12, 13-dibutyrate (PDBu). Only those PDBu-treated cells showing robust podosome formation were selected for evaluation. A minimum of 6 cells were studied in each group. F- and G-actin values are presented as percent of control. Ratios were calculated by dividing pixel counts from phalloidin fluorescence measurements by those from DNase I measurements. *$p < 0.05$ vs. control.

Table 3. A comparison of the effects of protein kinase C (staurosporine, bisindolymaleimide) inhibitors on PDBu-induced reorganization of α- and β-actin in A7r5 cells.

Inhibitor	Before PDBu	After PDBu
(A) α-Actin		
Staurosporine	30.2 ± 2.0*	9.1 ± 3.1*†
Bisindolymaleimide	4.3 ± 1.6*	12.2 ± 3.3*
Pooled Control	100.0 ± 3.5	100.0 ± 2.8
(B) β-Actin		
Staurosporine	71.1 ± 5.4*	74.6 ± 7.3*
Bisindolymaleimide	91.4 ± 2.8	74.5 ± 4.2*
Pooled Control	100.0 ± 5.8	100.0 ± 4.3

Note: Inhibitors were added 30 min before stimulation with PDBu (5 x 10^{-8} mol/L) to evaluate their effect on active cytoskeletal remodeling or 30 min after PDBu to evaluate their effect on PDBu-induced changes in remodeled structure. Results for α-actin are based on cell counts indicating the percent of cells showing PDBu-stimulated podosome formation. Results for β-actin are based on cell line scans measuring fluorescence intensity as an index of β-actin stress fiber structure. In each case, values are expressed as percent of values obtained from PDBu-stimulated inhibitor vehicle controls. *p < 0.05 vs. control, †p < 0.05 before vs. after PDBu.

Discussion

In previous work, we showed that the reorganization of the 2 major isoforms of actin was markedly different in response to low concentration (10^{-8} mol/L) phorbol ester stimulation and contraction in A7r5 smooth muscle cells (Fultz et al. 2000). α-Actin was observed to reform from stress fibers into peripheral bodies now identified as podosomes (Hai et al. 2002), whereas β-actin remained in stress fibers that shortened during the contraction of the cell. Differences in the modes of actin isoform remodeling could be important for implications regarding regulatory control and function in smooth muscle. However, a more recent report (Gimona et al. 2003) has demonstrated that at high phorbol ester concentration, β-actin also localizes at podosomes. The present results confirm these previous findings and further indicate a marked dichotomy in the concentration-response of actin isoform reorganization to PDBu. α-Actin showed robust podosome formation with a maximum in the percentage of cells exhibiting these structures observed at 10^{-7} mol/L PDBu. By comparison, PDBu concentrations at 10^{-6} mol/L or greater were required for similarly robust relocation of β-actin at podosomes (Fig. 2). We suggest 2 possible explanations of these results. Evidence has been reported suggesting that podosome formation is mediated through activation of PKCα (Gatesman et al. 2004). It has been further shown that PKCα exhibits a PDBu concentration-dependent translocation either to the subplasmalemma (10^{-7} mol/L) or to the perinuclear region (>10^{-7} mol/L) in A7r5 cells (Li et al. 2001*b*). Hence, differences in PDBu concentration-dependent localization of actin isoforms could reflect differences in the localization of the regulatory machinery controlling α- and β-actin structure.

Alternatively, increasing concentrations of PDBu could result in a higher level of PKC activation or the activation of additional regulatory pathways. PDBu has been shown to bind and activate conventional and novel PKC isozymes with high affinity (K_d, 10^{-9} mol/L) (Kazanietz et al. 1993) that leaves little reason to believe that concentrations ranging at 10^{-6} mol/L would be required for full activation of the enzyme. Moreover, our work has shown that cells stimulated at 10^{-8} mol/L PDBu may contract profoundly, with shortening of β-actin fibers to the point that the resolution of individual fibers is lost in the absence of β-actin localization at podosomes (Fultz et al. 2000). On the other hand, there is substantial evidence that phorbol esters at high concentration may utilize other receptors than PKC (Kazanietz 2000) and activate PKC-independent pathways (Rapuano and Bockman 1997). Hence, whereas the physiological significance of biological responses at high concentrations of phorbol ester is uncertain, the results raise the interesting possibility that α- and β-actin contractile remodeling is regulated by different pathways.

Consistent with previous observations of PDBu-induced stress fiber loss, quantification of whole cell fluorescence in cells simultaneously stained with phalloidin (F-actin) and DNase I (G-actin) indicated a significant decrease in the ratio of filamentous to monomeric actin in PDBu-treated cells (Table 2). However, this change occurred without significant increases in G-actin, suggesting there was no net depolymerization of actin despite the obvious loss of stress fibers in these cells. A possible explanation is the net incorporation of stress fiber actin into the tightly packed filaments forming the podosome core (Gimona et al. 2003), which would be expected to stain less completely than stress fibers. Consistent with this idea, Western blot analysis

indicated effectively identical F-actin content in PDBu-treated (76.7% ± 2.2%) compared with unstimulated control cells (80.0% ± 3.2%). How such a translocation of actin to podosomes occurs is not certain. Verkhovsky et al. (1997) has described actin polarity sorting with direct translocation of filaments to podosome-like structures in contracted fibroblasts. However, more recent evidence suggests that podosome formation is initiated by de novo actin polymerization at the stress fiber/focal adhesion interface (Kaverina et al. 2003). Interestingly, we observed the redistribution of G-actin to the periphery and about the podosomes in PDBu-treated cells (Fig. 3). A likely explanation of this pattern of localization is that the podosomes are focal points for high rates of actin filament turnover. Because of evidence suggesting that phorbol-induced podosome formation is mediated by PKC (Gatesman et al. 2004; Hai et al. 2002), we investigated the effects of selected protein kinase inhibitors on α- and β-actin structure when added either before or after 10^{-7} mol/L PDBu. Two PKC inhibitors were studied: staurosporine, an inhibitor of PKC (Chabannes et al. 2001; Matsumoto and Sasaki 1989) that may affect various serine/threonine kinases at high concentration (Peet and Li 1999) and bisindolymaleimide I, a selective inhibitor of PKC (Toullec et al. 1991). Both staurosporine and bisindolymaleimide have been shown to suppress force development in smooth muscle (Wright and Hurn 1994; Yamamoto et al. 1995; Yang et al. 2001). Inhibition of PKC blocked or reversed α-actin localization at podosomes leaving stress fibers intact (Table 3A), which is consistent with a direct role of PKC in destabilization of stress fibers and the stabilization of podosome formation. Unlike the effect of the inhibitors to stabilize α-actin stress fibers, these compounds caused detectable losses in β-actin stress fibers (Table 3B), particularly when added after PDBu stimulation. This

suggests that PKC contributes, at least in part, to the stability of β-actin stress fiber structure during PDBu stimulation.

In summary, lack of similarity in the organizational response of α- and β-actin to different concentrations of PDBu or the introduction of protein kinase inhibitors indicates these isoforms could be subject to different regulatory influences. In turn, this raises the possibility that the 2 isoforms could serve different functions in the contracting smooth muscle cells. In the present study we demonstrate reversal of podosome formation and re-establishment of α-actin stress fibers with partial loss of β-actin stress fiber structure in the presence of PKC inhibitors. These results suggest that the destabilization of stress fibers and transport and (or) maintenance of α-actin at podosomes is actively regulated by PKC. In contrast, the stability of β-actin stress fiber structure is at least partially dependent on PKC activity during PDBu-induced cytoskeletal remodeling.

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Chapter III

Role of the Kinase Domain of Myosin Light Chain Kinase in Actin Organization in the Contracting A7r5 Cell

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Abstract

Previous work in our laboratory has suggested a role for myosin light chain kinase in the crosslinking of actin filaments (Thatcher *et al*., 2007). This role for MLCK was attributed to non-kinase domains within the protein. However, the possibility remained that the kinase activity of MLCK and the resulting tension generated in tension bearing structures within the cell could contribute directly to the remodeling of the cytoskeleton during contraction. In the present study, we investigated the role of the kinase domain of MLCK in the crosslinking of actin filaments. Experiments were conducted using two inhibitors of the kinase domain of MLCK, ML-7 and ML-9, added prior to phorbol 12, 13-dibutyrate (PDBu) and after PDBu contraction of cells to determine the effects on active cytoskeletal remodeling and on PDBu-induced changes in cytoskeletal structure, respectively. The effects of these inhibitors were examined in both α- and β-actin. Inhibition of the kinase domain of MLCK caused a decrease in podosome formation in cells stained for α-actin when added prior to PDBu or after PDBu indicating kinase activity was essential for resting and PDBu-induced α-actin structure. By comparison, the MLCK inhibitors had no effect on β-actin structure when added before PDBu stimulation or after PDBu stimulation. The results allow no conclusions regarding the role of the kinase domain of MLCK in the crosslinking of actin filaments by MLCK. However, the results indicate that MLCK kinase activity is necessary for α-actin but not β-actin cytoskeletal structure during the PDBu-induced contraction.

Introduction

Myosin light chain kinase (MLCK) is a 130-150 kDa protein with a primary function in smooth muscle of regulating contraction (Stull *et al*., 1998). MLCK is activated by Ca^{2+}/calmodulin (CaM) binding to specific sites on the protein (Kamm and Stull, 1985). Once activated, MLCK phosphorylates the regulatory light chain of myosin thereby activating the myosin ATPase and allowing the contraction to occur (Aksoy *et al*., 1976; Chacko *et al*., 1977; DiSalvo *et al*., 1978; Gorecka *et al*., 1976; Ikebe *et al*., 1977; Sobieszek 1977).

More recently, MLCK has been found to play a role in bundling actin filaments (Hayakawa *et al*., 1994). MLCK contains two sites within its N-terminal region that bind to actin (Ye *et al*., 1997) and can bind two different actin filaments to cross-link the actin filaments into bundles (Hayakawa *et al*., 1994). The actin binding site between amino acids 1-41 overlaps with a Ca^{2+}/CaM binding site that regulates the interaction of MLCK with actin at this site (Olson *et al*., 1990; Ye *et al*., 1997; Gao *et al*., 2001) and has been referred to as the Ca^{2+}/CaM sensitive binding site (Ye *et al*., 1997). The second actin binding site is found between amino acids 138-218 (Ye *et al*., 1997; Gao *et al*., 2001). Because this site is not affected by binding of Ca^{2+}/CaM, it is referred to as the Ca^{2+}/CaM insensitive site (Ye *et al*., 1997; Gao *et al*., 2001). In the presence of Ca^{2+}/CaM, actin is released from the Ca^{2+}/CaM sensitive site, which then allows it to interact with myosin in the contractile process.

Previous work in our laboratory has indicated that MLCK is associated with both α- and β-actin isoforms (Thatcher *et al*., 2007). MLCK was found to be closely associated with α-actin along stress fibers in control cells as well as in podosomes of

PDBu-induced contracted cells. MLCK was associated with β-actin stress fibers in control cells and continued to be associated with β-actin in a diffuse manner around the perinuclear region of the cell upon contraction of the cell with PDBu. Taken together, this data indicates interaction of MLCK with both actin isoforms known to be present in the A7r5 cell line, not merely the α-actin isoform thought to be involved in contraction of smooth muscle cells.

The focus of this earlier work (Thatcher *et al*., 2007) centered on the effects of inhibiting the N-terminal region of MLCK on actin organization. Through downregulating MLCK using siRNA or by the addition of peptide containing amino acids 1-41 of MLCK either by microinjection or by peptide-mediated uptake, inhibition of the N-terminal region of MLCK caused dissolution of α-actin stress fibers in the central region of control A7r5 cells, while cells induced to undergo contraction by the addition of phorbol 12, 13-dibutyrate (PDBu) exhibited a 70% reduction in podosome formation. β-actin was found to disassemble with diffuse distribution of this isoform throughout the cell when N-terminal inhibitors were introduced into control cells and PDBu treated cells.

The present study examines the role of the kinase domain of MLCK in the structural organization of α- and β-actin in control and PDBu treated A7r5 cells. The inhibitors ML-7 and ML-9, which inhibit MLCK by binding at the kinase domain, were utilized to study the impact on actin cytoskeletal structure. α- and β-Actin structure were examined with inhibitors added alone to determine the effects on control cells as well as cells in which the inhibitors were added prior to and after the addition of PDBu to induce contractile cytoskeletal remodeling. The idea behind the study was to determine if the inhibition of kinase activity leading to changes in the internal tension within the cell had

similar effects on actin structure to that seen during inhibition of N-terminal actin binding domains.

Materials and Methods

Cell Culture

A7r5 smooth muscle cells, derived from embryonic rat aorta and shown to maintain the ability to contract to phorbol esters (Fultz et al. 2000; Nakajima et al. 1993), were obtained from American Type Culture Collection (Manasass, Va.). Cells were plated on 75-cm^2 flasks and grown to approximately 85% confluence at 37^oC in a humidified atmosphere of 5% CO_2 in air. The cells were maintained in Dulbecco's modified Eagles medium (DMEM) supplemented with 10% fetal bovine serum, 100 U/mL penicillin G, and 100 μg/mL streptomycin. Media was changed every other day and cells were passaged at least once a week. Passaging was accomplished by addition of trypsin-EDTA solution in PBS and collection of cells by centrifugation.

Immunocytochemistry and Confocal Microscopy

In experiments employing immunostaining, cells were seeded onto glass coverslips, placed in 6-well culture plates and returned to the incubator for a minimum of 24 h to allow for attachment and spreading. After experimental treatment, cells were fixed and permeabilized by addition of ice cold acetone for 1 min. The cells were washed with PBS containing 0.5% Tween-20 (PBS-T), pH 7.5, followed by a 30-min incubation in blocking solution containing 5% nonfat dry milk in PBS. α-Actin staining was accomplished by incubation of cells in a 1:500 dilution of monoclonal-α-smooth muscle actin, clone 1A4 FITC-labeled antibody (Sigma Chemical Co., St. Louis, Mo.) for 60 min at room temperature. In order to visualize β-actin, a β-actin-EGFP expression plasmid was purchased from Clontech (Palo Alto, CA). The green fluorescent protein-

actin fusion protein has been successfully utilized in a wide range of cell types (Aizaws *et al.*, 1997; Faix *et al.*, 1998; Noegel and Schleicher, 2000; Okada *et al.*, 1999) and has been shown to be a suitable probe for the study of actin organization and dynamics in several mammalian cell lines (Choidas *et al.*, 1998). Cells were transfected in culture flasks with 2-6 μg of plasmid using lipofectamine (Life Technologies, Rockville, MD) according to the manufacturer's standard protocol. Fluorescence of the β-actin-GFP fusion protein was typically observed within 2 days and experiments were performed within 3 or 4 days after transfection. Cells were imaged by mounting on a Nikon Diaphot Microscope and confocal microscopy performed with a Bio-Rad Model 1024 scanning system with a Krypton/Argon laser. Micrographs were constructed by projection of Z-plane acquisitions and analyzed by Lasersharp and Confocal Assistant Software (BioRad, Hercules, Calif.).

Cell Treatments and Analysis of Actin Remodeling

MLCK inhibitors ML-7 and ML-9 were added at a final concentration of 10^{-5}, 10^{-6}, 10^{-7}, or 10^{-8} M. The effect of the compounds were evaluated in their ability to alter α-actin and β-actin structure both during PDBu-stimulated (10^{-7} M) reorganization and after PBDu-induced reorganization was established. For the former experiments, an inhibitor was added 30 min before PDBu with the cells fixed for imaging 30 min after PDBu stimulation. For the latter, the inhibitor was added 30 min following PDBu with cells fixed for imaging at 30 min after the introduction of inhibitor. Vehicle-control cells were fixed at identical intervals as the inhibitor-treated cells.

To estimate losses in α- and β-actin stress fibers, confocal images of treated and control cells were obtained at fixed confocal settings and analyzed using Image J

software (NIH). Using the line scan tool, the cell was bisected in the long axis at mid-cell and the resulting plot was imported into Microsoft Office Excel (Fig. 1). The point of lowest intensity was selected as baseline and the graph was then analyzed using Peakfit 4.0 software to determine the area under the curve (I = total intensity). This value was then divided by line pixel number (p) to normalize for line length. The final value, β-actin filament intensity = I/p was calculated using Excel.

Cell counts were conducted on α-actin stained cells to determine changes in podosome formation over the various concentrations and treatment groups. In each treatment group, a minimum of 100 cells were counted in total noting the number of cells that exhibited podosome formation. The counts were reported as a percentage of cells that exhibited podosome formation. Cell counts were not conducted on β-actin cells. Previous work (Brown *et al*., 2006) has indicated that cells treated with PDBu at a concentration of 10^{-7} M and stained for β-actin or transfected with the β-actin construct exhibited podosomes in less than 20% of the cells. For this reason, cell counts were not conducted, but rather stress fiber loss was evaluated.

Data Analysis

Analysis of differences between treatment means was performed by 1-way ANOVA followed by Student's unpaired t test (Sigma Stat, SPSS, Chicago, Ill.). Differences between means were considered significant if $p \leq 0.05$. Averaged results are presented as mean ± SE throughout the text.

Results

Previous work has revealed a marked difference in the response of α- and β-actin to PDBu stimulation at a concentration of 10^{-7} M (Figure 1) (Fultz *et al*., 2000; Brown *et al*., 2006). In control cells, both α- and β-actin are present in stress fibers that run the length of the cell. Upon stimulation with PDBu, α-actin is found to lose stress fiber structure with the majority of the stress fibers remodeling into podosomes around the periphery of the cell. β-actin does not remodel into podosome structures but rather the stress fibers appeared to shorten with the change in the size of the cell.

MLCK inhibitors were added prior to or after PDBu stimulation to evaluate their effect on cytoskeletal remodeling or their effect on PDBu-induced changes in remodeled structure, respectively. In order to examine the effects of ML-7 and ML-9 on α-actin cytoskeletal structure, podosome formation was evaluated (data not shown). In cells treated with inhibitors prior to PDBu stimulation, the percentage of cells exhibiting podosomes decreased (Figure 2). The same trend was seen in cells treated with the MLCK inhibitors after stimulation with PDBu (Figure 3). These results suggest that ML-7 and ML-9 prevent podosome formation when added prior to PDBu stimulation and reverse podosome formation when the inhibitors were added after PDBu stimulation.

To evaluate the effects of the inhibitors on β-actin stress fiber structure, filament intensity of the cells was determined using line scan analysis (Table 1). In all treatment groups except ML-7 at a concentration of 10^{-8} M, no significant differences were found in the β-actin stress fiber structures when inhibitors were added before or after PDBu

stimulation (Figure 4 and 5). It is unclear why 10^{-8} M ML-7 would result in a loss in β-actin structure when added both before and after PDBu. The K_i for ML-7 is 300 nM and 10^{-8} M of the inhibitor would fall well below this concentration suggesting the results reflect experimental artifact.

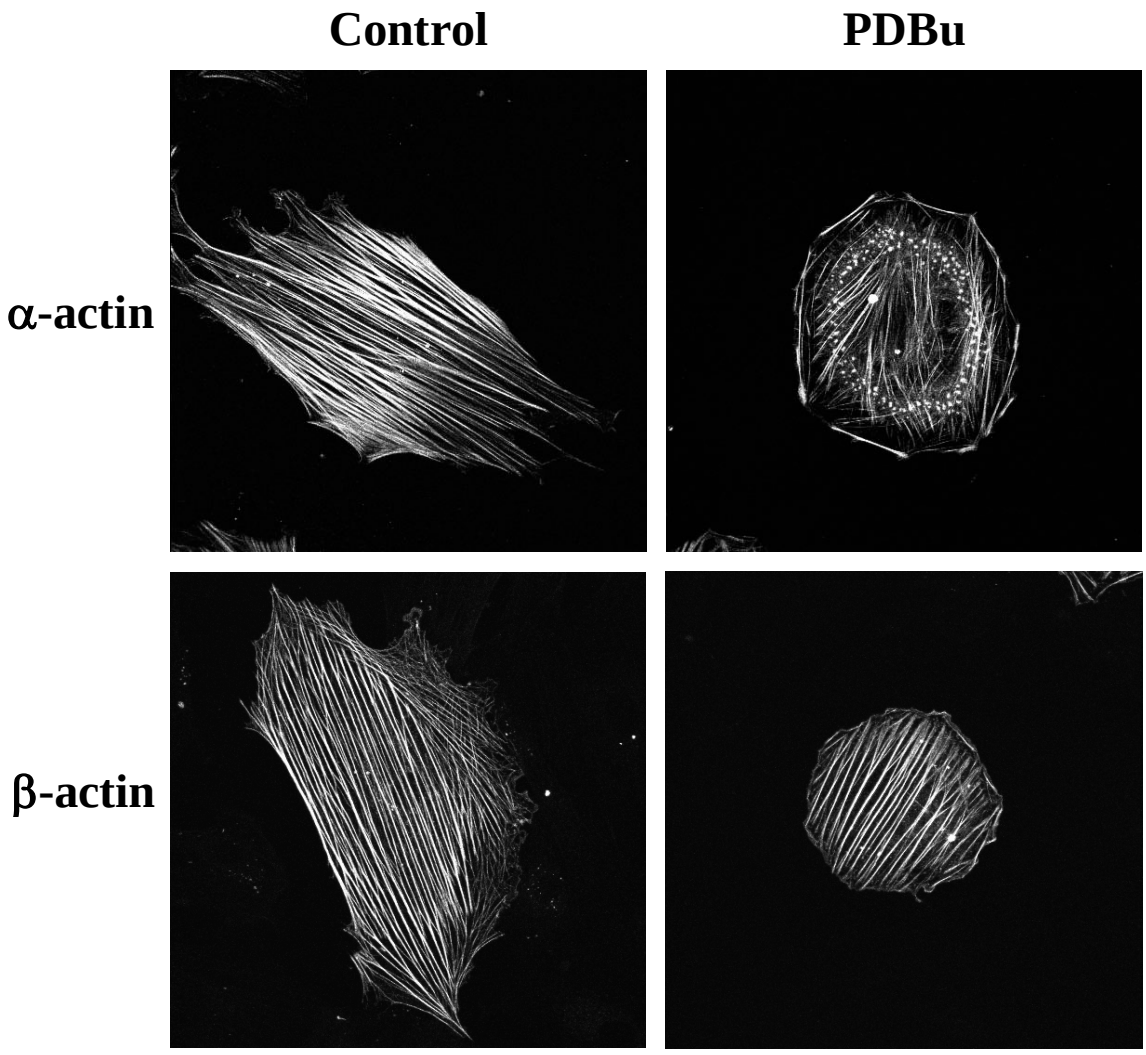

Figure 1. Comparison of control and PDBu treated cells exhibiting α-actin or β-actin. Control cells stained for α-actin exhibit numerous stress fibers running the length of the cell. α-Actin stained cells treated with PDBu (10^{-7} M) exhibit robust podosome formation around the periphery of the cell with loss of stress fiber structure. β-Actin control cells also exhibit numerous stress fibers running the length of the cell. Cells treated with PDBu (10^{-7} M) continue to show β-actin stress fibers running the length of the cell without the presence of podosome formation.

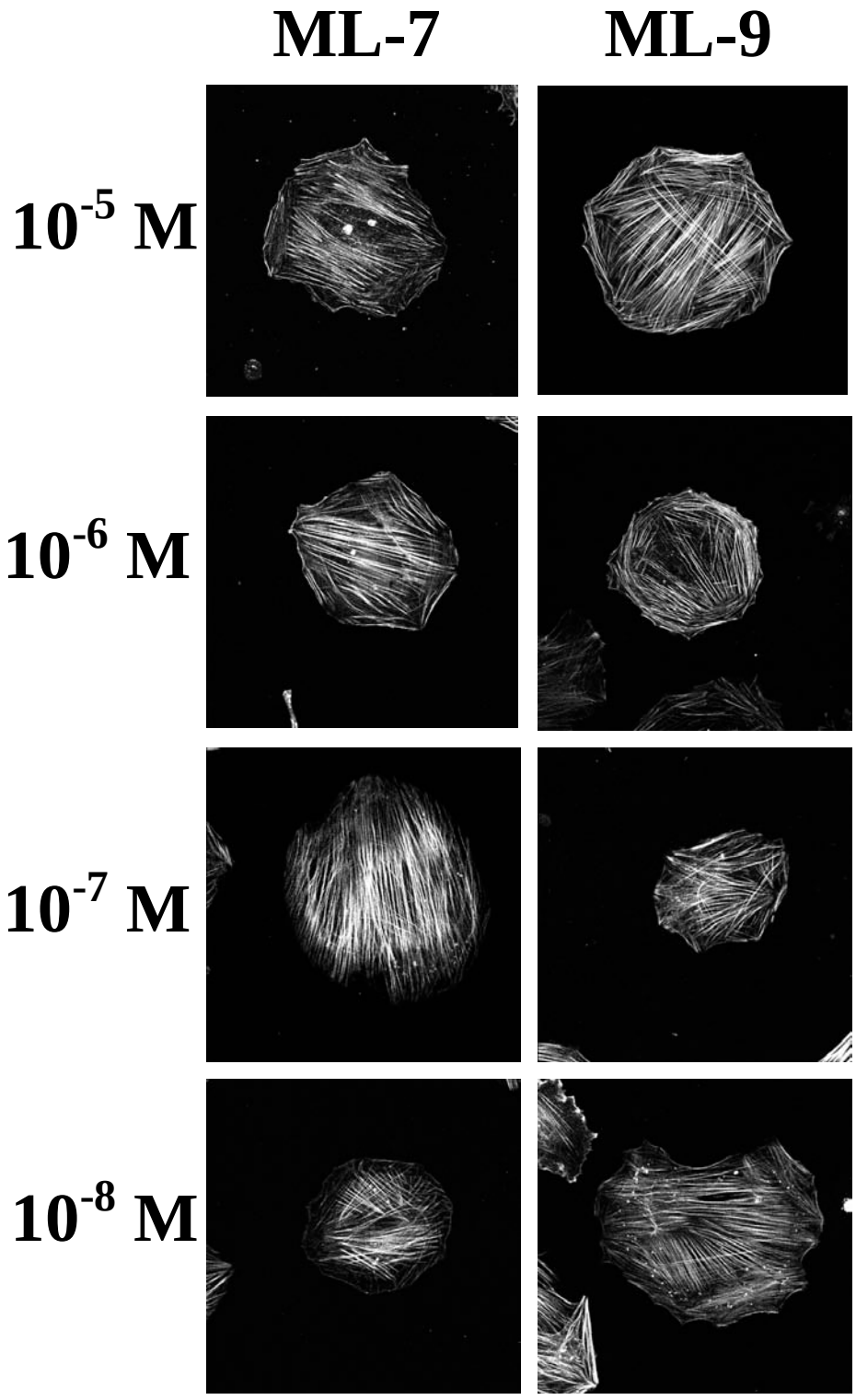

Figure 2

Figure 2. Cells stained for α-actin and treated with the MLCK inhibitors ML-7 or ML-9 30 minutes prior to PDBu stimulation. There is a trend for a decrease in podosome formation within these cells suggesting the prevention of podosome formation.

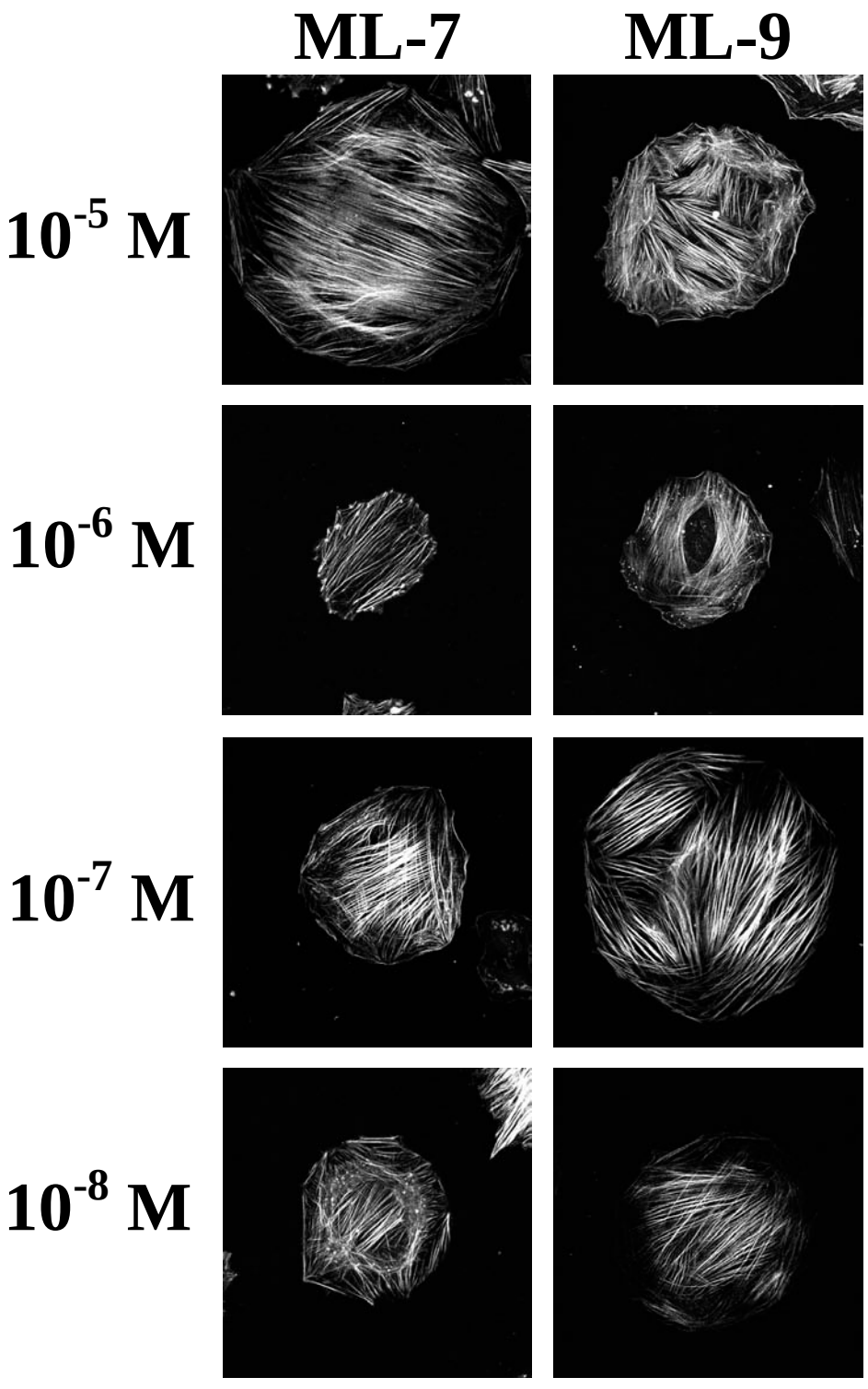

Figure 3

Figure 3. Cells stained for α-actin and treated with the MLCK inhibitors ML-7 or ML-9 for 30 minutes following PDBu stimulation. There is a trend for a decrease in podosomes in these cells suggesting a reversal in podosome formation.

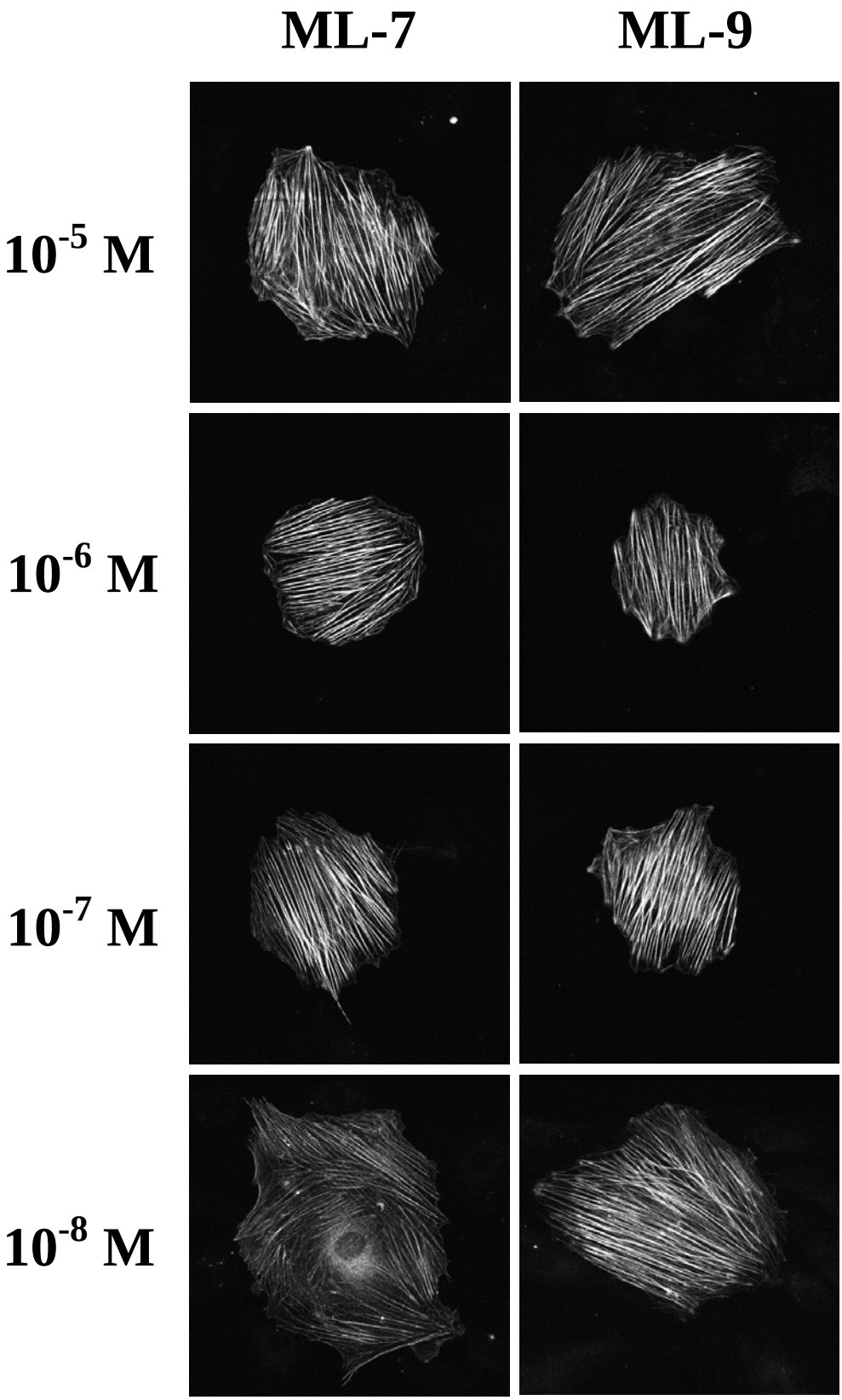

Figure 4

Figure 4. Cells stained for β-actin and treated with the MLCK inhibitors ML-7 or ML-9 for 30 minutes prior to PDBu stimulation. As indicated in table 1, with the exception of 10^{-8} M treatment, ML-7 had no effect on β-actin stress fibers.

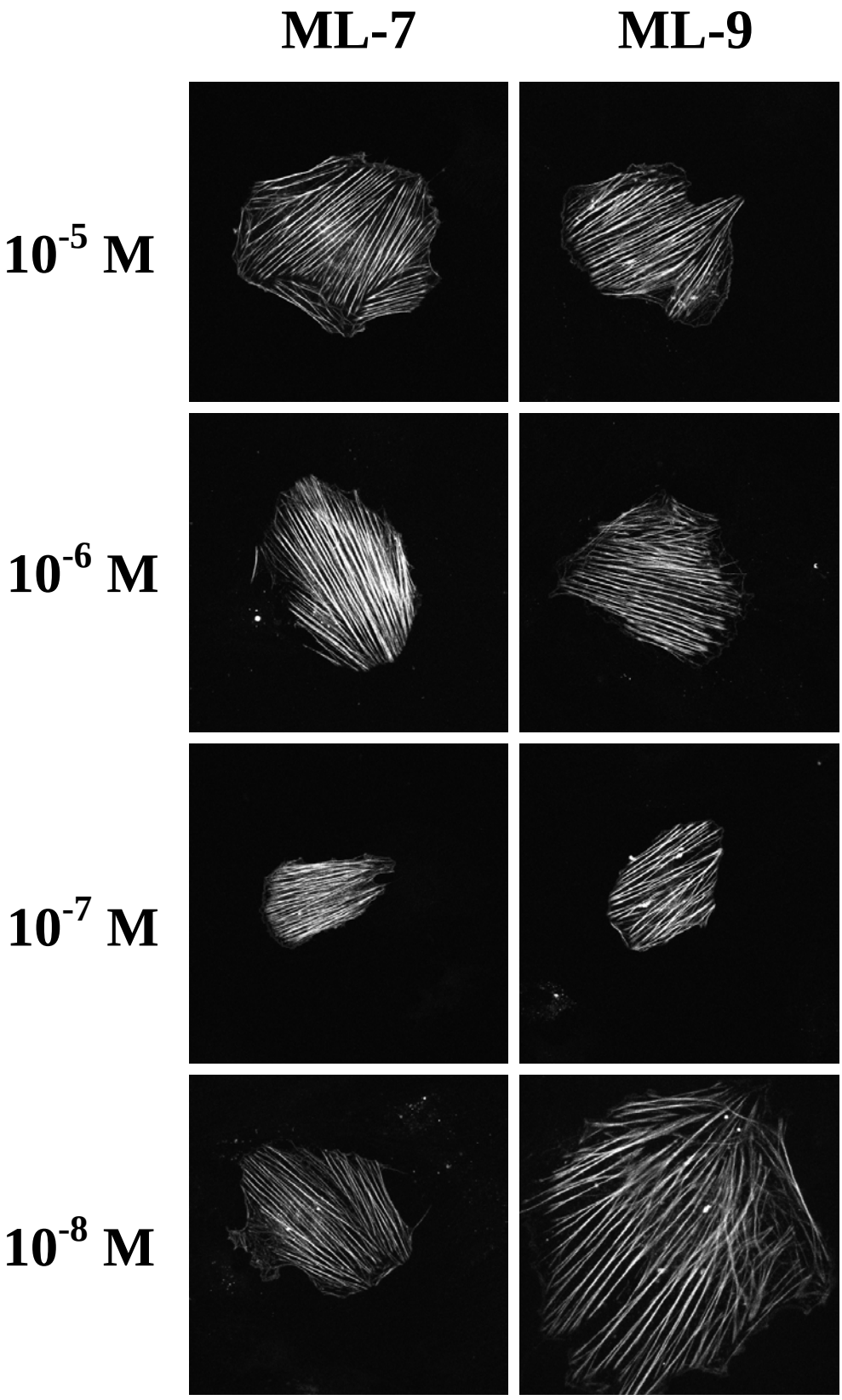

Figure 5

Figure 5. Cells stained for β-actin and treated with the MLCK inhibitors ML-7 or ML-9 for 30 minutes after PDBu stimulation. As indicated in table 1, only with the exception of 10^{-8} M treatment, ML-7 had no effect on β-actin stress fibers.

Table 1. A comparison of the effects of ML-7 and ML-9 at varying concentrations on β-actin structure of A7r5 cells when added before or after stimulation with PDBu.

	Before PDBu	**After PDBu**
Pooled Control	100.0 ± 2.3	100.0 ± 2.3
PDBu	93.3 ± 2.2	93.3 ± 2.2
ML-7 10^{-8} M	84.6 ± 2.8*	84.6 ± 2.5*
ML-7 10^{-7} M	95.2 ± 2.5	101.6 ± 3.5
ML-7 10^{-6} M	107.4 ± 3.1	108.1 ± 3.7
ML-7 10^{-5} M	106.6 ± 3.3	101.8 ± 3.2
ML-9 10^{-8} M	92.9 ± 3.1	96.9 ± 3.8
ML-9 10^{-7} M	95.1 ± 3.0	106.4 ± 3.0
ML-9 10^{-6} M	92.2 ± 3.0	89.9 ± 2.6
ML-9 10^{-5} M	90.8 ± 3.2	92.0 ± 2.9

Note: Inhibitors were added 30 min before stimulation with PDBu (10^{-7} M) to evaluate their effect on active cytoskeletal remodeling or 30 min after PDBu to evaluate their effect on PDBu-induced changes in remodeled structure. Results are based on cell line scans measureing fluorescence intensity as an index of β-actin stress fiber structure. Values are expressed as percent of values obtained from PDBu-stimulated inhibitor vehicle controls. * $p < 0.05$ vs. pooled control

Discussion

The concept of a dual role of MLKC in smooth muscle contraction was a novel hypothesis first proposed by our laboratory. In addition to its kinase properties enabling force development through the interaction of myosin and actin filaments, we proposed that MLCK actin binding could maintain the actin/myosin contractile machinery in opposition in the resting cell and release tension bearing actin filaments upon activation of MLCK, allowing sliding filament dynamics and contractile remodeling. Because of the novelty of this hypothesis very little information is available in the literature.

In initial studies examining MLCK actin binding (Thatcher *et al*., 2007), MLCK was shown to be closely associated with both α- and β-actin in control and PDBu-treated cells by means of colocalization and fluorescence resonance energy transfer (FRET) analysis. Next, MLCK was downregulated 40-55% with the introduction of MLCK-siRNA. Upon downregulation of MLCK, cells demonstrated a loss in stress fiber structure and rounding of the cell. In cells stained with α-actin, the central region of the cells were devoid of stress fibers, while the periphery of the cells demonstrated a network of α-actin stress fibers. β-Actin stained cells, on the other hand, generally lacked stress fibers all together with β-actin forming a network in the perinuclear region of the cell. Cells treated with PDBu demonstrated both isoforms relocating to podosomes around the periphery of the cell in negative control cells. However, cells treated with MLCK-siRNA showed a nearly 70% reduction in podosome formation and resembled cells treated with MLCK-siRNA without being stimulated with PDBu.

Another set of experiments were conducted introducing peptides containing sequences overlapping the actin binding domains of MLCK into A7r5 cells to

competitively inhibit actin binding. Two sets of peptides were used. One set contained the amino acids 1-41, while the other set only contained the amino acids 1-25. The second set of peptides had no effect on control or PDBu treated cells stained for α- actin. However, introduction of the 1-41 peptides into cells either by microinjection or by peptide-mediated uptake caused the same results found in the MLCK-siRNA experiments. This work indicated the Ca^{2+}/CaM insensitive actin binding site of MLCK showed to cause crosslinking of actin stress fibers played a role in determining actin structure. The crosslinking role of MLCK appears to be important in maintaining the actin structure in resting cells possibly by stabilizing the actin stress fibers. It is thought that during Ca^{2+}-dependent contraction MLCK releases actin at the Ca^{2+}/CaM sensitive binding site to allow actin to be moved by myosin contributing to force development. In a Ca^{2+}-independent contraction, such as that produced by phorbol, actin is not released from MLCK, which may be important to remodeling of actin during PDBu induced contractions. In any case, the results from Thatcher *et al.*, (2007) provided compelling evidence that actin filament binding by MLCK plays a major role in actin structural arrangement in the cell before and during contraction by PDBu.

While our early results indicated MLCK contributes to the stabilization and remodeling of actin in control and PDBu treated cells, whether this effect was due exclusively to non-kinase properties of MLCK was not certain. As mentioned by Thatcher *et al.*, (2007), it is possible that a loss in basal kinase activity of MLCK could lead to loss of stress fiber structure if MLCK is responsible for maintaining some internal strain in the cell. Chrzanowska-Wodnicka and Burridge (1996) have found stress fiber formation in the presence of internal strain. Therefore, it was of interest to determine if

inhibition of MLCK kinase activity and possible tension development caused changes in actin cytoskeletal structures similar or different from the results of Thatcher *et al*., (2007). If the results were found to be similar, it would suggest that MLCK binding of actin filaments was essential for tension development and through this mechanism, influenced cytoskeletal structure.

In the current studies, MLCK inhibitors showed a trend in decreasing the number of cells that exhibited α-actin remodeling to podosomes, which is in agreement with the findings in Thatcher *et al*., (2007) where inhibition of the Ca^{2+}/CaM insensitive actin binding site caused a decrease in podosome formation by approximately 70%. The two sets of data taken together suggest that the crosslinking of α-actin by MLCK combined with tension development could influence α-actin remodeling during PDBu-induced contraction.

However, inhibition of the kinase domain had no consistant effect on β-actin, structure. This result differs from the results of Thatcher *et al*., (2007) concerning β-actin and the inhibition of the Ca^{2+}/CaM insensitive actin binding site which caused dissolution of β-actin fibers resulting in diffuse staining of the cell for β-actin. And while the interaction of MLCK and α-actin could play an important role in tension development and cytoskeletal remodeling during PDBu-induced contraction, it appears that the interaction of MLCK and β-actin does not require kinase activity or tension development for structural integrity.

The results taken together seem to point to a role for the crosslinking of α-actin by MLCK in tension development and cytoskeletal remodeling in contraction of the A7r5

cell. Thatcher *et al*. (2007) suggests a model for the crosslinking of actin by MLCK and the role it plays in Ca^{2+} dependent smooth muscle contraction. The model proposes that MLCK binds to α-actin by the Ca^{2+}/CaM sensitive actin binding site and to β-actin by the Ca^{2+}/CaM insensitive binding site. With the efflux of Ca^{2+}, Ca^{2+} complexes with calmodulin to interact with MLCK causing release of α-actin by MLCK. The release of actin in close association with myosin and simultaneous phosphorylation of myosin allows crossbridge cycling in the absence of sarcomere structure found in other muscle types. The present results suggest this model may be feasible for explaining the interaction of MLCK and α-actin but not β-actin.

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Chapter IV

Summary and Conclusions

General Discussion

Two isoforms of actin have been found to be present in vascular smooth muscle cells. It has long been thought that only the α-actin isoform takes part in the contraction of the cell, while β-actin is present only to support structure within the cell. However, if the concentration of PDBu is high enough, β-actin will remodel into podosomes around the periphery of the cell in the same manner α-actin remodels at much lower concentrations of PDBu. At a concentration of 10^{-7} M PDBu, which activates a physiological level of PKC to cause contraction, α-actin is found to remodel into podosomes in about 70% of the cells, while β-actin was found to remodel into podosomes in only 20% of the cells. Staining the cells with phalloidin to label all actin filaments and DNase I to label globular actin showed a decrease in filamentous actin with no change in globular actin. Examining the amount of filamentous and globular actin in control and PDBu treated cells by Western Blot analysis indicated no change in filamentous actin in PDBu-treated cells, suggesting phalloidin is unable to reach and be detected in the tightly packed actin found in the core of the podosome. This marked dichotomy in actin isoform remodeling suggests differential regulation of the actin isoforms during smooth muscle contraction.

Treating the cells with PKC inhibitors, staurosporine and bisindolymaleimide, prior to and after PDBu stimulation, showed an inhibition of α-actin remodeling to podosomes when added prior to PDBu stimulation and reversed podosome formation

with re-establishment of stress fibers when added after PDBu stimulation. β-actin was evaluated based on stress fiber structure and exhibited a loss in stress fiber structure when the PKC inhibitors were added prior to or after PDBu stimulation. The results from these studies indicate differential isoform reorganization during PDBu contraction, as well as different modes of regulation of the two actin isoforms structure.

Another protein important in smooth muscle contraction is MLCK. MLCK is well known as the enzyme involved in the initiation of smooth muscle contraction in Ca^{2+}- dependent contractions. MLCK has also recently been found to crosslink actin filaments. The crosslinking of actin is considered a non-kinase function of the enzyme. We wanted to determine if the kinase domain and tension development played a role in MLCK binding to actin and contractile remodeling of actin structure by using two inhibitors of MLCK, ML-7 and ML-9. These inhibitors were found to cause a decrease in podosome formation in cells stained with α-actin when added prior to or after PDBu-induced contraction. The use of the inhibitors in cells expressing β-actin did not show a change in β-actin structure when the inhibitors were added prior to or after PDBu stimulation. Therefore, the kinase domain of MLCK and tension development appears to play a role in cytoskeletal remodeling of α-actin in the contracting A7r5 cell. However, the kinase domain of MLCK with tension development does not seem to have an effect on β-actin structure during contraction of the A7r5 cell.

Future Studies

With so many avenues left to explore in smooth muscle contraction, there are an endless number of proteins that could play a role in the regulation of α- and β-actin.

Another protein that we have done a little work with is Rho kinase. Rho kinase is a member of a signaling cascade that is thought to play a role in actin filament assembly and possibly a role in bundling actin filaments (Tang and Anfinogenova, 2008).

Another avenue to explore is to determine how α-actin remodels into podosomes. Our lab has done some live cell, time course studies examining β-actin during contraction of the cell since it is a GFP-labeled protein. Attempts have been made to replicate these experiments using a tagged α-actin with no success because of the inability to make a construct to express the tagged protein. It is uncertain whether α-actin depolymerizes and repolymerizes at podosomes, or if the actin is taken into the podosome core as a filamentous actin. Such a construct could shed light on this subject.

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"Principles of Biology"
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RESEARCH EXPERIENCE

Marshall University
Cell Culture: Maintenance and contractility studies of passaged A7r5 smooth muscle cells. **2002 - 2008**

Imaging: Confocal microscopy, FRET analysis, fluorescence microscopy, and related computer software.

Molecular Biology: Transformation of competent bacteria with plasmid DNA, extraction of plasmid and total DNA, mammalian cell plasmid transfection, single and double labeling immunocytochemistry, immunoprecipitation, SDS-PAGE, Glycerol-PAGE, and Western Blot analysis

Animal Surgery: Removal of rat aorta.

Tissue Extraction: Harvesting of smooth muscle medial layer of rat aorta.

Contractility: Rat aorta.

Gunma University Graduate School of Medicine, Maebashi, Japan
Protein Purification: Actin from rabbit skeletal muscle powder and recombinant myosin light chain kinase from transformed bacteria. **Feb. & Mar. 2004**

Actin Binding and Bundling Assays: Centrifugation assays, SDS-PAGE analysis, and densitometry.

Morehead State University
Radiolabels: Labeling of rat skeletons *in vivo* with ^{3}H-tetracycline, determination of bone resorption by collecting urine and determining the quantity of ^{3}H-tetracycline present using a scintillation counter. **2001-2002**

PUBLICATIONS AND PAPERS

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Oral presentation for Biomedical Science Seminar Series at Marshall University Joan C. Edwards School of Medicine entitled "The Role of Leptin in Obesity." March 24, 2003.

Invited Talk for Biomedical Science Seminar Series at Marshall Unitversity Joan C. Edwards School of Medicine entitled "My experience in Japan" August, 2004.

Guest Lecture for Cell Physiology Class at Marshall University Joan C. Edwards School of Medicine entitled "Cytoskeleton." August 29, 2005.

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POSTER PRESENTATIONS AND ABSTRACTS

Brown, Dawn, Ava Dykes, Jason Black, Sean Thatcher, Mike Fultz, and Gary Wright. Differential Actin Isoform Reorganization in the Contracting A7r5 Cell. FASEB Experimental Biology Conference, 2006.

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