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Diverse Roles of PKCa in Vascular Smooth Muscle Contraction

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Diverse Roles of PKCα in Vascular Smooth Muscle Contraction

by

Ava Caudill Dykes

Dissertation submitted to
the Graduate College
of
Marshall University
in partial fulfillment of the requirements
for the degree of

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in
Biomedical Sciences

Approved by

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ABSTRACT

DIVERSE ROLES OF PKCα IN A7r5 SMOOTH MUSCLE CELLS

by Ava Caudill Dykes

A complete understanding of the mechanisms underlying smooth muscle contractility has proven elusive due to the many interacting factors thought to influence contractile behavior in this muscle type. One such factor, protein kinase Cα (PKCα), is proposed to play a central role in both the activation and modulation of smooth muscle contraction. PKCα is activated through a G-protein coupled receptor and is subsequently translocated to its site of action. Although the mechanisms regulating PKCα site specific translocation are not completely understood, it is thought that this process is essential for PKCα specificity of action within the cell. In chapter II we discuss our research demonstrating a PKCα/β-tubulin relationship in the contracting smooth muscle cell. Here, we show for the first time that PKCα translocation is dependent on an intact system of microtubules and propose the hypothesis of microtubular involvement in PKCα translocation. Activation of PKCα in contracting smooth muscle is thought to result in the phosphorylation of several proteins which could be important in contraction. One such protein, calponin, is known to influence smooth muscle contractility but its mechanism of action is not clear. In chapter III, we discuss our research showing the formation of a PKCα/calponin complex in contracting smooth muscle with associated losses in α-actin stress fibers and cytoskeletal remodeling. Based on this work, we
propose that calponin plays a major role in the stabilization of stress fibers and that the release from calponin cross-linking of actin filaments provides a mechanism enabling actin filament sliding and subsequent cytoskeletal remodeling.
DEDICATION

I would like to dedicate this work to my boys, Jarod and Alec, for all the years of waiting patiently while Mommy was working late;

To my Mom and Dad for all the love and support - I never could have come this far without you;

To my brother, Eric, and my sister-in-law, Ellen, thanks for all the help when the kids were little;

And to my grandmother and my aunts and uncle, Ava, Mean, Donna and Gene, who were always willing to lend a hand.
ACKNOWLEDGMENTS

I would like to thank the faculty of the physiology department for their unwavering support and encouragement.

I would also like to thank my labmates, Sean Thatcher, Dawn Brown, and Jason Black, and fellow grad students Michele Herdman, Aileen Marcelo, and Ryan Morrison. I’m glad you were here with me!

To my committee members:

Dr. Mike Fultz, as a former lab member, you taught me everything I know! As a committee member, your guidance has continued to be a great help to me.

Dr. Michael Norton, as always, your wisdom and insight has proven invaluable.

Dr. Elsa Mangiarua, thanks so much for taking the time to be a friend as well as a mentor.

Dr. William McCumbee, your commitment and integrity are inspiring, but your choice of journal club topics could use a little work.

And to Dr. Gary Wright, perhaps the most important thing you’ve taught me is self-reliance. What can I say except, I owe it all to Dr. Wright!
# TABLE OF CONTENTS

TITL E PAGE........................................................................................................................................................... i
ABSTRACT.................................................................................................................................................................... ii
DEDICATION ................................................................................................................................................................... iv
ACKNOWLEDGMENTS ...................................................................................................................................................... v
TABLE OF CONTENTS............................................................................................................................................... vi
LIST OF FIGURES........................................................................................................................................................ viii
LIST OF TABLES.......................................................................................................................................................... xi
LIST OF SYMBOLS / NOMENCLATURE.................................................................................................................... xii

CHAPTER I .............................................................................................................................................................. 1
GENERAL INTRODUCTION ......................................................................................................................................... 1

D ISSERTATION ORGANIZATION ............................................................................................................................. 1
LITERATURE REVIEW ................................................................................................................................................. 2
SMOOTH MUSCLE CONTRACTION .......................................................................................................................... 2
CYTOSKELETAL REMODELING ............................................................................................................................... 7
PODOSOMES ............................................................................................................................................................ 11
PROTEIN KINASE C .................................................................................................................................................... 12
THE ROLE OF PKC IN SMOOTH MUSCLE CONTRACTION ...................................................................................... 14
PROTEIN KINASE Cα ............................................................................................................................................... 16
MICROTUBULES .......................................................................................................................................................... 17
CALPONIN .................................................................................................................................................................. 19

ATOMIC FORCE MICROSCOPY ................................................................................................................................ 20
LIST OF FIGURES

CHAPTER I. GENERAL INTRODUCTION

Figure 1. Diagram of resting and contracted sarcomere structure 3

Figure 2. Mechanism of smooth muscle contraction 4

Figure 3. Comparison of length-tension relationships of smooth and striated muscle 6

Figure 4. Structural domains of PKC isoforms 13

Figure 5. The role of phosphoinositide hydrolysis in activation of PKC 15

Figure 6. Depiction of AFM components 21

Figure 7. Example of FRET process 24

Figure 8. The Förster distance 25

Figure 9. Spectral overlap of donor and acceptor fluorophores 26

Figure 10. Depiction of parallel dipole orientations 26

CHAPTER II. MICROTUBULE-DEPENDENT PKC-α LOCALIZATION IN A7r5 SMOOTH MUSCLE CELLS

Figure 1. Effect of colchicine on the translocation of PKCα in A7r5 smooth muscle cells 51

Figure 2. Confocal images showing microtubular, microfilament, and intermediate filament cytoskeletal structure in unstimulated and PDBu-activated A7r5 smooth muscle cell 52
Figure 3. Dual immunostaining of microtubules and PKCα in unstimulated and PDBu-activated A7r5 cells 54

Figure 4. Evaluation of microtubule and PKCα co-localization by FRET microscopy in unstimulated, PDBu-activated, and colchicine-treated A7r5 cells 56

Figure 5. Line scan analysis showing β-tubulin emission intensity before and after photo-bleaching of PKCα in an unstimulated control cell 58

Figure 6. Line scan analysis showing β-tubulin emission intensity before and after photo-bleaching of PKCα in a PDBu-stimulated cell 60

Figure 7. The effect of colchicine on microfilaments and intermediate filaments 62

Figure 8. The time course of colchicine-induced dissolution of microtubular structure in A7r5 cells 63

Figure 9. Topographical evaluation of the dense network of microtubules surrounding the nucleus in an untreated control cell using AFM 64

Figure 10. Topographical evaluation of a colchicine-treated control cell using AFM 66

Figure 11. Topographical evaluation of the peri-nuclear microtubular sleeve in a PDBu-contracted A7r5 cell using AFM 68

Figure 12. Topographical evaluation of the peri-nuclear microtubule sleeve in a PDBu-contracted A7r5 cell incubated with colchicine 70

Figure 13. A model developed from measurements of control and colchicine-treated cells describing the three-dimensional structure of the peri-nuclear microtubule sleeve 72
CHAPTER III. DOWN-REGULATION OF CALPONIN DESTABILIZES ACTIN CYTOSKELETAL STRUCTURE

Figure 1. Immunostaining of α-actin, PKCα, and calponin in unstimulated and PDBu-activated A7r5 cells 98

Figure 2. Dual immunostaining of α-actin and calponin in unstimulated and PDBu-activated A7r5 cells 100

Figure 3. Dual immunostaining of calponin and PKCα in unstimulated and PDBu-activated A7r5 cells 102

Figure 4. Western blot analysis of PKCα, α-actin, and calponin content in A7r5 cells treated with TGF-β1 and PDGF-BB 104

Figure 5. Immunostaining of α-actin in unstimulated and PDBu-activated A7r5 cells incubated with TGF-β1 or PDGF-BB 105

Figure 6. A proposed role of calponin in the controlled release of bundled actin filaments for interaction with myosin and subsequent remodeling during cell contraction 107
LIST OF TABLES

CHAPTER II. MICROTUBULE-DEPENDENT PKCα LOCALIZATION IN A7r5 SMOOTH MUSCLE CELLS

Table 1. FRET distances calculated from whole cell emission scans and line scans of selected cell regions in unstimulated, PDBu-stimulated, and colchicine-treated cells

CHAPTER III. DOWN-REGULATION OF CALPONIN DESTABILIZES ACTIN CYTOSKELETAL STRUCTURE

Table 1. FRET analysis of the association of calponin with α-actin and PKCα in control and PDBu-treated A7r5 smooth muscle cells

Table 2. Western blot analysis of PKCα, α-actin, and calponin content in A7r5 cells at different time intervals during treatment with cytokine TGF-β1 or PDGF-BB to up-regulate or down-regulate calponin expression

Table 3. Effect of TGF-β1 and PDGF-BB induced changes in cell expression of calponin on cytoskeletal fiber density in control cells or podosome formation in PDBu-treated cells
<table>
<thead>
<tr>
<th>Symbol</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Å</td>
<td>angstrom</td>
</tr>
<tr>
<td>ADP</td>
<td>adenosine diphosphate</td>
</tr>
<tr>
<td>AFM</td>
<td>atomic force microscopy</td>
</tr>
<tr>
<td>ANOVA</td>
<td>analysis of variance</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>CaM</td>
<td>calmodulin</td>
</tr>
<tr>
<td>CaP</td>
<td>calponin</td>
</tr>
<tr>
<td>CH</td>
<td>calponin homology</td>
</tr>
<tr>
<td>CPI-17</td>
<td>protein kinase C-dependent phosphatase inhibitor – 17 kDa</td>
</tr>
<tr>
<td>CO₂</td>
<td>carbon dioxide</td>
</tr>
<tr>
<td>DAG</td>
<td>diacylglycerol</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modified Eagle’s medium</td>
</tr>
<tr>
<td>E</td>
<td>efficiency of energy transfer</td>
</tr>
<tr>
<td>EM</td>
<td>electron microscopy</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>ERK1</td>
<td>extracellular signal-related kinase 1</td>
</tr>
<tr>
<td>ERK2</td>
<td>extracellular signal-related kinase 2</td>
</tr>
<tr>
<td>FITC</td>
<td>fluorescein isothiocyanate</td>
</tr>
<tr>
<td>FRET</td>
<td>fluorescence resonance energy transfer</td>
</tr>
<tr>
<td>GAPDH</td>
<td>glyceraldehyde-3-phosphate dehydrogenase</td>
</tr>
<tr>
<td>GDP</td>
<td>guanosine diphosphate</td>
</tr>
<tr>
<td>GTP</td>
<td>guanosine triphosphate</td>
</tr>
<tr>
<td>HSP27</td>
<td>heat shock protein 27</td>
</tr>
<tr>
<td>I</td>
<td>intensity</td>
</tr>
<tr>
<td>I₀</td>
<td>intensity of donor in presence of acceptor</td>
</tr>
<tr>
<td>I₀DNA</td>
<td>intensity of donor in absence of acceptor</td>
</tr>
<tr>
<td>IP₃</td>
<td>inositol-3, 4, 5-triphosphate</td>
</tr>
<tr>
<td>κ</td>
<td>transition dipole orientation</td>
</tr>
<tr>
<td><strong>MAP</strong></td>
<td>microtubule-associated protein</td>
</tr>
<tr>
<td><strong>MLCK</strong></td>
<td>myosin light chain kinase</td>
</tr>
<tr>
<td><strong>Myosin-LC</strong></td>
<td>myosin-light chain</td>
</tr>
<tr>
<td><strong>PBS</strong></td>
<td>phosphate-buffered saline</td>
</tr>
<tr>
<td><strong>PBS-T</strong></td>
<td>PBS with 0.5% TWEEN-20</td>
</tr>
<tr>
<td><strong>PDBu</strong></td>
<td>phorbol-12, 13-dibutyrate</td>
</tr>
<tr>
<td><strong>PDGF</strong></td>
<td>platelet-derived growth factor</td>
</tr>
<tr>
<td><strong>PI3K</strong></td>
<td>phosphatidyl inositol-3-kinase</td>
</tr>
<tr>
<td><strong>PIP2</strong></td>
<td>phosphatidyl-4, 5-bisphosphate</td>
</tr>
<tr>
<td><strong>PKC</strong></td>
<td>protein kinase C</td>
</tr>
<tr>
<td><strong>PP2A</strong></td>
<td>protein phosphatase 2A</td>
</tr>
<tr>
<td><strong>r</strong></td>
<td>distance between donor and acceptor within a FRET system</td>
</tr>
<tr>
<td><strong>R₀</strong></td>
<td>Förster distance or radius</td>
</tr>
<tr>
<td><strong>ROCK</strong></td>
<td>Rho-associated kinase</td>
</tr>
<tr>
<td><strong>SEM</strong></td>
<td>standard error of the mean</td>
</tr>
<tr>
<td><strong>SKM</strong></td>
<td>skeletal muscle</td>
</tr>
<tr>
<td><strong>SM</strong></td>
<td>smooth muscle</td>
</tr>
<tr>
<td><strong>TGF</strong></td>
<td>transforming growth factor</td>
</tr>
<tr>
<td><strong>TRITC</strong></td>
<td>tetramethylrhodamine isothiocyanate</td>
</tr>
</tbody>
</table>
Chapter I.

General Introduction

Dissertation Organization

This dissertation is divided into four chapters. The first chapter represents a general review of the literature of relevant topics. Chapter two is a manuscript previously published in the American Journal of Physiology, Cell Physiology, reproduced with the kind permission of the American Physiological Society. This chapter describes the association between β-tubulin and PKCα and reports a novel cell structure dependent on this interaction. Chapter three is a manuscript recently submitted to Acta Physiologica. This paper examines PKCα’s association with one of its substrates, calponin (CaP), and the impact of changes in CaP expression levels on α-actin cytoskeletal structure. Here we show that calponin may have a novel role in contraction, contributing to actin structural stability through cross-linking of actin filaments. Chapter four contains a brief discussion of the results and implications of both manuscripts, as well as recommendations for future research.

In addition to a discussion of the factors contributing to the unique smooth muscle (SM) contraction, a review of certain techniques will follow. These methods were pioneered for use in the study of SM within our laboratory. FRET (fluorescence resonance energy transfer) microscopy and AFM (atomic force microscopy) were novel physiological tools at the time of publication and represent the visionary integrative approaches utilized within the field of cell physiology. Besides a brief review of the theory behind these tools, their current use and applications will be evaluated.
Literature Review

Smooth Muscle Contraction

The mechanism of contraction in smooth muscle (SM) is different from other muscle types. Although the fundamental actin-myosin interaction is the same, with force production the result of the cyclic interaction of crossbridges formed between actin and myosin causing these filaments to slide past one another, smooth muscle exhibits a number of distinctive properties that cannot be accounted for on the basis of models devised to explain striated or skeletal muscle (SKM) contraction (Gunst and Tang 2000). One such model is the sliding filament theory. As illustrated in Figure 1, this mechanism is dependent upon the organization of the contractile protein into a sarcomere. In the relaxed sarcomere, overlapping actin and myosin filaments lie parallel to each other. In the contracted state, these filaments greatly overlap and the Z lines appear to have moved to the ends of the myosin filaments. This configuration is due to the sliding of actin filaments past the myosin filaments, hence the name sliding filament. Perhaps the most obvious difference between SM and SKM is the lack of this structured sarcomere. It is the ordered sarcomere that gives SKM its striated appearance. It is the lack of which that bestows SM’s “smooth” appearance and therefore its name (Guyton and Hall 2000). It has been assumed that sarcomere-like structures adjacent to dense bodies constitute the basic contractile unit in SM. In one such proposed model, Bond and Somlyo (1982) contend that α-actinin-containing dense bodies are analogous to Z-lines in SKM and thus demarcate the boundaries of an equivalent sarcomere. However, a repeating, regular arrangement of actin and myosin has yet to be demonstrated (Draeger et al. 1990).
One distinguishing feature of SM is that its biochemical sequence of activation differs from that of SKM (Figure 2). SM lacks the regulatory protein troponin (found in SKM). Instead, SM contains the Ca$$^{++}$$-sensitive regulatory proteins calmodulin (CaM) and calponin (CaP) (Murphy 1994), to be discussed in a subsequent section. Following an increase in [Ca$$^{++}$$], 4 free Ca$$^{++}$$ form a complex with calmodulin. This complex (Ca$$^{++}$$-CaM) activates myosin light chain kinase (MLCK), enabling its kinase activity. Active MLCK can then phosphorylate the regulatory light chain of the myosin molecule to induce a conformation change in the myosin head, so that it is positioned to interact with the actin filament. In the presence of ATP, the Mg$$^{++}$$-ATPase activity of the myosin head will hydrolyze ATP to ADP, producing energy for the actin-myosin power stroke. This maneuver results in the movement of myosin along the actin filament (Driska et al. 1981). The contributions of calponin (CaP) and PKCa shown in Figure 2 will be discussed below.
Another significant disparity stems from the variable ratio of actin to myosin among different smooth muscle types. Whereas SKM consistently exhibits a 6:1 ratio of actin:myosin, smooth muscle shows a range of values for this ratio. For example, the ratio in chicken gizzard is 8:1, while that in vascular SM is approximately 15:1 (Gunst and Tang 2000). Ratios as high as 50:1 have been reported in amphibian visceral muscle (Gunst and Tang 2000). This increased ratio arises, in part, from the reduced myosin content in SM. Repeated measurements have found that SM has only 1/5 the myosin content of SKM (Murphy et al. 1974). What makes this notable is the ability of smooth muscle to produce even greater contractile force than that of skeletal muscle. Moreover, SM contractile force is produced at markedly reduced energy expenditure, approximately 1/10 to 1/300 that of SKM (Paul 1983). VanBuren et al. (1994) have reported that
smooth muscle myosin exerts three times greater average force per cross-bridge head (0.6pN) than SKM myosin (0.2pN), suggesting the mechanism by which high SM force is achieved. However, their findings have not been verified.

In addition to these differences SM has the remarkable ability to slowly develop force (Wright and Hurn 1994). Vascular smooth muscle contraction has been shown to be delineated into two distinct phases, the initial (fast) phase and a secondary (slow) phase. When studied separately, the fast phase was shown to have a length-tension curve similar to SKM; whereas, increasing the tissue preload had little effect on active tension developed in the slow phase of contraction. Cytochalasin, an actin polymerization inhibitor, selectively prevented tension generation in the slow phase when this tissue was stimulated by either elevated K⁺ or PDBu. Similarly, the PKC inhibitor, staurosporine, also blocked the slow response. These results suggest different mechanisms regulate the SKM-like fast phase and the unique slow phase of the smooth muscle contraction (Wright and Hurn 1994). Once established, the tension generated by SM can be maintained for extended periods at a minimal energy cost, about 0.35% of that expended by SKM in a comparable contraction (Paul 1983). It has been estimated that the maintenance of blood pressure would double basal metabolic rate if the energetics of vascular SM matched those of SKM (Murphy 1994). There is also evidence supporting the fact that force development and tension maintenance are dissociated from myosin light chain phosphorylation and myosin ATPase activity (Driska et al. 1981; Merkel et al. 1990). The mechanism of these unique properties of SM is unknown.
SM may be stretched through a much broader range of lengths than SKM without significantly affecting active force development. This is illustrated in Figure 3, showing that at even very short lengths or with high levels of stretch, SM maintains the capability to develop near optimal levels of active tension. Because the length-tension relationship of muscle is thought to reflect stretch-induced changes in the relative positions of actin and myosin filaments within the sarcomere (Davis and Gore 1989), the factors determining this parameter in SM are not certain.

![Figure 3. Comparison of length-tension relationships of smooth muscle with striated (SKM) muscle.](www.ucl.ac.uk/uro-neph/ppt/is0304/sm11103.ppt)

Finally, SM shows the ability to undergo stress relaxation, the release of tension following tissue stretch. Aortic rings contracted with either K⁺ or PDBu exhibited an approximate 30% decrease in tension generated by passive tissue stretch (Wright and Battistella-Patterson 1998). The stress-relaxation response could be abolished by the removal of extracellular Ca²⁺ or treatment with cytochalasin B, an actin polymerization inhibitor. However, colchicine, a microtubule depolymerizing agent, had no effect. These results indicated that stress-relaxation in contracted tissue is calcium-dependent
and may be explained by the remodeling of tension-bearing portions of the actin cytoskeleton (Wright and Battistella-Patterson 1998).

**Cytoskeletal Remodeling**

As discussed above, many of the contractile properties of SM indicate that its mechanism(s) of contraction differ from other muscle types. Unique characteristics such as slow force development (Wright and Hurn 1994), prolonged tension maintenance at low energy cost (Paul 1983), a broad length-tension relationship (Battistella-Patterson et al. 1997), and the property of stress-relaxation (Wright and Battistella-Patterson 1998) each indicate that contraction of SM cannot be explained solely on the basis of sliding filament theory. One hypothesis gaining increasing acceptance is that SM contractile properties are determined by cytoskeletal remodeling. The origins of cytoskeletal remodeling as a concept in SM contractility can be traced to the work of D. M. Warshaw (1987). Intrigued by the observation that the velocity of shortening in contracting SM was much slower than in SKM, he studied the force-shortening velocity relationship in single SM cells isolated from toad stomach muscularis (Warshaw 1987). The need for this study arose from the fact that all previous SM studies had been performed on tissue preparations. Hence the results from these earlier studies represented the average of many single cell responses coupled with the influence of surrounding ground tissue and could differ from that of a single cell. Warshaw’s work showed that SM slow shortening velocity occurs independent of time after stimulation and was not due to reduced force-generating capacity. Ultimately, it was concluded that slowing of velocity during shortening in a single SM cell was the result of an internal load that opposes shortening.
and increases as cell length decreases (Harris and Warshaw 1990). Importantly, it was further concluded that, in the single cell, cell length was disassociated from contractile element length. In opposition to the sliding filament theory, it was speculated that stretching a relaxed single smooth muscle cell would not change the position of the contractile units on the length-force curve. It was proposed that a viscous element may exist in SM cells that is connected in series with the contractile element thus allowing changes in cell length to occur without corresponding length changes in the contractile unit. In the stimulated cell, structural changes could occur to couple the contractile unit length to cell length. This proposal was the first suggestion of cytoskeletal remodeling and predicts that stretching (relaxed) smooth muscle would not alter the degree of myosin–actin overlap, as occurs in SKM (Harris and Warshaw 1991).

Later studies by Gunst et al. (1993) further challenged the sliding filament model of contraction in smooth muscle. According to the sliding filament model, the amount of force produced at any length should be a function of the number of crossbridges contributing to the force production and the rate of shortening should reflect the rate of crossbridge cycling. It follows that as muscle length increases, force would be increased but that, given a constant load, shortening velocity should remain constant or increase. However, it was found that shortening velocity decreases with increasing muscle length in the single cell. In canine smooth muscle, shortening velocity was higher when the muscle was stimulated to contract at shorter lengths. The inverse relationship between velocity of shortening and tissue length at activation was concluded to be the result of “cytostructural reorganization” of the cell in response to stretch. Such reorganization was proposed to optimize the contractile force developed at differing lengths, but could also
create an internal load due to compression of cytoskeletal structure, yielding a system in which shortening velocity is inversely related to muscle length (Gunst et al. 1993). Additional studies in canine tracheal smooth muscle focused on changes in force during oscillations in muscle length (Shen et al. 1997). The surprising finding was that the active force generated was greatly decreased below isometric force, but returned to this level upon lengthening. After ruling out other possible cellular mechanisms responsible for this phenomenon, it was concluded that the decrease in force is due to the plastic properties of the contractile filaments of smooth muscle cells. During the lapse in force, the lengths of the contractile elements were proposed to reset in relation to the new cell length to optimize functionality (Shen et al. 1997). These findings were corroborated by Battistella-Patterson et al. (1997) by using cytochalasin B, an actin polymerization inhibitor, to show that the contractile domain of the actin cytoskeleton in aortic rings could contribute to force development by undergoing remodeling during cell shortening. This study also suggested that remodeling of actin tension bearing fibers could contribute to the low energy requirement for tension maintenance by holding the cell in a contracted state (Battistella-Patterson et al. 1997). It has also been suggested that crosslinking between actin and intermediate filaments by filamin also contributes to tension maintenance (Small et al. 1986).

There are several limitations to studying smooth muscle cell structure in tissue. These include the disordered structure of the differentiated smooth muscle cell cytoskeleton, the random nature of cell orientation in tissue, as well as extracellular matrix and connective tissue dynamics. One way of overcoming these complications is to examine the cytoskeleton of isolated cells from lines that retain the ability to contract.
in culture. One such cell line is the A7r5 embryonic rat aorta cell line. These cells have been shown to exhibit an adult smooth muscle phenotype, and have retained the ability to contract (Firulli et al. 1998). The A7r5 cell line was first used in a study by Fultz et al. (2000) to conclusively demonstrate actin cytoskeletal remodeling during contraction. Through the use of immunocytochemistry and confocal microscopy, A7r5 cells were shown to undergo cellular contraction after stimulation with $10^{-8}$M phorbol-12, 13-dibutyrate (PDBu). Prior to this stimulation both $\alpha$- and $\beta$-actin were localized in stress fibers that traversed the long axis of the cell. With stimulation, cells underwent cell constriction characteristic of contraction and both actin isoforms were subject to structural reorganization. $\beta$-actin fibers retained stress fiber formation, but appeared to shorten during cellular contraction. By comparison the majority of $\alpha$-actin stress fibers appeared to reform into dense columns at the periphery of the cell.

Immunocytochemistry revealed that these dense bodies contained not only $\alpha$-actin, but $\alpha$-actinin (Fultz et al. 2000), an actin crosslinking protein known to localize in dense bodies of highly differentiated smooth muscle cells in tissue. Dense bodies, identified (Fay and Delise 1973; Fay et al. 1983) and known to contain $\alpha$-actinin as early as 1983, are thought to serve as anchorage points for contractile proteins analogous to the Z line in the sarcomere of striated muscle. The dichotomy of actin isoform remodeling has led to the hypothesis that these isoforms serve different functions (Fultz et al. 2000; Li et al. 2001); $\beta$-actin shortens but maintains filamentous structure, holding the cell in the contracted configuration while $\alpha$-actin remodels to optimize contractile efficiency and to allow force development from several foci. It was subsequently determined that the peripheral bodies reported by Fultz et al. (2000) were podosomes, $\alpha$-actinin, actin and vinculin-
containing adhesion structures, likely to function as a molecular scaffold (Hai et al. 2002) or serve as an analogue of dense bodies (Fultz et al. 2000) in the contracting A7r5 cell.

**Podosomes**

The term podosome is most often associated with osteoclasts, where they play a role in bone resorption. They have also been found in macrophages, dendritic cells, transformed fibroblasts and carcinoma cell lines (Linder and Aepfelbacher 2003). More recently, podosomes have been identified in leukocytes (Calle et al. 2006), epithelial cells (Spinardi et al. 2004), endothelial cells (Moreau et al. 2003), and smooth muscle cells (Gimona et al. 2003; Linder and Aepfelbacher 2003). Although podosome composition and function can be cell-type specific, most are comprised of an actin core surrounded by associated proteins and participate in cell adhesion or motility (Linder and Aepfelbacher 2003).

First identified as dynamic actin-containing structures referred to as “dense bodies,” in A7r5 smooth muscle cells by Fultz et al. (2000), smooth muscle podosomes have now been recognized as an important component of cytoskeletal remodeling and the smooth muscle contraction. Besides an actin-rich core, podosomes in this cell type have consistently been shown to contain α-actinin, an actin-bundling protein, and vinculin, a focal adhesion-associated protein (Linder and Aepfelbacher 2003). Additionally, A7r5 vascular smooth muscle cell podosomes have been shown to incorporate smooth muscle myosin (Fultz and Wright 2003), calponin, and PKCα (present study). It should be noted that A7r5 podosome formation is reported primarily in the isolated cell and is considered an adaptation necessary for tension development. This reasoning follows the observation
that very few podosomes are found in cells grown to confluence or in aortic tissue where multiple cell-to-cell contacts have been formed. These contacts form cell restraints and provide an anchorage site for actin filaments during contraction. Podosomes therefore likely play roles in both cellular adhesion and tension generation (Kaverina et al. 2003). In addition to these functions, smooth muscle podosomes have recently been suggested to function in extracellular matrix resorption and vascular cell motility (Burgstaller and Gimona 2005).

**Protein Kinase C**

Currently there are 12 known isoforms of PKC. Summarized in Figure 4, they are classified depending upon their requirements for activation. In general, PKC isozymes possess a regulatory domain and a catalytic domain. The catalytic domain contains the substrate binding site as well as a phospholipid-binding domain, which allows for interaction with the plasma membrane. The conventional or classical PKCs have a Ca\(^{++}\)-binding and a diacylglycerol (DAG)-binding domain, located within their regulatory domains. Accordingly, these isoforms require phospholipids, Ca\(^{++}\), and DAG (or DAG analogs like phorbol esters) for activation. Conventional PKCs include the \(\alpha\), \(\beta_1\), \(\beta_{II}\), and \(\gamma\) isoforms. Novel PKCs also have a phospholipid-binding domain and a DAG domain, but lack the Ca\(^{++}\) domain prevalent in conventional forms. Novel PKCs, such as the \(\delta\), \(\varepsilon\), \(\eta\), \(\theta\), and \(\mu\) isoforms, require only phospholipids and DAG for their activation. Atypical PKCs, including \(\zeta\), \(\iota\), and \(\lambda\), do not possess a regulatory domain, and require only phospholipid binding for activation (Webb et al. 2000).
The past decade has seen an upsurge in the study of PKC regulation factors. Biochemical studies have shown that acidic phospholipids are cofactors for PKC activation. PKC binding to DAG or phorbol esters in the presence of phospholipids induces a conformational change in PKC that results in its activation. Similarly, the presence of free fatty acids lowers the threshold for DAG activation of PKC. PKC is prone to autophosphorylation and phosphorylation by other serine/threonine kinases and so is subject to regulation by serine/threonine phosphorylation (Ron and Kazanietz 1999).

Aside from means of stimulation, PKC’s mode of activation is also dependent upon compartmentalization within the cell, which is achieved through the translocation of the enzyme to different parts of the cell. It is thought that translocation to the plasmalemma provides a site where DAG, a PKC activator, is produced. Similarly, translocations may occur to specialized cellular compartments such as the nucleus, focal adhesions and other structures containing specific anchoring proteins to localize the kinase to its site of action. These anchoring proteins can include cytoskeletal elements and their associated proteins (Webb et al. 2000). Of particular interest to the present
work, the conventional PKCα has been shown to mediate cytoskeletal remodeling and podosome formation in A7r5 smooth muscle cells (Hai et al. 2002; Gatesman et al. 2004) Subsequent portions of this review will discuss PKCα’s interaction with two proteins: microtubules (β-tubulin) and calponin, an actin-binding protein, molecules now thought to play an important role in PKCα translocation and PKCα-mediated smooth muscle contraction, respectively.

The Role of PKC in Smooth Muscle Contraction

PKC is able to exert a variety of effects upon smooth muscle contraction, and is thought to possess the ability to phosphorylate myosin directly (Sutton and Haeberle 1990) (Figure 2). This action may bypass the need for the Ca++/calmodulin cascade and represents an alternate pathway for activation of contraction. PKC may also phosphorylate other elements of the contractile cascade to expedite or modify events in the process of contraction. For example, PKC is known to activate a 17 kDa protein designated protein kinase C-dependent phosphatase inhibitor -17, or CPI-17. CPI’s most notable function is to inhibit myosin phosphatase. Myosin phosphatase serves to dephosphorylate myosin light chain and without this important “off switch,” myosin will remain phosphorylated and contraction will continue presumably until the exhaustion of ATP stores occurs. Hence, PKC-mediated inhibition of myosin phosphatase may serve to enhance the level of contractile response to a given stimuli in SM. However, under physiological conditions, activation of contraction utilizes both the Ca++/CaM and PKC pathways (Figure 5). Binding of norepinephrine at its receptor results in activation of phospholipase C (PLC), which acts on phosphatidyl-4, 5-bisphosphate (PIP₂) to produce
inositol-3, 4, 5-triphosphate (IP₃) and diacylglycerol (DAG) (Newton 1995). IP₃ acts on calcium channels in the sarcoplasmic reticulum and the sarcolemma to cause the release of intracellular calcium and enhance the influx of extracellular calcium, respectively. Hence, the elevation of [Ca²⁺], results in activation of CaM and may also serve, in combination with DAG, to precipitate the activation of conventional PKCs.

Figure 5. The role of phosphoinositide hydrolysis in activation of PKC. Phospholipase C (PLC), activated through a G-protein-coupled receptor, hydrolyzes PIP₂ (phosphatidylinositol-4,5-bisphosphate) into DAG (diacylglycerol) and IP₃ (inositol-3,4,5-triphosphate) (Siegel et al. 1999).
Protein Kinase C α

Unlike other PKC isoforms, PKCα is ubiquitously expressed and can be activated in a number of ways. Besides direct activation by Ca++ and DAG analogues, this isotype can also be activated by cell contact and physical stresses (Nakashima 2002). Regardless of the stimulus, activation of PKCα requires the translocation from the cytosol to specific domains within the cell. Li et al. (2002) showed that translocation could be selectively directed by varying phorbol-12, 13-dibutyrate concentration in the A7r5 cell, suggesting differential regulation of mechanisms of transport or PKCα tethering at specific sites. Similar to results obtained by stimulation with the calcium ionophore A23187 or the intracellular releasing compound thapsigargin, low phorbol concentrations (≤10^{-7}M) promoted PKCα translocation to the plasmalemma. Conversely, higher concentrations of phorbol (≥10^{-6}M) promoted translocation to the perinuclear region of the cell. Perinuclear translocation could be blocked by microtubular disruption by colchicine, but plasmalemma translocation was not (Li et al. 2001). Besides showing concentration-dependent responses to a stimulus, the fact that one of these responses could be selectively inhibited demonstrated the likelihood that PKCα is translocated by different pathways.

PKCα has also been found to mediate the formation of podosomes (Hai et al. 2002; Gatesman et al. 2004) in the contracting A7r5 SM cell. As noted earlier, podosome formation is a hallmark of cytoskeletal remodeling in the contracting A7r5 SM cell. This remodeling was attributed to the α isoform based on several observations. First, PKCα is the predominant conventional PKC isoform in this cell type. Secondly, podosome formation was induced by the addition of 1μM PDBu, a known DAG analogue and
conventional PKC activator. In addition, cytoskeletal remodeling and podosome formation was blocked by treatment with Gö6976, a PKC inhibitor known to be highly selective for PKC\(\alpha\) and to have no effect on other PKC classes (Hai et al. 2002).

**Microtubules**

The term microtubule refers to tubules formed from a mixture of \(\alpha\)- and \(\beta\)-tubulin and microtubule-associated proteins (MAPs). \(\alpha\)- and \(\beta\)-tubulin monomers pair to form dimers, from which a polar protofilament is formed. Subsequently, thirteen such protofilaments join in parallel to form a polar, hollow tube. Microtubules exhibit polarity with distinct ends: a fast-growing plus end and slow-growing minus end. Both \(\alpha\)- and \(\beta\)-tubulin bind guanosine triphosphate (GTP). However, only \(\beta\)-tubulin can hydrolyze this molecule to guanosine diphosphate (GDP) (Kreis and Vale 1999). This hydrolysis weakens \(\beta\)-tubulin’s binding affinity for other molecules and therefore results in depolymerization of the filament. Tubulin molecules on the minus end of the filament are continually lost while tubulin bound to GTP is continually added to the plus end. This is the basis for the phenomenon known as treadmilling (Margolis and Wilson 1998).

The drug colchicine blocks microtubule polymerization, while another drug, taxol, blocks depolymerization and thus stabilizes the filament. Both drugs are classified as anti-mitotic agents and are used as anti-cancer agents directed at rapidly growing cells (Margolis and Wilson 1998).

Microtubules originate near the center of a cell at the centriole and project radially toward the periphery. Extending through the cell in this manner enables these filaments to serve a variety of functions in the organization of the cell. Positioning of membrane-
bound organelles is dependent upon microtubular configuration (Duden et al. 1990). Additionally, this radial arrangement is ideal for another microtubule function, that of intracellular transport. Cytoplasmic microtubules associate with two families of motor proteins that shuttle cell components within the cell. The kinesins move toward the plus end of the filament (periphery of the cell), while the dyneins move toward the minus end at the center of the cell. Both kinesins and dyneins have globular heads with ATP-hydrolyzing activity. Hydrolyzing ATP to ADP provides the energy for a cycle of conformational changes that enable the molecule to move along the tubule. The tails of these proteins bind to certain cellular components or organelles and transport them in this manner (Sheetz 1999). Besides anchoring to cell components via motor proteins, tubulin has also been shown to bind directly to other key proteins, such as phosphofructokinase, glyceraldehyde-3-phosphate, phosphatidyl inositol-3-kinase (PI-3K) (Janmey 1998), actin (Sider et al. 1999), and PKCα (Schmalz et al. 1996). These associations provide the basis for a signaling role for microtubule proteins, although this proposed role of microtubules has not been widely studied.

There is also evidence to suggest that the microtubular system plays an indirect role in the contraction of excitable cells. When microtubules in non-muscle cells (fibroblasts, endothelial cells) are disrupted by treatment with an anti-mitotic agent such as colchicine, there is an increase in contractility. Similarly, several studies have described a colchicine-mediated increase in force in intact arteries (Paul et al. 2000). The work of Battistella-Patterson et al. (1997) showed that colchicine-induced microtubule disruption produced an increase in force in the initial fast phase of tension development, but caused a reduction in force and a delay in development of the secondary, slow phase.
of tension development. Ingber (1993) proposed a theory explaining this phenomenon. Cellular tensegrity is a model in which microtubules are postulated to function as rigid struts that oppose the force generated by myosin. Disruption of this resistive force with the drug colchicine is predicted to allow a greater degree of the force generated by the actin-myosin contraction to be transmitted throughout the cell, thus augmenting the contraction (Paul et al. 2000).

**Calponin**

Calponin (CaP) is a 34kDa actin-binding protein first isolated from chicken gizzard by Takahashi et al. (1986). To date, four isoforms have been described. Basic or h1 calponin is most well characterized and is known to be SM specific. Calponin β is a product of alternative splicing from the h1 calponin gene. Neutral or h2 calponin and acidic calponin are expressed in non-muscle cells, as well as SM (Winder et al. 1998). h1 Calponin has been shown to bind to α-actin (Takahashi et al. 1986), β-actin (Szymanski 2004), HSP27, PKCα (Patil et al. 2004), tubulin, desmin, myosin (Szymanski 2004), and other proteins through its versatile calponin homology (CH) domains and several protein-specific binding sites (Czurylo et al. 2000). This characteristic of CaP is suggestive of the wide utility of this multifunctional protein.

Calponin’s most prominent role is regulated by PKCα. In the resting SM cell, calponin has a high binding affinity for filamentous actin. This binding prevents actin from interacting with the myosin head, and thus prevents cellular contraction. With cellular stimulation, PKCα is also activated and is able to translocate to and phosphorylate calponin. This phosphorylation induces a conformation change in
calponin, causing it to release actin which is then free to interact with myosin and the contraction can proceed. As shown in Figure 3, this disinhibition can be reversed by protein phosphatase 2A (PP2A) (Winder and Walsh 1990).

The ability to crosslink actin and to bind other cytoskeletal proteins has led to calponin’s implication in cytoskeletal organization and actin stabilization. Based on several pieces of evidence, calponin may also have a signaling function. Namely, the calponin homology domain is homologous to those found in several signaling molecules. More promising proof stems from the fact that calponin has been found to be a direct target for Rho-associated kinase (ROCK) (Kaneko et al. 2000), a serine/threonine kinase involved in SM contraction. Additionally, Leinweber et al. (1999) have shown direct interaction between the CH (calponin homology) domain of calponin and the MAP kinase signaling molecules ERK1 and ERK2 (Leinweber et al. 1999).

**Atomic Force Microscopy**

Gerd Binnig received the Nobel Prize in 1986 for his work in scanning probe microscopy and is credited with inventing the atomic force microscope (AFM). The AFM is a topographic instrument, used for studying surface properties of materials. A sensitive technique, AFM can reach atomic level resolution. An AFM consists of a tiny tip, usually only 2-3µm in length, suspended from a cantilever (Figure 6). The cantilever is best described as a diving board. Only 100 to 200µm long, the cantilever bends as the tip comes in contact with attractive and repulsive forces on the surface of the sample. These cantilever deflections can be measured as a laser signal is reflected off the back of the cantilever and onto a split photo-diode, divided into four quadrants, A, B, C, and D.
By measuring the differences in signal between these quadrants, the cantilever’s position can be measured. (Dykes 2001).

AFM scanning is typically performed in one of three modes: contact, non-contact, or intermittent. Contact AFM, also known as repulsive mode, occurs when the tip actually makes soft contact with the sample. As the tip rasters over the sample the contact force causes the cantilever to bend with changes in topography. This mode is not recommended for biological samples since even slight contact may damage the sample. Non-contact AFM is a technique where the cantilever vibrates near the surface of the sample (within angstroms). This mode is ideal for imaging soft samples. The lack of physical contact ensures the avoidance of sample degradation. However, the presence of liquid will distort the resulting image by imaging the surface of the liquid layer rather than the surface of the sample itself. Therefore imaging within a fluid cell, as for live
imaging, cannot be performed in this mode. The third AFM mode incorporates elements of both contact and non-contact AFM. Intermittent-contact mode operates like non-contact AFM in that the cantilever vibrates near the sample surface. However, in this mode, also called “tapping mode” the cantilever is so near the sample that the tip will barely hit or “tap” the surface. An image is produced by monitoring the tip’s oscillating amplitude changes in response to the distance between the tip and the sample. Tapping mode is the most commonly used since it overcomes the limitations of both contact and non-contact imaging. Due to the reduced physical contact, there is less sample damage than contact AFM, and tapping mode gives better resolution of large samples than non-contact because of the decreased tip-to-sample distance (Howland and Benatar 1993).

AFM has proven to be a great tool in the biological sciences and offers several advantages over previously employed methods of studying biological tissue, enabling for the first time the imaging of the three-dimensional surface structure of biological specimens. Also, samples can be imaged in a fluid cell, so physiological processes can be viewed in real time at molecular resolution. Although earlier methods, such as electron microscopy (EM) and X-ray diffraction could offer molecular resolution, extensive sample preparation was a drawback. Besides prolonging an experiment, these methods call for sample dehydration, fixation and/or embedding. All are harsh to delicate tissues and may alter the native configuration of the sample. Importantly, samples can not be studied live under these conditions (Lal and John 1994).

Basic AFM operation can be modified to reveal even more information about a sample. For example, the viscoelastic properties of several biological materials have been reported. Radmacher et al. (1992) imaged elasticity and viscosity of platelets. Lal
and John (1994) proposed the study of localized viscoelastic properties of molecular motors during muscle contraction. In other studies, the morphological changes of cultured glial cells have been recorded and AFM was used to observe the growth of the cell edge and the widening of space between filamentous structures (Henderson et al. 1992). More recently, the real-time contractility of cultured atrial myocytes was observed. As ionotrophic calcium was increased, the cells underwent rapid contraction and a shortening of cytoplasmic fibers. Additionally, it was predicted that the force sensitivity of AFM could be used to obtain direct information about localized length-tension relationships in a contracting cell (Lal and John 1994).

**Fluorescence Resonance Energy Transfer**

Fluorescence resonance energy transfer (FRET) has been described as a distance-dependent interaction between two fluorescent molecules in which the stimulated emission of one molecule, the donor, is transferred to a second, acceptor molecule, where it serves as excitation energy and results in emission at a longer wavelength (Haugland 2002) (Figure 7). In a conventional fluorescent system, light excites a fluorescent molecule at its characteristic excitation wavelength. When the molecule becomes “excited,” one of the molecule’s electrons is promoted to a higher energy level, and the molecule is said to be in an electronic excited state. The electron quickly decays to a lower vibrational level, and then more slowly decays back to the ground state as a photon of light is emitted. The wavelength of the emitted photon is longer than the excitation wavelength. Within a FRET system, the donor molecule is excited in this way. However, when FRET occurs, a photon is not emitted from the donor. Instead that
energy resonates, or is transferred, to the neighboring acceptor fluorophore and is able to repeat this excitation process in the new molecule. The resulting photon of light will be of a wavelength longer than the excitation wavelength of the donor or the acceptor (Haugland 2002).

![FRET Diagram](image)

Figure 7. Example of FRET process. Modified from [meds.queensu.ca/qcri/greer/ri_pag.htm](meds.queensu.ca/qcri/greer/ri_pag.htm).

There are three primary conditions that must be met in order for FRET to occur. Foremost, the donor and acceptor pair must be in close proximity. Specifically, they must be close enough for the described energy transfer to occur. For most donor-acceptor pairs this distance falls between 10 and 100 Å. Another characteristic of the donor-acceptor pair is the Förster radius, R₀ (Figure 8.) When transfer efficiency is plotted as a function of the distance between the donor and acceptor, the Förster distance is defined as the distance between fluorophores at which energy transfer is 50%.
Figure 8. The Förster distance is defined as the distance between two fluorophores at which a 50% efficiency of energy transfer occurs (Haugland 2002).

A second requirement is illustrated in Figure 9. Here, the excitation spectrum of the acceptor overlaps the emission spectrum of the donor. According to the FRET process described above, this physical trait is essential for observing the secondary acceptor emission (Haugland 2002). The third and final requirement for FRET to occur involves the fluorophores’ transition dipole orientations. This orientation factor, denoted as $\kappa$, is simply a measure of the angle between the two fluorophores. Efficiency of energy transfer is most efficient when the fluorophores’ orientations are approximately parallel, as shown in Figure 10. Because this is a dipolar system, $\kappa^2$ is directly proportional to $R^6$, the sixth power of the distance between the two fluorophores (probability of energy transfer $\alpha \kappa^2 / R^6$). $\kappa$ can vary between 0 and 4, but when orientation cannot be determined it is assigned a value of 2/3 for randomly oriented donors and acceptors.
Figure 9. Spectral overlap of a donor fluorophore (Alexa 488) and an acceptor fluorophore (Alexa 546) (Haugland 2002).

Figure 10. Depiction of parallel dipole orientations, denoted $\kappa^2$. Red arrows indicate electronic field (Fish 2005).
The FRET phenomenon was first described by T. Förster (Forster 1948). Known as Förster’s theory of resonance transfer, this first paper illustrates how the probability of resonance depends on the local configuration of fluorophores (Stephens 2006). From quantum mechanics it can be shown, as mentioned above, that the energy transfer efficiency \( E \) varies inversely with the sixth power of the distance between the donor and the acceptor:

\[
E = \frac{1}{(r / R_0)^6}
\]

where \( r \) is the distance between the donor and the acceptor (Stephens 2006). In some cases, these calculations can not be ciphered directly and the energy transfer efficiency must first be determined experimentally. If FRET conditions are met, the result will be a quenching of donor fluorescence accompanied by an increase in acceptor fluorescence. In acceptor photobleaching FRET, donor quenching is measured by the extent of “dequenching” after acceptor photobleaching. The increase in donor fluorescence is used to calculate the energy transfer efficiency using the equation:

\[
E = 1 - \frac{I_D}{I_{DNA}}
\]

where \( I_D \) is the intensity of the donor in the presence of the acceptor and \( I_{DNA} \) is the intensity of the donor with no acceptor (Kenworthy 2001; Stephens 2006).

Giving spatial resolution beyond the scope of conventional light microscopy, the sensitivity of FRET has allowed for the advancement of basic science research in several fields. Examples of FRET applications now in common use include the study of protein structure, spatial distribution of proteins, immunoassays, real-time PCR (polymerase chain reaction), and membrane fusion assays (Haugland 2002). More specifically, FRET has enabled greater understanding of the SM contraction process. The distance between
actin and myosin was measured long ago (dos Remedios et al. 1987). Since then, FRET has been used for Ca\textsuperscript{++}-imaging (Truong et al. 2001) and contractile protein localization in cell and tissue studies (Black et al. 2006). In addition, this sensitive technique was used to analyze conformational changes in the myosin head during ATP hydrolysis (Mizukura and Maruta 2002).

References


CHAPTER II

Microtubule-Dependent PKCα Localization in A7r5 Smooth Muscle Cells

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Abstract

Utilizing laser scanning confocal, fluorescence resonance energy transfer (FRET), and atomic force (AFM) microscopy, we have investigated the association of PKCα with microtubules during stimulus-induced relocalization in A7r5 smooth muscle cells. Confocal microscopy using standard immunostaining techniques confirmed earlier observations that colchicine disruption of microtubules blocked PKCα localization in the peri-nuclear region of the cell caused by phorbol-12, 13-dibutyrate (PDBu, 10⁻⁶M). Dual-immunostaining suggested co-localization of PKCα and β-tubulin in both unstimulated and PDBu-treated cells. This finding was verified by FRET microscopy which indicated that the association of PKCα was heterogeneous in distribution and confined primarily to microtubules in the peri-nuclear region. FRET analysis further showed that the association between the molecules was not lost during colchicine-induced dissolution of microtubules, suggesting the formation of tubulin/PKCα complexes in the cytosol. Confocal imaging indicated that peri-nuclear microtubular structure was more highly sensitive to colchicine dissolution compared to other regions of the cell. Topographical imaging of fixed cells by AFM indicated a well-defined elevated structure surrounding the nucleus which was absent in colchicine-treated cells. It was calculated that the volume of the nuclear sleeve-like structure of microtubules increased approximately 5-fold in PDBu-treated cells, suggesting a probable increase in microtubular mass. Based on PKCα localization, increased colchicine sensitivity, and their volume change in stimulated cells, the results suggest that peri-nuclear microtubules...
form a specialized structure which may be more dynamically robust than in other regions of the cell. PKCα could contribute to this dynamic activity. Alternatively, peri-nuclear microtubules could act as a scaffold for regulatory molecule interaction at the cell center.

**Key Words:** cytoskeleton, microtubules, PKCα, translocation, A7r5
Introduction

The protein kinase C (PKC) family consists of a group of serine-threonine kinases separated into three subgroups based on their structure and activation requirements. Cells are commonly found to express several PKC isoforms (4) and PKC may be involved in the regulation of multiple cell functions within a single cell type. This suggests that individual isoforms phosphorylate specific substrate(s) and that compartmentalization of isoform activity occurs under physiological conditions (19). One way this is thought to occur is through translocation to isoform-specific sites located adjacent to target substrate. The inactive form of PKC is diffusely distributed throughout the cytosol or may be localized to specific regions or structures in the cell. Following stimulation of the cell, isoforms may relocate from inactive pools to their active cell loci. Hence, the intracellular translocation of PKC may represent an important mechanism for targeting specific substrates and control of isoform function.

There is evidence that the translocation of an individual PKC isoform may be varied in a single cell type through use of different stimulatory agents (18), whether highly differentiated (14, 10, 7) or less highly differentiated passaged cells (1) are utilized, and depending on cell culture conditions (8). In addition, our laboratory has reported that PKC\(\alpha\) may be selectively relocated at the plasmalemma or the peri-nucleus of A7r5 smooth muscle cells, depending on the concentration of phorbol ester (16). The A7r5 clonal cell line was chosen for these studies because it exhibits an adult smooth muscle phenotype (5). This cell line was originally derived from embryonic rat aortic smooth
muscle (15) and shows expression and promoter activity of several highly restricted smooth muscle cell markers (5). In addition to these indications of smooth muscle phenotype, A7r5 cells retain the ability to contract by both Ca\(^{2+}\)-dependent and -independent mechanisms (20, 6, 17) which is lost in passaged smooth muscle cells. Interestingly, our studies with A7r5 cells indicated that translocation of PKC\(\alpha\) to the peri-nucleus but not the subplasmalemma was blocked by the use of colchicine to disrupt cell microtubules (16). These studies indicated that concentrations of PDBu at or below 10\(^{-7}\)M resulted in colchicine-insensitive translocation of PKC\(\alpha\) to the subplasmalemma, whereas PDBu concentrations above 10\(^{-7}\)M caused colchicine-sensitive relocation of the enzyme in the peri-nuclear region. By comparison, concentrations of PDBu ranging from 10\(^{-9}\)M to 10\(^{-5}\)M resulted only in colchicine-sensitive translocation of PKC\(\alpha\) to the peri-nuclear region of multi-passaged cells derived from rat aortic smooth muscle (1). These observations suggest that in the highly proliferative, dedifferentiated passaged smooth muscle cells, only the colchicine-sensitive mechanism for peri-nuclear translocation of PKC\(\alpha\) is operative or sensitive to PDBu activation, whereas high concentrations of PDBu are required to unmask this mechanism in A7r5 cells. Hence, it was concluded that multiple pathways are available for the redistribution of active PKC\(\alpha\) in A7r5 cells which could utilize different mechanisms for the movement or docking of the isoform at specific target sites. However, due primarily to interference from the intensity of PKC\(\alpha\) immunostaining, this early study was unable to show clear evidence for a direct association of PKC\(\alpha\) with microtubules. Hence the role of the microtubular cytoskeleton on PKC\(\alpha\) localization at the peri-nucleus following phorbol ester stimulation remained unclear.
In the present study, we investigated the association of PKC\(\alpha\) with microtubular structure in A7r5 smooth muscle cells stimulated with \(10^{-6}\)M PDBu, the concentration previously demonstrated to cause colchicine-sensitive PKC\(\alpha\) translocation to the peri-nucleus. The results confirm earlier observations that peri-nuclear translocation of PKC\(\alpha\) requires an intact microtubular cytoskeleton. The results further indicate at least a partial association of PKC\(\alpha\) with microtubules in the unstimulated and PDBu-stimulated cell.
Materials and Methods

Cell culture

A7r5 cells, originally derived from embryonic rat aorta and exhibiting an adult smooth muscle phenotype (5) were obtained from American Type Culture Collection (Manassas, VA) and cultured in Dulbecco’s modified Eagle’s medium (DMEM) that was supplemented with 10% fetal bovine serum, 100 units/ml penicillin G, and 100µg/ml streptomycin. Cells were plated onto 75cm² culture flasks and grown to approximately 85% confluence. Cultures were maintained at 37°C in a humidified atmosphere of 5% CO₂ in air. The medium was changed every two days and the cells were passaged at least once a week. Cells were detached from the culture flask by the addition of a 1:10 0.5%/5.3mM trypsin/EDTA solution in phosphate-buffered saline (PBS).

Cell plating and treatment

A7r5 cells were plated onto 22mm by 22mm #1 glass coverslips placed in six well culture plates and returned to the incubator for a minimum of 24 hours to allow for cell attachment and spreading. Cells were treated prior to fixation according to one of five protocols: (1) control cells received vehicle but no treatment before fixation; (2) PDBu cells were treated with 10⁻⁶M phorbol-12, 13-dibutyrate (PDBu) (Sigma Chemical Co., St. Louis, MO) for up to 30 minutes; (3) control/colchicine cells were treated with 40µg/ml colchicine (Sigma Chemical Co., St. Louis, MO) for 30 minutes; (4) PDBu/colchicine cells were treated with 10⁻⁶ M PDBu for 30 minutes followed by treatment with 40µg/ml colchicine for 30 minutes; and (5) colchicine/PDBu cells were
treated with 40µg/ml colchicine for 30 minutes followed by treatment with 10⁻⁶ M PDBu for 30 minutes. Cells were fixed and permeabilized by the addition of ice-cold acetone for 1 minute. Fixed cells were preserved in 1X PBS buffer.

Confocal and FRET microscopy

In preparation for experiments using laser scanning confocal microscopy, A7r5 cells were plated and fixed as previously described. Following fixation the cells were washed multiple times (3X) with PBS containing 0.5% TWEEN-20 (PBS-T), pH 7.5, and incubated for 30 minutes in a blocking solution (5% nonfat dry milk in PBS). Cells were stained for β-tubulin by incubation in a 1:500 dilution of monoclonal anti-β-tubulin clone TUB 2.1 FITC-labeled antibody (Sigma Chemical Co., St. Louis, MO) for a minimum of 2 hours at room temperature. For α-actin staining, fixed cells were incubated with a 1:500 dilution of monoclonal anti-α-smooth muscle actin clone 1A4 FITC-labeled antibody (Sigma Chemical Co., St. Louis, MO) for 30 minutes at room temperature. For total F-actin staining, cells were incubated with 2.5 x 10⁻⁶M TRITC-labeled phalloidin (Sigma Chemical Co., St. Louis, MO) for 30 minutes at room temperature. Cells were stained for vimentin via incubation with a 1:300 dilution of monoclonal anti-vimentin clone V9 (Sigma Chemical Co., St. Louis, MO) at room temperature for 2 hours. Fixed cells were stained for PKCα using a 1:500 dilution of anti-PKCα-clone M4 (Upstate Biotechnology, Lake Placid, NY). Treatment with anti-vimentin and anti-PKCα solutions was followed by the addition of Alexa Fluor 488-labeled secondary antibody (Molecular Probes, Inc., Eugene, OR). In studies utilizing fluorescence resonance energy transfer (FRET) microscopy, Alexa Fluor 546 (Molecular Probes, Inc., Eugene, OR) was
employed as the secondary antibody to anti-PKCα.

Stained cells were surveyed by mounting on a Nikon Diaphot Microscope. Confocal microscopy was performed with a BioRad Model 1024 Scanning system equipped with a krypton/argon laser. Cells stained with Alex Fluor 488 or FITC conjugates were excited with the 488nm laser line at 10% power. The resulting emission was visualized using a 522 DF 32 band pass filter. For colocalization studies, the BioRad Scanning system’s multichannel capacity was engaged. When both an FITC conjugate and Alexa Fluor 594 were present, a HQ 598/40 band pass filter was used to observe the secondary fluorophore simultaneously on a second channel. Similarly, multiple channels were necessary in FRET microscopy. Within a FRET system two fluorophores with overlapping emission and excitation spectra are implemented. Here FITC (excitation, 488nm; emission, 520 nm) and Alexa Fluor 546 (excitation, 546nm; emission, 580nm) were the donor/acceptor pair employed. The donor molecule (FITC) was directly excited and the resulting emission is visualized using the 522 DF 32 band pass filter. However, a portion of energy given off by the fluorophore is not released as light but is transferred to a neighboring fluorophore that can be excited at that wavelength. Thus the FITC emission induced the Alexa Fluor 546 molecule to release energy as light at its characteristic emission wavelength which was visualized on a second channel using an HQ 598/40 band pass filter. The resonance energy transfer can only occur if the donor/acceptor molecules are close enough to each other for the transfer to occur efficiently. The Förster distance ($R_0$) is the distance at which fluorescence resonance energy transfer from the donor dye to the acceptor dye is 50%. Distances greater than $R_0$
will result in less intense emission while distances less than $R_0$ will yield higher intensities. The Förster distance calculated for this pair is 64 angstroms. Resultant images were analyzed using Lasersharp and Confocal Assistant software (BioRad, Hercules, CA).

In order to calculate the distance between PKCα and β-tubulin, images of cells were acquired using laser scanning confocal microscopy with excitation at the 488nm-laser line. To obtain an internal control, the imaging area was then excited at the 568nm-laser line at 100% power to photo-bleach the acceptor molecule (PKCα). Subsequently, a second set of images was acquired again using 488nm-laser line excitation and the appropriate multi-channel filter set to measure β-tubulin FITC emission (522 DF 32) and to verify the absence of anti-PKCα secondary antibody Alexa 546 emission (HQ598/40). The images were analyzed by two methods; both the determination of whole cell emission intensity and by a single line scan emission intensity measurement. By selecting the entire scan area, an intensity profile was generated for each whole cell image (Image J Software, NIH) with the resulting plot analyzed using Peakfit v4.11 software (SPSS Science, Inc., Chicago, IL) to obtain the area under the curve. These values were then utilized in calculating the efficiency of energy transfer ($E$) and the distance between the donor and acceptor pair ($r$) using the equations below (a, b) (13) where: $I_D$ is the intensity of the donor molecule in the presence of acceptor molecule; $I_{DNA}$ is the intensity of the donor molecule in the absence of acceptor (after photo-bleaching); $r$ is the FRET distance between donor and acceptor molecules; and $R_0$ is the Förster distance.
a) \[ E = 1 - \frac{I_D}{I_{DNA}} \]

b) \[ E = \frac{1}{[1 + (r/R_0)^6]}. \]

The resulting distances were analyzed by Sigma Stat 2.03 (SPSS Inc., Chicago, IL) in comparisons of values obtained for control cells, those stimulated with PDBu, and colchicine-treated cells. Images analyzed by line scan emission intensity were also evaluated using Image J (NIH). For each set of images (I_D, before photo-bleaching; I_{DNA}, after photo-bleaching), a line was arbitrarily drawn to bisect the cell nucleus and extending to the visible peripheral borders of the cell. The resulting plot profile was then utilized to evaluate two aspects of the intracellular regional distribution of the association between β-tubulin and PKCα. Initially we compared the FRET distance in the perinuclear versus the peripheral region of the cell. This was accomplished by averaging the two highest values within the twenty pixel distance of the nuclear mass and the cell peripheral border. Secondly, we identified the point of maximum efficiency of energy transfer (E) on the line scan as a focal area in which PKCα interaction with tubulin might approach saturating conditions.

**Atomic Force Microscopy**

In experiments employing atomic force microscopy (AFM) fixed cells were rinsed with deionized water and dried with compressed air prior to study. Coverslips were mounted.
to 20mm AFM metal specimen discs (TMMicroscopes, Sunnyvale, CA) using 12mm carbon conductive tabs (Ted Pella, Inc., Redding, CA). Cells were imaged in contact mode using a TMMicroscopes Explorer atomic force microscope (2). All images were acquired with either TMMicroscopes Model No. 1520-00 or Model No. 1930-00 cantilevers. Subsequent analysis of images was performed using SPMLab version 5.01 analysis software (TMMicroscopes, Sunnyvale, CA). Based on the whole cell scan of each cell a line scan was performed bisecting the nucleus in an axis arbitrarily chosen to provide measurement outside the visible perimeter of the cell. The reference point for each cell height measurement was the default point assigned as zero by the SPM Lab Analysis software. Because the height of the nuclear sleeve was the object of study, and to avoid sampling bias, the measurement of cell height (untreated) or ground substance (colchicine-treated) was obtained by using the whole cell scan to visually set measurement points on the sleeve rim or immediately adjacent to the nuclear mass, which were then identified for analysis on the line scan. The two measurements on opposite sides of the nucleus were then averaged to give a single value for height.

AFM measurements showed a well-defined, elevated structure surrounding the nucleus (Figs 9, 11) that was absent in colchicine-treated cells (Fig. 10, 12). Based on these observations it was concluded that the central network of microtubules formed a sleeve-like structure surrounding the nucleus. The sleeve diameter and thickness were directly measured and sleeve height was obtained by subtracting the average cell height of colchicine-treated cells (ground substance) from the cell height of individual untreated cells (Fig. 13). Based on these measurements the average volume (V) of the microtubular
sleeve was then determined using the formula for calculation of the volume of a hollow cylinder indicated below, where; \( V \) is the volume of the microtubular sleeve, \( h \) is the sleeve height, \( r_0 \) is the outside radius, and \( r_i \) is the inside radius of the cylinder.

\[
V = h \times \pi r_0^2 - h \times \pi r_i^2
\]

**Statistics**

Differences in FRET distances and AFM obtained topographical measurements were analyzed by one- or two-way ANOVA followed by Student’s t-test (Sigma Stat 2.03, SPSS Science, Inc., Chicago, IL). Differences were considered significant if \( p<0.05 \) in all cases. The data are presented as mean ± SEM throughout the text.
Results

Previous work (16) has indicated that stimulation of A7r5 cells with PDBu (>10^-7M) results in the peri-nuclear translocation of PKC\(\alpha\) with evidence that this movement is blocked by treatment with colchicine. PKC\(\alpha\) appears to be diffusely distributed in the unstimulated cell (Fig. 1a). However, the addition of 10^{-6}M PDBu resulted in intense staining for PKC\(\alpha\) in the peri-nuclear/nuclear region of the cell (Fig. 1b). Addition of colchicine (40\(\mu\)g ml^{-1}) prior to PDBu blocked the translocation of PKC\(\alpha\) (Fig. 1c), while its addition to the media after stimulation with PDBu caused a punctate, diffuse redistribution of PKC\(\alpha\) back into the cytosol (Fig. 1d). These results suggest that activated PKC\(\alpha\) is associated in some fashion with peri-nuclear microtubules or other microtubule-dependent cell structure.

Figure 2 shows the structural distribution of the microtubular (\(\beta\)-tubulin), actin (\(\alpha\)-actin, phalloidin) and intermediate filament (vimentin) components of the cytoskeleton in unstimulated (control) and PDBu-activated A7r5 cells. Unlike the distribution of actin filaments, the dense network of microtubular and intermediate filament structure surrounding the nucleus provided an early suggestion of these two filament types as candidates for a role in PKC\(\alpha\) localization. Dual-immunostaining suggested a significant degree of co-localization of microtubules and PKC\(\alpha\) in both control and PDBu-stimulated cells but particularly in perinuclear region of the PDBu-treated cells (Fig. 3). The conclusion of tubulin/PKC\(\alpha\) interaction was strengthened by FRET microscopic
evaluation of whole cell scans which indicated an association between microtubules and PKCα in both stimulated and unstimulated cells as well as colchicine-treated control and PDBu-stimulated cells (Fig. 4). These images further suggested that the tubulin/PKCα association was largely confined to the central region of cells having an intact microtubular system.

Consistent with results from whole cell emission scans, FRET analysis of line scans from control (Fig. 5) and PDBu-stimulated (Fig. 6) cells indicated a heterogeneous distribution of tubulin/PKCα association with evidence of high levels in the efficiency of energy transfer (E) in the peri-nuclear region compared to that at the periphery of the cell.

The calculation of FRET distances from whole cell emission scans yielded similar values among control, PDBu-stimulated, and colchicine-treated groups (Table 1) supporting the conclusion of significant tubulin/PKCα association in cells both before and after disruption of microtubular structure. Further, FRET distances were significantly increased in the periphery compared to the peri-nucleus in both control and PDBu-stimulated cells, verifying visual observations of differences in the regional distribution of tubulin/PKCα interaction within the cell. Interestingly, the FRET distances calculated for points of peak efficiency of energy transfer (E) obtained from each line scan (Table 1) were very similar among the different groups (60.1 ± 0.4 Å) and fell at the upper end of the range of measurements reported between known loci on actin and myosin (22). These latter findings further suggest the occurrence of discrete “hot spots” of tubulin/PKCα interaction within the cell.
Because colchicine caused dispersal of PKCα from the peri-nuclear region in cells preactivated with PDBu (Fig. 1), it was of interest to evaluate the effect of the drug on the major cytoskeletal elements. Colchicine treatment had no detectable effect on actin microfilaments or on intermediate filaments (Fig. 7), greatly reducing the likelihood of their involvement in PKCα localization. By comparison, the drug caused the complete disruption of all but the very peripheral microtubular structure within 20 min. after addition to the medium (Fig. 8). Interestingly, the colchicine-induced dissolution of microtubules was clearly observed to occur at variable rates in different regions of the cell. The organization of microtubules was consistent among the great majority of A7r5 cells imaged, forming a dense network about the nucleus from which cables of microtubules radiated to a second dense network at the subplasmalemma. Upon treatment with colchicine, we invariably noted initial dissolution of the central network, which was generally complete before loss in the structural integrity of radiating cables or peripheral structure was noticeable.

Topographical imaging of fixed cells by AFM revealed a well-defined, elevated structure surrounding the nucleus (Fig. 9), which was absent following treatment with colchicine (Fig. 10). A similar analysis of the PDBu-activated cell (Fig. 11, 12) indicated that the contraction of the cell significantly altered the dimensions and increased the volume of this structure by roughly 5-fold (Fig. 13). The results suggest that the dense network of microtubules at the center of the cell forms a sleeve-like structure which, based on enhanced sensitivity to colchicine and the regional association with PKCα, may represent
a more dynamic and specialized subgroup of microtubules. The significant change in the dimensions and volume of the microtubular sleeve could reflect compression of the structure during cell contraction or an artifact of sample preparation. Alternatively, these results could be suggesting active polymerization and an increase of microtubular structure in the central region of the activated A7r5 cell.
Figure 1. Effect of colchicine on the translocation of PKCα in A7r5 smooth muscle cells. PKCα appeared diffusely distributed throughout the cell prior to stimulation (a). Within 30 minutes after the addition of phorbol 12, 13 dibutyrate (PDBu, 10⁻⁶M), PKCα translocated to the peri-nuclear region of the cell (b). The addition of colchicine (40µg/ml) to the medium 20 minutes prior to PDBu blocked PKCα translocation (c). The addition of colchicine 30 minutes after PDBu resulted in the dispersal of PKCα from the peri-nuclear region back into the cytosol (d). Cells were fixed with acetone and stained with anti-PKCα clone M4 primary antibody followed by Alexa 488-labeled secondary antibody prior to confocal imaging. Images represent those obtained in 3 individual experiments in which a total in excess of 120 cells were evaluated from each treatment group.
$\beta$-tubulin

$\alpha$-actin

phalloidin

vimentin
Figure 2. Confocal images showing microtubular (β-tubulin), microfilament (α-actin, phalloidin), and intermediate filament (vimentin) cytoskeletal structure in unstimulated (control) and PDBu-activated A7r5 smooth muscle cells. Microtubules were visualized using a monoclonal anti-β-tubulin clone TUB2.1 FITC-labeled antibody. Actin was visualized by use of a monoclonal anti-α-smooth muscle actin clone 1A4 FITC-labeled antibody or TRITC-labeled phalloidin. Intermediate filaments were imaged through use of a monoclonal anti-vimentin clone V9 primary antibody followed by Alexa 488-labeled secondary antibody. Images are representative of those obtained in 3 individual experiments in which a total in excess of 120 cells were evaluated in each group.
Figure 3. Dual immunostaining of microtubules and PKCα in unstimulated (control) and PDBu-activated A7r5 cells. Thirty minutes following the addition of PDBu (10^{-6}M) to the medium cells were fixed with acetone and prepared for confocal imaging. Microtubules were visualized using a monoclonal anti-β-tubulin clone TUB2.1 FITC-labeled antibody. PKCα was visualized by staining with monoclonal anti-PKCα clone M4 primary antibody followed by Alexa 594-labeled secondary antibody. Yellow color indicates co-localization of the two proteins. Images shown are exemplary examples of those obtained in 4 individual experiments in which a total of 173 cells were evaluated.
**Figure 4.** Evaluation of microtubule and PKCα co-localization by fluorescence resonance energy transfer (FRET) microscopy in unstimulated (Control), PDBu-activated, and colchicine-treated A7r5 cells. Cells received vehicle or 10⁻⁶M PDBu for 30 minutes and were fixed with acetone for FRET imaging. Colchicine (40µg/ml) was added to the medium 30 minutes after the injection of PDBu. Cells were stained for β-tubulin using monoclonal anti-β-tubulin clone TUB2.1 FITC-labeled antibody and for PKCα using monoclonal anti-PKCα clone M4 primary antibody followed by Alexa 546-labeled secondary antibody. The FRET effect was observed by excitation at 488 nm only. At this wavelength FITC (β-tubulin) was directly excited to fluoresce, with emission capture at 522nm. The excitation of Alexa 546 (emission capture at 598nm) and PKCα visualization therefore indicates protein-protein interaction. Images represent those obtained in 4 or 5 individual experiments in which a total of 15 cells were evaluated.
Figure 5. Line scan analysis showing β-tubulin emission intensity before (A, a) and after (B, b) photo-bleaching of PKCα in an unstimulated control cell. The line scan was performed in an axis arbitrarily chosen to bisect the nucleus, extending to the visible borders of the cell (top panel); whereas, the bottom panel shows the results. β-tubulin was visualized using a monoclonal anti-β-tubulin clone TUB2.1 FITC-labeled antibody. PKCα was visualized using a monoclonal anti-PKCα clone M4 primary antibody followed by
Alexa 546-labeled secondary antibody. The basal image of β-tubulin emission was obtained by excitation with the 488nm-laser line at 10% power (A). The cell and surrounding area were then excited with the 568nm-laser line at 100% power to photo-bleach all traces of PKCα-Alexa 546 emission. Immediately following photo-bleaching, a second β-tubulin image was captured with the 488nm-laser line (B) at the exact settings utilized in basal imaging. According to FRET theory, donor molecule (β-tubulin) emission is expected to increase in the absence of the acceptor molecule (PKCα). The images represent results obtained in 3 individual experiments in which a total of 20 cells were evaluated.
Figure 6. Line scan analysis showing β-tubulin emission intensity before (A, a) and after (B, b) photo-bleaching of PKCα in a PDBu-stimulated cell. The line scan was performed in an axis arbitrarily chosen to bisect the nucleus, extending to the visible borders of the cell (top panel); whereas, the bottom panel shows the results. β-tubulin was visualized using a monoclonal anti-β-tubulin clone TUB2.1 FITC-labeled antibody. PKCα was visualized using a monoclonal anti-PKCα clone M4 primary antibody followed by Alexa 546-labeled secondary antibody. The basal image of β-tubulin
emission was obtained by excitation with the 488nm-laser line at 10% power (A). The cell and surrounding area were then excited with the 568nm-laser line at 100% power to photo-bleach all traces of PKCα-Alexa 546 emission. Immediately following photo-bleaching, a second β-tubulin image was captured with the 488nm-laser line (B) at the exact settings utilized in basal imaging. According to FRET theory, donor molecule (β-tubulin) emission is expected to increase in the absence of the acceptor molecule (PKCα). The images represent results obtained in 3 individual experiments in which a total of 20 cells were evaluated.
Figure 7. The effect of colchicine on microfilaments and intermediate filaments.

Unstimulated A7r5 cells were incubated with vehicle (Control) or 40µg/ml colchicine for 20 minutes prior to fixation and staining. Actin stress fibers were visualized by staining with a monoclonal anti-α-smooth muscle actin clone 1A4 FITC-labeled antibody. Intermediate filaments were imaged using a monoclonal anti-vimentin clone V9 primary antibody followed by Alexa 488-labeled secondary antibody. The results indicate that colchicine had no significant effect on actin or intermediate filament structure. Images are representative of those obtained in 3 individual experiments in which a total of 150 to 175 colchicine-treated cells were evaluated.
Figure 8. The time course of colchicine-induced dissolution of microtubular structure in A7r5 cells. Cells were untreated (vehicle control) or were incubated with 40µg/ml colchicine for 5, 10, or 20 minutes prior to fixation and staining. Microtubules were imaged using a monoclonal anti-β-tubulin clone TUB2.1 FITC-labeled antibody. Images are typical of those obtained in four separate experiments in demonstrating the dissolution of the dense network of microtubules surrounding the nucleus (arrows) in advance of significant losses in peripheral structure.
**Figure 9.** Topographical evaluation of the dense network of microtubules surrounding the nucleus in an untreated control cell using atomic force microscopy (AFM). The top panel shows a three-dimensional view of a contact AFM image obtained from an unstimulated, fixed A7r5 cell. The bottom panel shows a line scan of the cell indicating cross-sectional structure and representative of cell height in the nuclear area. The image is representative of those obtained in 3 individual experiments in which a total of 10 cells were evaluated.
**Figure 10.** Topographical evaluation of a colchicine-treated control cell using atomic force microscopy (AFM). The top panel shows a three-dimensional view of a contact AFM image of an unstimulated control cell incubated with 40µg/ml colchicine prior to fixation. The bottom panel shows a line scan of the cell indicating cross-sectional structure and representing cell height in the nuclear area. The image is representative of those obtained in 3 individual experiments in which a total of 10 cells were evaluated.
Figure 11. Topographical evaluation of the peri-nuclear microtubular sleeve in a PDBu-contracted A7r5 cell using atomic force microscopy (AFM). The cell was incubated with $10^{-6}$M PDBu prior to fixation. The top panel shows a three-dimensional view of a contact AFM image of the cell after PDBu treatment. The bottom panel shows a line scan of the image indicating cross-sectional structure and representative of cell height in the nuclear area. The image is representative of those obtained in 3 individual experiments in which a total of 10 cells were evaluated.
**Figure 12.** Topographical evaluation of the peri-nuclear microtubule sleeve in a PDBu-contracted A7r5 cell incubated with colchicine. The cell was incubated with $10^{-6}$M PDBu after which 40µg/ml colchicine was added for an additional 30 minutes prior to fixation. The top panel shows a three-dimensional view of a contact AFM image of the cell. The bottom panel shows a line scan of the image indicating cross-sectional structure and representative of cell height in the nuclear area. The image is representative of those obtained in 3 individual experiments in which a total of 10 cells were evaluated.
<table>
<thead>
<tr>
<th></th>
<th>1 Cell Height (nm)</th>
<th>2 Sleeve Height (nm)</th>
<th>3 Sleeve Thickness (µm)</th>
<th>4 Sleeve Diameter (µm)</th>
<th>5 Ground Substance (nm)</th>
<th>Sleeve Volume (µm$^3$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>302.4±12.8</td>
<td>38.6±12.8</td>
<td>3.7±0.2</td>
<td>14.6±0.5</td>
<td>---</td>
<td>4.9</td>
</tr>
<tr>
<td>Control/Colchicine</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>PDBu</td>
<td>419.2±22.4*</td>
<td>213.4±22.4*</td>
<td>2.8±0.2*</td>
<td>16.4±1.2</td>
<td>---</td>
<td>25.6</td>
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<td>PDBu/Colchicine</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>205.8±23.0</td>
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</tr>
</tbody>
</table>

* denotes significant difference from control
Figure 13. A model developed from measurements of control and colchicine-treated cells describing the three-dimensional structure of the peri-nuclear microtubule sleeve. The cartoon depicts untreated (a, b) and colchicine-treated (c, d) cells indicating the absence of the microtubular structure after colchicine. The table provides a summary comparison of cell dimensions in control and PDBu-activated cells. Direct measurements include cell height in untreated (control) (1) and colchicine-treated (5) cells, sleeve diameter (4) and sleeve thickness (3). The sleeve height was obtained by subtracting the average ground substance height of colchicine-treated cells (5) from the height of individual control cells (1). Sleeve volume was calculated using the averaged cell measurements and assuming the shape of the structure was similar to a hollow cylinder. With the exception of sleeve volume, the values presented represent the mean ± SEM of 10 cells.
Table 1. FRET distances calculated from whole cell emission scans and line scans of selected cell regions in unstimulated (control), phorbol 12, 13 dibutyrate (PDBu)-stimulated, and colchicine-treated cells.
### A. Whole Cell

<table>
<thead>
<tr>
<th></th>
<th>N</th>
<th>Fret Distance, Å</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>15</td>
<td>89.2 ± 6.8</td>
</tr>
<tr>
<td>Control + Colchicine</td>
<td>15</td>
<td>80.7 ± 2.8</td>
</tr>
<tr>
<td>PDBu</td>
<td>15</td>
<td>99.9 ± 4.6</td>
</tr>
<tr>
<td>PDBu + Colchicine</td>
<td>15</td>
<td>90.2 ± 2.9</td>
</tr>
</tbody>
</table>

### B. Regional

1. **Peri-Nucleus vs. Periphery**

<table>
<thead>
<tr>
<th></th>
<th>N</th>
<th>Peri-Nucleus</th>
<th>Periphery</th>
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</thead>
<tbody>
<tr>
<td>Control</td>
<td>20</td>
<td>72.8 ± 1.7</td>
<td>87.3 ± 2.6*</td>
</tr>
<tr>
<td>PDBu</td>
<td>20</td>
<td>72.9 ± 1.8</td>
<td>95.8 ± 4.3*</td>
</tr>
</tbody>
</table>

2. **Tubulin/PKCa “Hot Spots”**

<table>
<thead>
<tr>
<th></th>
<th>N</th>
<th>FRET Distance, Å</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>20</td>
<td>61.4 ± 1.1</td>
</tr>
<tr>
<td>Control + Colchicine</td>
<td>20</td>
<td>59.6 ± 1.8</td>
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<tr>
<td>PDBu</td>
<td>20</td>
<td>59.8 ± 4.4</td>
</tr>
<tr>
<td>PDBu + Colchicine</td>
<td>20</td>
<td>59.7 ± 2.3</td>
</tr>
</tbody>
</table>

An (*) indicates significant difference peri-nucleus vs. periphery (p<0.05 or greater). “Hot Spots” refer to points of maximal efficiency of energy transfer (E) obtained from line scans. N = number of cells evaluated.
Discussion

Our results confirm earlier findings that treatment of cells with colchicine to disrupt microtubules blocks the translocation of PKC\(\alpha\) to the peri-nucleus (23, 1, 18). Based on this finding, it was suggested that microtubules could influence both the movement of PKC\(\alpha\) to the peri-nucleus and/or the docking mechanism in this region of the cell (1). We further show that treatment of preactivated cells in which translocation is complete with colchicine causes dispersal of PKC\(\alpha\) back into the cytosol (Fig. 1). This suggests that the principal impact of the microtubules is not on the actual movement of PKC\(\alpha\) but that they act to stabilize the aggregation of the enzyme in different regions of the cell. In previous work utilizing a PKC\(\alpha\)-GFP fusion protein expression, we were not able to clearly demonstrate a direct relationship between PKC\(\alpha\) and microtubules in A7r5 cells (18). However, we now show that dual immunostaining for PKC\(\alpha\) and \(\beta\)-tubulin with confocal microscopic imaging (Fig. 3) and analysis of overlapping emission/excitation spectra by FRET microscopy (Fig. 4-6, Table 1) indicate significant co-localization and close association of PKC\(\alpha\) with microtubules in both unstimulated control and PDBu-stimulated A7r5 cells. The present finding that association of PKC\(\alpha\) with tubulin does not require cell stimulation suggests that the activation of the enzyme is not necessary for interaction between the two molecules. Alternatively, these results may reflect a basal level of PKC\(\alpha\) activity in resting cells. Evidence that PKC\(\alpha\) is associated with tubulin in colchicine-treated cells (Fig. 4, Table 1) further indicates that PKC\(\alpha\) may complex with tubulin in the cytosol.
It is noteworthy that both confocal imaging (Fig. 3) and FRET analysis (Fig. 4) indicate a heterogeneous distribution of PKCα in association with microtubules. Visual inspection of whole cell images suggests that the interaction is primarily confined to the central region whereas the microtubular structure at the cell periphery appears devoid of PKCα. FRET measurements (Table 1) verified a predominance of tubulin/PKCα interaction in the region immediately adjacent to the nucleus and further indicated the existence of discrete focal points of intense resonance energy transfer. The FRET distances calculated for these “hot spots” of maximal tubulin/PKCα interaction are within the range of values reported for other inter-molecular interactions (22) and could be indicating the direct binding of PKCα with tubulin. However, the magnitude of the FRET distance recorded could also be consistent with the association of β-tubulin and PKCα via a linker protein. In view of the highly restricted compartmentalization of the tubulin/PKCα interaction, we consider it unlikely that PKCα is constitutively bound to tubulin. It must be emphasized that the results do not allow quantitation of PKCα/microtubule association and the possibility remains that a portion of PKCα is associated with other structure in the peri-nuclear region. Taken together, however, the results suggest that the microtubules could act as a scaffold for PKCα binding. This function is primarily confined to the peri-nuclear microtubular structure and occurs through an interaction of PKCα with microtubule-associated compounds.

In comparison with other cytoskeletal components, the microtubules of the A7r5 cell form a unique structural configuration, as seen with confocal microscopy (Fig. 2). A dense network about the nucleus is connected by thick cables radiating across the cell...
body to a dense network of microtubules lying under the plasmalemma. Interestingly, a highly reproducible sequence of colchicine-induced dissolution of microtubular structure was observed in these various regions of the cell (Fig. 8). There was initial loss of the central peri-nuclear network with complete disassembly realized by 5 min. after colchicine addition. By comparison, significant loss of radiating cables occurred at 10 to 20 min, whereas the subplasmalemma network of microtubules was often at least partially intact after 20 min. of colchicine treatment. Because colchicine is thought to prevent polymerization by binding tubulin subunits, the high level of colchicine sensitivity suggests that microtubular structure in the peri-nuclear region is more dynamically robust than in other regions of the cell.

Topographical analysis of fixed cells by AFM indicated that the peri-nuclear network of microtubules formed an elevated sleeve-like structure enveloping the nucleus (Fig. 9, 10). A similar evaluation of PDBu-activated cells indicated that the dimensions of the peri-nuclear sleeve were significantly altered during cell shortening (Fig. 11, 12). By utilizing measurements of height, thickness and diameter (Fig. 13), we calculated the volume of the sleeve assuming a form roughly the shape of a hollow cylinder. This estimate indicated that the volume of the microtubular sleeve was approximately 4.9 µm$^3$ in control cells and 25.5 µm$^3$ in cells treated with PDBu. Differences in microtubular volume between control and PDBu-stimulated cells could reflect an artifact of colchicine treatment or sample preparation for imaging. Alternatively, the 5-fold increase observed in PDBu-treated cells could represent an increase in microtubular mass in the activated cells. As previously noted, the interaction between PKC$\alpha$ and $\beta$-tubulin was retained.
after treatment with colchicine, suggesting that PKCα may associate with monomeric or small aggregates of tubulin in the cytosol. The possibility occurs that such a tubulin/PKCα complex is translocated to the peri-nucleus during expansion of the microtubular structure in this region during cell activation.

Activation of PKCs has been shown to result in changes in cytoskeletal structure in association with the phosphorylation of an array of cytoskeletal proteins (12). It is well documented that PKCs may interact with each of the cytoskeletal components with some evidence to suggest increased activity in the activated cell (9). Hence, there is a large literature on the role of PKCs in cytoskeletal regulation. However, the great bulk of research has focused on regulation of actin microfilaments (12) and relatively little is known of the role of PKC in microtubule dynamics. Kabir et al. (2001) have reported the interesting finding that activation of PKC by phorbol esters in Aplysia bag cell neurons in primary cell culture resulted in a doubling in the length of a typical microtubule growth episode while increasing and decreasing rescue and catastrophe frequencies, respectively. They concluded that PKC may play an important role in regulating cellular processes involving directed microtubular growth. This group (21) has subsequently shown phorbol ester-induced translocation of PKCs to microtubules, suggesting a direct role of these kinases in the regulation of distal microtubular advance in neural growth cones. Their findings open the possibility that the translocation of PKCα to the peri-nuclear microtubules in A7r5 cells could serve a regulatory function directed toward microtubular growth and stability in this region of the cell. This function, in turn, would appear consistent with increased dynamic activity evidenced by colchicine sensitivity and
the increase in volume of peri-nuclear microtubular structure in the present study.

Alternatively, the translocation of PKCα could reflect a specialized role of peri-nuclear microtubules as a scaffold for interaction of regulatory molecules influencing a variety of cell functions in the central region of the cell. There is evidence from a variety of cell types suggesting a link between PKC-mediated function and the capacity for microtubule reorganization. For example, the prevention of microtubular depolymerization with taxol blocked PKC (phorbol ester)-induced NF-kappa B activation in murine NIH3T3 cells (24) while causing hyperphosphorylation of vimentin and reorganization of the intermediate filament cytoskeleton via a PKC signaling pathway in 9L rat brain tumor cells (3). Others (25) have proposed that microtubule catastrophe may be obligatory for PKC (phorbol ester)-mediated MCL1 gene expression in the MCL1 human myeloblastic leukemia cell line. As a matter of speculation, a dynamic microtubular scaffold could serve to compartmentalize regulatory interactions while providing for rapid and potentially directional expansion during growth of structure and the reduction of interaction or compartmentalized release of compounds during catastrophe and loss of structure.

In summary, the results confirm earlier conclusions that the microtubular cytoskeleton is required for PDBu-induced localization of PKCα at the peri-nuclear region in A7r5 smooth muscle cells. The results suggest that this is due to an association of activated PKCα with microtubule-associated compounds confined to the microtubules in this region of the cell. In addition to the regional limitation of PKCα localization, the distribution of microtubular structure in the cell and differences in colchicine sensitivity
suggest that microtubules in the peri-nuclear region represent a distinct subpopulation which may differ in their dynamic growth characteristics from those in other regions. PKC could be an important factor in determining these characteristics. Alternatively, the peri-nuclear microtubules could serve as a scaffold for interaction of regulatory proteins at the cell center.
References


phorbol ester via inhibition of I kappa B alpha phosphorylation and degradation.  

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CHAPTER III

Down-regulation of calponin destabilizes actin cytoskeletal structure

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Abstract

Aim: The effects of changes in the expression levels of h1 calponin (CaP) on actin cytoskeletal organization were studied in control and phorbol-ester-treated A7r5 smooth muscle cells.

Methods: Protein association and expression in control and stimulated A7r5 smooth muscle cells were evaluated by Western blotting, laser scanning confocal microscopy (LSCM) and fluorescence resonance energy transfer (FRET) microscopy in cells treated with either 2x10^{-6}M TGF-β1 or 2x10^{-5}M PDGF-BB to alter h1 calponin expression.

Results: Single immunostained samples showed that CaP and α-actin, localized in fibers in unstimulated control A7r5 smooth muscle cells, were translocated to podosomes following treatment with phorbol-12, 13-dibutyrate (PDBu). Confocal colocalization imaging and FRET analysis both indicated substantial association of CaP with α-actin in stress fibers of control cells and in podosomes of PDBu-treated cells. PKCα, which showed evidence of only slight association with CaP in control cells, exhibited markedly increased (293%) association in PDBu-contracted cells. Platelet-derived growth factor (PDGF)-BB down-regulated (non-detectable) while transforming growth factor (TGF)-β1 up-regulated (424%) the expression of CaP without affecting the levels of α-actin or PKCα. PDGF-BB resulted in a significant loss in α-actin stress fibers (-47%) and prevented podosome formation (-69%). By comparison, TGF-β1 had no effect on stress fibers in control cells but also reduced (-70%) podosome formation.

Conclusions: The results suggest that CaP could play a major role in the stabilization of actin stress fibers in resting cells and may contribute to podosome formation in PDBu-treated cells.
Introduction

Calponin (CaP) is a 34-kDa protein expressed in smooth muscle but not in skeletal or cardiac muscle types (Takahashi et al. 1987). CaP has been linked to the regulation of smooth muscle contractility through its ability to inhibit Mg\textsuperscript{2+}-ATPase activity (Winder and Walsh 1990) and reduction of unloaded shortening velocity without effect on isometric force (Jaworowski et al. 1995; Obara et al. 1996). Conversely, increased velocity of shortening is seen in smooth muscle from CaP-gene knockout mice (Matthew et al. 2000). Attempts to understand the mechanism underlying the influence of CaP on contractility have focused on the protein binding properties of the molecule. CaP contains binding sites for both actin and myosin as well as calmodulin and desmin (Szymanski 2004). The combination of desmin and actin binding has prompted speculation that CaP could play a structural role serving in cytoskeletal organization which influences the mechanical properties of the cell (Lehman 1986; Kolakowski et al. 1995). It is further proposed that actin/myosin binding properties of CaP could tether these filaments, imposing an internal load to brake velocity of contraction which, combined with the inhibitory effect of CaP on Mg\textsuperscript{2+}-ATPase activity, could underlie latch-bridge activity in smooth muscle (Szymanski 2004).

Kolakowski et al. (1995) have reported evidence indicating that CaP contains two actin binding sites; one at amino acid residues 145-182 (El-Mezgueldi et al. 1992) and another at residues 48-134 which exhibits sequence similarity to the actin binding region of several actin-binding proteins (Vancompernolle et al. 1990). Interestingly, in the presence of CaP actin filaments formed highly ordered actin bundles similar in
appearance to stress fibers and at a CaP: actin molar ratio of about 0.5: 1.0. As observed by Kolakowski et al. (1995), formation of actin bundles could be related to CaP oligomerization and precipitation but was more likely due to direct CaP cross-linking of filaments. Moreover, it was observed that CaP binding and actin bundling were reversed by Ca\textsuperscript{2+}/calmodulin. These results suggest that CaP could play an active role in the stabilization/destabilization of actin cytoskeletal structure during contractile remodeling of the cell. Consistent with this idea, Gimona et al. (2003) have reported that two functionally distinct populations of actin fibers occur in phorbol ester-contracted A7r5 smooth muscle cells. Actin stress fibers decorated with h1 CaP remained stable; whereas, those decorated with SM22-\(\alpha\) disassembled and reorganized at podosomes, suggesting a direct role of CaP in actin remodeling dynamics.

In the present study, we examined the effects of changes in the expression levels of CaP on actin cytoskeletal structure in unstimulated and PDBu-treated A7r5 smooth muscle cells. The results suggest that CaP is required for stability of actin stress fibers in resting cells; whereas, excessive CaP concentrations inhibit actin remodeling in PDBu-stimulated cells. Based on these findings, we propose the hypothesis that actin bundling by CaP represents an important mechanism for stabilization of actin structure and the controlled release of actin filaments for sliding filament force development and actin cytoskeletal remodeling in the contracting cell.
Material and Methods

Cell Culture: A7r5 cells, originally derived from embryonic rat aorta and exhibiting an adult smooth muscle phenotype (Firulli et al. 1998) were obtained from American Type Culture Collection (Manassas, VA) and cultured in Dulbecco’s modified Eagle’s medium (DMEM) that was supplemented with 10% fetal bovine serum and 100 units/ml penicillin G. Cultures were maintained in a humidified atmosphere of 5% CO₂ in air and allowed to grow to approximately 85% confluence. The medium was changed every two days and cells were passaged at least once a week. Cells were detached from culture plates by the addition of a trypsin/EDTA solution in phosphate buffered saline (PBS).

Cell Treatments: A7r5 cells were incubated with either PDGF-BB or TGF-β1 to down-regulate (Hautmann et al. 1999) or up-regulate (Sugenoya et al. 2002) the expression of calponin h1, respectively. Cells were plated on Integrid™ style tissue culture dishes (Becton, Dickinson and Co., Cockeysville, MD) and prior to growth factor addition, growth was arrested by replacement of media with DMEM supplemented with 0.5% fetal bovine serum. PDGF-BB (2x10^{-5}M) and TGF-β1 (2x10^{-6}M) were added and in initial experiments, cells were harvested at selected intervals for Western blotting analysis to determine the optimal time for study of changes in CaP, α-actin, and PKCα expression. Based upon these experiments, PDGF-BB-treated cells at 48 hours and TGF-β1 cells at 12 hours were selected for further study.

For imaging studies, cells were plated onto 22mm x 22mm #1 glass cover slips placed in six well culture plates and returned to the incubator for a minimum of 24h to
allow for attachment and spreading. Cells were growth arrested (24h) followed by 48h incubation with PDGF-BB or 12h incubation with TGF-β1. During these intervals control cells received vehicle. After incubation cells were left unstimulated or were contracted by the addition of PDBu (10⁻⁵M). The cells were then fixed and permeabilized for imaging by addition of ice-cold acetone for 1.0 minute.

**Confocal/FRET Microscopy:** Following fixation in acetone, the cells were repeatedly (3X) rinsed with PBS containing 0.5% TWEEN-20 (PSB-T), pH 7.5, and were then incubated for 1h in blocking solution (5% nonfat dry milk in PBS). α-Actin was imaged using a monoclonal anti-α-smooth muscle actin clone 1A4 antibody (Sigma Chemical CO., St. Louis, MO). PKCα was imaged using a whole antiserum anti-PKCα antibody (Sigma Chemical Co.) and CaP was visualized using a monoclonal anti-CaP clone hCP antibody (Sigma Chemical Co.). Incubation with unlabeled primary antibodies was followed by incubation with a 1:150 dilution of the appropriate IgG or IgM secondary Alexa Fluor 488 or Alexa Fluor 546 antibody (Molecular Probes, Inc., Eugene, OR) for 1h at room temperature.

Immunostained cells were mounted on a Nikon Diaphot microscope and confocal microscopy was performed with a BioRad Model 1024 Scanning system equipped with a krypton/argon laser. For FRET analysis CaP was labeled with Alexa 488 (excitation 488nm; emission 520nm) and served as the donor component of the system. α-Actin and PKCα were labeled with Alexa 546 (excitation 546nm; emission 580nm) and served as the acceptor molecules. The donor molecule (CaP) was directly excited and the resulting emission obtained with a 522 DF 32 band-pass filter. However, a portion of the energy
of emission was not released as light but was transferred to neighboring Alexa 546 fluorophore resulting in emission that was captured on a second channel with an HQ 598/40 band pass filter. Subsequently, the sample was excited at the 568nm laser line at 100% power to photobleach the acceptor (PKCα, α-actin) molecule and a second image of the cell was acquired again at the 488nm laser line with the multichannel set to obtain CaP fluorescence (522 DF 32) and to verify the absence of acceptor label Alexa 546 emission (HQ 598/40). An intensity profile was generated for each image (Image J Software, NIH) and the resulting plot was analyzed with Peakfit v4.11 software (SPSS Science, Richmond, CA) to obtain the area under the curve. The values were then used to calculate the percent increase in fluorescence after photobleaching. Because resonance energy transfer can only occur if the donor and acceptor molecules are sufficiently close to one another for efficient transfer, the resulting values were analyzed in comparisons of treated and control cells as an index of the association between CaP and the other proteins in unstimulated and PDBu-contracted cells.

α-Actin stress fiber density was evaluated as previously described (Black et al. 2006). Images of treated and control cells were obtained at fixed confocal settings and analyzed using Image J software (NIH). Utilizing the line scan tool, the cell was bisected perpendicular to the long axis at mid-cell and the resulting plot was imported into Microsoft Office Excel. The point of lowest intensity was selected as baseline and its plot was then analyzed using Peakfit v411 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 (I/p) was calculated using Excel. To evaluate cells responding to PDBu by formation of podosomes, cell counts were performed in three
independent experiments totaling a minimum of 300 cells. Cells were scored by two viewers and the counts were averaged.

**Western Blot Analysis:** Treated cells were detached from tissue culture dishes by the addition of a 1:10 trypsin/EDTA solution in phosphate-buffered saline (PBS). The solution was placed in a 15ml conical tube and subjected to centrifugation in a Fisher Scientific Centrifuge Model 228 clinical centrifuge (Pittsburgh, PA). The resulting pellet was collected and resuspended in 500µl RIPA buffer containing a Complete Mini protease inhibitor cocktail tablet (Roche Diagnostics, Mannheim, Germany). These lysates were further disrupted by 10 second bursts from a Teckman sonic disrupter (Cole Parmer Instrument Co., Vernon Hills, IL) at power setting 60. The samples were then centrifuged at 5,000 rpm/min for 2 minutes to remove cellular debris and protein concentrations in the supernatant were determined by the BCA® (Pierce, Rockford, IL) protein assay. Fifty µl of each treatment sample were loaded onto an 8% sodium dodecyl sulfate polyacrylamide gel (SDS-PAGE) and the separated proteins electrically transferred to 0.45µm polyvinylidene difluoride (PVDF) transfer membrane (Pierce, Rockford, IL). The membrane was incubated in a 5% non-fat dry milk/PBS blocking solution for 1 hour at room temperature and after multiple 5-minute washes with PBS/0.5% TWEEN (3X), the membrane was incubated for 1 hour with either a 1:500 dilution of monoclonal anti-α-smooth muscle actin clone 1A4 antibody (Sigma Chemical Co., St. Louis, MO), a 1:200 dilution of whole antiserum anti-PKC α antibody (Sigma Chemical Co., St. Louis, MO), a 1:500 dilution of monoclonal anti-calponin clone hCP antibody (Sigma Chemical Co., St. Louis, MO), or a 1 µg/ml solution of a monoclonal
antibody to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Ambion, Inc., Austin, TX). This was followed by incubation with the appropriate secondary antibody: peroxidase-labeled goat anti-rat IgM affinity purified antibody (Kirkegaard and Perry Laboratories, Gaithersburg, MD), anti-mouse Ig, horseradish peroxidase-linked whole antibody (sheep), or ECL™ anti-rabbit IgG horseradish peroxidase-linked whole antibody (donkey) (Amersham Biosciences UK Limited, Buckinghamshire, England). Reactive bands were visualized by the ECL™ chemiluminescence method (Amersham, Piscataway, NJ) using CL-Xposure™ Clear Blue X-ray film (Pierce, Rockford, IL). Resultant bands were evaluated via densitometry using Image J Software (NIH) with the resulting plot analyzed using Peakfit v4.11 software (SPSS Science, Inc., Richmond, CA) to obtain the area under the curve. To standardize these areas the values obtained were divided by the corresponding GAPDH values and the respective control groups were normalized to a value of 1.00.

**Statistical Analysis:** Results were analyzed by Sigma Stat 2.03 (SPSS Inc., Richmond, CA) using one-way ANOVA and Student’s t-test in comparisons of values obtained for control cells with those stimulated with PDBu in untreated and TGF-β1 or PDGF-BB treated groups. Values are presented as mean ± SEM throughout the text.
Results

Single immunostaining for individual proteins in unstimulated control cells indicated that α-actin and CaP localized in stress fibers while PKCα was distributed in a network pattern previously shown to be in association with β-tubulin (Dykes et al. 2003)(Fig. 1). PDBu-stimulated cells showed a loss in stress fibers with translocation of α-actin and CaP to podosomes, while PKCα appeared to be diffusely distributed in the perinuclear region of PDBu-treated cells (Fig. 1). Co-localization imaging suggested a strong association of α-actin and CaP in stress fibers of control cells and in podosomes of PDBu-treated cells (Fig. 2). There was little or no evidence of co-localization of PKCα and CaP in control cells. However, these proteins appeared strongly associated in podosomes but not stress fibers of PDBu-contracted cells (Fig. 3). FRET analysis confirmed the association of α-actin and CaP seen in co-localization images of control and PDBu-treated cells, further indicating a slight (-22%) decrease in α-actin/CaP complex in PDBu compared to control cells (Table 1). Similar to co-localization results FRET analysis indicated insignificant PKCα/CaP association in control cells. However, the association of these proteins was significantly increased (293%) in PDBu-contracted cells.

Incubation of A7r5 cells with TGF-β1 resulted in an approximate 5-fold increase in CaP protein content at 12 hours (Fig. 4, Table 2A) without significant changes in α-actin or PKCα levels. Conversely, PDGF-BB caused the down-regulation of CaP to levels below detection (Fig. 4, Table 2B) without significant changes in α-actin or PKCα content. Although the CaP content of stress fibers increased nearly 2-fold, α-actin fiber density was unchanged in TGF-β1-treated control cells (Fig. 5, Table 3A). In contrast,
PDGF-BB down-regulation of CaP resulted in significant loss in \( \alpha \)-actin stress fiber intensity (-47%).

Approximately 85% of PDBu-treated cells showed relocalization of \( \alpha \)-actin at podosomes (Fig. 5, Table 3B). By comparison, cells treated with either TGF-\( \beta \)1 or PDGF-BB showed reduced (-70%) podosome formation (Table 3B). TGF-\( \beta \)1-treated cells maintained a robust system of \( \alpha \)-actin stress fibers in the presence of PDBu; whereas, this structure was only weakly developed in PDGF-BB incubated cells (Fig. 5).
Figure 1. Immunostaining of α-actin, PKCα, and calponin in unstimulated (control) and PDBu-activated A7r5 cells. Cells were treated with $10^{-5}$M PDBu for 20 minutes before acetone fixation. α-Actin was visualized with a monoclonal anti-α-smooth muscle actin clone 1A4 antibody. Calponin was visualized using a monoclonal anti-calponin clone hCP antibody. These treatments were followed by anti-mouse Alexa 488 secondary antibody. PKCα was visualized with whole antiserum anti-PKC α antibody, followed by anti-rabbit Alexa 546 secondary antibody.
**Figure 2.** Dual immunostaining of \(\alpha\)-actin and calponin in unstimulated (control) and PDBu-activated A7r5 cells. \(\alpha\)-Actin was visualized with a monoclonal anti-\(\alpha\)-smooth muscle actin clone 1A4 antibody followed by Alexa 488 secondary antibody. Calponin was visualized using a monoclonal anti-calponin clone hCP antibody followed by Alexa 546 secondary antibody. Yellow color in merged panels indicates colocalization of the two proteins.
**Figure 3.** Dual immunostaining of calponin and PKCα in unstimulated (control) and PDBu-activated A7r5 cells. Calponin was visualized with a monoclonal anti-calponin clone hCP followed by Alexa 488 secondary antibody. PKCα was visualized using a whole antiserum anti-PKCα antibody followed by Alexa 546 secondary antibody. Yellow color in merged panels indicates colocalization of the two proteins.
**Figure 4.** Western blot analysis of PKCα, α-actin, and calponin content in A7r5 cells treated with TGF-β1 and PDGF-BB. A7r5 cells were grown to confluence, then growth arrested for 24 hours. TGF-β1-treated cells were exposed to 2x10⁻⁶M TGF-β1 for 0 (control), 6 or 12 hours before collection. PDGF-BB-treated cells were exposed to 2x10⁻⁵M PDGF-BB for 0 (control), 24 or 48 hours before collection. Sample preparation included homogenization and BCA protein concentration determination prior to SDS-PAGE. After the proteins were electrically transferred to PVDF membrane, calponin, α-actin, PKCα, and GAPDH bands were probed with monoclonal anti-calponin clone hCP, monoclonal anti-α-smooth muscle actin clone 1A4, whole antiserum anti-PKCα, or monoclonal antibody to glyceraldehyde-3-phosphate dehydrogenase primary antibodies, respectively. This was followed by incubation with the appropriate secondary antibody: anti-mouse Ig, horseradish peroxidase-linked whole antibody (sheep), or anti-rabbit IgG horseradish peroxidase-linked whole antibody (donkey). Reactive bands were visualized by the ECL™ chemiluminescence method.
Figure 5. Immunostaining of α-actin in unstimulated (control) and PDBu-activated A7r5 cells incubated with TGF-β1 or PDGF-BB. TGF-β1-treated cells were exposed to 2 x 10^{-6} M TGF-β1 for 12 hours. PDGF-BB-treated cells were exposed to 2 x 10^{-5} M PDGF-BB for 48 hours. Cells were stimulated to contract by addition of 10^{-5} M PDBu for 20 minutes. α-Actin was visualized with a monoclonal anti-α-smooth muscle actin clone 1A4 antibody followed by anti-mouse Alexa 488 secondary antibody.
Cell Contractile Activation

↑ Ca^{2+}, Activation of calmodulin (CaM)

MLCK
Initiation of force development

Force development, Sliding filament

Cytoskeletal Structural Remodeling
Figure 6. A proposed role of CaP in the controlled release of bundled actin filaments for interaction with myosin and subsequent remodeling during cell contraction. Stimulation with activation of myosin light chain kinase (MLCK) or PKC-mediated activation of the Rho cascade to inhibit myosin light chain phosphatase would initiate force development. In addition, CaP disassociation from actin would occur at Ca\textsuperscript{2+}/CaM sensitive sites or phosphorylation at Ser\textsuperscript{175} resulting in the reversal of Mg\textsuperscript{2+}-ATPase activity and unbundling of actin filaments. The combined activities would ensure the strategic availability of actin filaments for interaction with myosin and enable subsequent actin remodeling during cell constriction.
Table 1. FRET Analysis of the association of calponin with α-actin and PKCα in Control and PDBu-treated A7r5 smooth muscle cells. In each case calponin served as the donor molecule. Data are presented as percent increase in fluorescence intensity after photobleaching of the acceptor molecule. Values reflect the average from a minimum of ten cells in three independent experiments.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>α-Actin</th>
<th>PKCα</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>41.6 ± 12.2</td>
<td>2.9 ± 0.6</td>
</tr>
<tr>
<td>PDBu</td>
<td>32.8 ± 7.0</td>
<td>11.4 ± 2.5*</td>
</tr>
</tbody>
</table>

An asterisk (*) indicates significant difference from the control value, p < 0.05 or better.
Table 2. Western blot analysis of PKCα, α-actin, and calponin content in A7r5 cells at different time intervals during treatment with cytokine TGF-β1 (A) or PDGF-BB (B) to up-regulate or down-regulate calponin expression, respectively. Data are presented as percent of control values and represent the average of three independent experiments.

A) TGF-β1

<table>
<thead>
<tr>
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<th>Control</th>
<th>6 hr</th>
<th>12 hr</th>
</tr>
</thead>
<tbody>
<tr>
<td>PKCα</td>
<td>100 ± 0</td>
<td>130 ± 14</td>
<td>202 ± 78</td>
</tr>
<tr>
<td>α-actin</td>
<td>100 ± 0</td>
<td>102 ± 21</td>
<td>101 ± 20</td>
</tr>
<tr>
<td>Calponin</td>
<td>100 ± 0</td>
<td>377 ± 47*</td>
<td>524 ± 163*</td>
</tr>
</tbody>
</table>

B) PDGF-BB

<table>
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<tr>
<th></th>
<th>Control</th>
<th>24 hr</th>
<th>48 hr</th>
</tr>
</thead>
<tbody>
<tr>
<td>PKCα</td>
<td>100 ± 0</td>
<td>71 ± 15</td>
<td>90 ± 10</td>
</tr>
<tr>
<td>α-actin</td>
<td>100 ± 0</td>
<td>94 ± 6</td>
<td>99 ± 11</td>
</tr>
<tr>
<td>Calponin</td>
<td>100 ± 0</td>
<td>85 ± 7</td>
<td>0*</td>
</tr>
</tbody>
</table>

An asterisk (*) indicates significant difference from the control value, p < 0.05 or better.
Table 3. Effect of TGF-β1 and PDGF-BB induced changes in cell expression of calponin on A) α-actin and calponin fiber density in control cells B) podosome formation in PDBu-treated cells. Cell density data reflects fluorescent intensity per pixel whereas data from PDBu-contracted cells indicates the percentage of cells showing podosome formation. Values represent the average of fiber density measurements in a minimum of 10 cells and podosome counts in a minimum of 300 cells obtained in three independent experiments.

A) Cell Fiber Density

<table>
<thead>
<tr>
<th>Treatment</th>
<th>α-actin</th>
<th>Calponin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>85.7 ± 7.8</td>
<td>33.0 ± 1.0</td>
</tr>
<tr>
<td>TGF-β1</td>
<td>84.3 ± 6.1</td>
<td>64.4 ± 4.7*</td>
</tr>
<tr>
<td>PDGF-BB</td>
<td>45.8 ± 5.2*</td>
<td>0*</td>
</tr>
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</table>

B) Podosome Formation

<table>
<thead>
<tr>
<th></th>
<th>PDBu Only</th>
<th>TGF-β1</th>
<th>PDGF-BB</th>
</tr>
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<tbody>
<tr>
<td>% Cells</td>
<td>84.0 ± 1.0</td>
<td>26.0 ± 4.0*</td>
<td>26.5 ± 2.5*</td>
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</tbody>
</table>

An asterisk (*) denotes significant difference from the control (A) or PDBu only (B) value, p < 0.05 or better.
Discussion

It is now generally accepted that the cytoskeletal structure of smooth muscle undergoes reorganization which is thought to impact positively on force development. However, the nature of this remodeling and the mechanisms by which it is regulated are not certain. Previous work has demonstrated that contraction of A7r5 smooth muscle cells with PDBu results in a loss of α-actin stress fibers with translocation of α-actin to podosomes (Fultz et al. 2000; Black et al. 2006), an event mediated by PKCα (Hai et al. 2002; Gatesman et al. 2004). Recent work has further shown that actin fibers decorated with CaP were stable during PDBu stimulation (Gimona et al. 2003), suggesting that this protein could play a regulatory role in stress fiber stabilization or the translocation of actin to podosomes.

In vitro studies indicate that CaP is phosphorylated by PKC or calmodulin-dependent kinase II (Winder and Walsh 1990) resulting in the loss of its ability to bind actin and the ability to inhibit Mg²⁺-ATPase activity. Although there is controversy surrounding how readily CaP is phosphorylated in vivo (Barany et al. 1991; Gimona et al. 1992) there is evidence that the molecule is transiently phosphorylated in contracting tissue (Winder et al. 1993; Gerthoffer and Pohl 1994). Recent evidence has further indicated a direct association of CaP and PKCα induced by acetylcholine in colonic smooth muscle (Patil et al. 2004). Consistent with these latter observations; we obtained evidence of significant PKCα/CaP association after PDBu addition. PKCα/CaP complex appeared to be concentrated at podosomes and was not apparent in remaining stress fibers. This coupled with our observation of only a slight decrease in α-actin/CaP in contracted cells (Table 1) suggests that only a select portion of CaP could have been
phosphorylated or otherwise induced to disassociate from α-actin. Alternatively, 
PKCα/CaP complex may be involved in the translocation or assembly of proteins at the 
podosome.

PDGF-BB has been shown to down-regulate CaP expression in non-muscle cells 
(Sugenoya et al. 2002) with resultant changes in cytoskeletal organization (Heldin and 
Westermark 1999). In A7r5 cells, treatment with this growth factor for 48h caused an 
absence of detectable CaP expression without significant change in the levels of α-actin 
or PKCα. It was expected that release from CaP inhibition would result in a degree of 
basal cell constriction with attendant reorganization of the actin cytoskeleton. Instead, 
we observed an approximate 50% loss in α-actin stress fibers in unstimulated control 
cells and apparent loss in ability to respond to PDBu with cell constriction and podosome 
formation. One explanation of these findings is that CaP is required for the structural 
integrity of α-actin structure and the disruption of the contractile apparatus blocks 
subsequent cell contraction and cytoskeletal remodeling.

TGF-β1 has been shown to promote the differentiation of several cell types to a 
smooth muscle phenotype, prompting the up-regulation of actin, myosin, and CaP 
(Hautmann et al. 1999; Sugenoya et al. 2002). In the A7r5 cell, 12h treatment with TGF-
β1 caused an approximate 5-fold increase in CaP protein without significantly affecting 
the expression levels of α-actin or PKCα. Both unstimulated and PDBu cells treated with 
TGF-β1 showed robust α-actin stress fiber structure. Similar to PDGF-BB-treated cells, 
however, these cells showed a marked decrease in PDBu-induced podosome formation. 
In light of our present findings suggesting that only a small portion of the total CaP is 
disassociated from α-actin during contraction, a possible explanation of reduced
responsiveness to PDBu is excessive CaP stabilization of contractile structure and inhibition of contraction.

CaP has long been known to influence the contractile properties of smooth muscle, its mechanism primarily attributed to an ability to bind actin and myosin as well as the inhibition on Mg\(^{2+}\)-ATPase. We suggest that in addition to these actions the capability of CaP to crosslink actin filaments plays a major physiological role in smooth muscle contraction. Our present results suggest that CaP is required for the structural integrity of at least a portion of the actin contractile cytoskeleton. Down-regulation of CaP expression resulted in disruption of α-actin stress fibers and blocked subsequent stimulation-induced remodeling of the cytoskeleton and podosome formation. In addition, we find that over-expression of CaP is also associated with the inhibition of actin remodeling and podosome formation, likely due to excessive CaP stabilization of stress fibers. Hence, the results suggest that a balanced content of CaP is necessary for stress fiber stability in the resting A7r5 smooth muscle cell and for normal constriction and actin cytoskeletal remodeling in PDBu-activated cells. As shown in Figure 6, the initiation of force development either by activation of myosin light chain kinase by Ca\(^{2+}\)/CaM complex or the Rho cascade by PKC to inhibit myosin light chain phosphatase may be expected to additionally reverse CaP inhibition of Mg\(^{2+}\)-ATPase and the crosslinking of actin filaments through disassociation of CaP from actin at Ca\(^{2+}\)/CaM sensitive sites (Kolakowski et al. 1995) or due to phosphorylation of CaP at Ser\(^{175}\) (Winder et al. 1993). Reversal of CaP effects simultaneous with initiation of force development would strategically enhance actin/myosin interaction at sites with individual filaments available for movement by myosin.
References


CHAPTER IV.

SUMMARY AND CONCLUSION

General Discussion

Early thinking on smooth muscle contraction centered on explaining the contractile properties of this muscle type within the framework of existing models developed to explain the mechanisms of skeletal muscle contraction. In recent decades, it has become increasingly clear that smooth muscle contractility cannot be completely explained by these comparisons. Although the notion of cytoskeletal remodeling has provided a viable hypothesis for understanding the mechanics of the smooth muscle contraction, many aspects of this phenomenon in terms of its nature and regulation also remain unexplained. The body of this work focuses on the contributions of the PKC family of proteins to cytoskeletal remodeling during the smooth muscle contraction process.

Microtubules and their associated motor proteins, kinesin and dynein, are important for intracellular transport. As the “tracks” for this efficient transfer system, the microtubular array extends from the center of a cell to the outer periphery. The distribution of these filaments in the cell gives microtubules the potential for interaction with many cellular components and the possibility for influence on a variety of functions within the cell through biologically active MAPs. Our present work demonstrated the direct association of PKCα with β-tubulin, a major component of microtubules. Surprisingly, this association remained intact in resting and stimulated cells, as well as cells in which microtubules had been disrupted by colchicine, indicating an unusually strong association. Additionally, it was also shown that PKCα’s translocation to its site
of action is dependent on the microtubular system. When microtubules were disrupted by
dissolution with colchicine, PKCα remained diffusely distributed and did not translocate
to the nucleus, as observed in intact cells. Additionally, treatment with colchicine after
PKCα was stimulated to nuclear translocation caused a redistribution of PKCα
throughout the cell further indicating the role of microtubules in “fixing” the distribution
of this protein. This work represents important new findings and allows the introduction
of the novel hypothesis of a microtubular scaffold for the interaction and
compartmentalized release of PKCα, with the possibility of the involvement of additional
signaling molecules. Also, the methods described here represent pioneering work using
FRET microscopy and AFM in fixed biological samples.

CaP has long been known to inhibit Mg$^{2+}$-ATPase activity and decrease the
velocity of shortening in smooth muscle. In vitro phosphorylation of calponin by PKCα
causes its dissociation from actin and relieves this inhibition. Previously, this
disinhibition by PKCα was viewed to be a switch, operating by a simple on or off
mechanism. The study here, however, has revealed that this is not necessarily the case.
In the present work, we obtained evidence indicating direct PKCα/calponin association in
A7r5 cells undergoing cytoskeletal remodeling and subsequent contraction. Moreover,
this work suggests that CaP is required for the structural integrity of at least a portion of
the actin cytoskeleton. Additionally, these results indicate that overexpression of CaP
prevents actin remodeling and podosome formation. These findings have led to the novel
hypothesis that CaP plays an important role in the stabilization of actin stress fibers in
resting cells and may contribute to podosome formation in PDBu-treated cells.
Future Work

Studies to further this project would include the use of both general and specific PKC inhibitors, such as staurosporine or GÖ6976, respectively, to examine PKCα’s effects on both microtubule and calponin structure and function. It would be important to ascertain if a PKC inhibitor could interrupt the static β-tubulin/PKCα interaction described here. Additionally, blocking the growth of the microtubule-based nuclear sleeve via a PKC inhibitor could ascribe a signaling complex involving PKCα and β-tubulin, as well as contribute to assigning a more definite function for this novel organelle. Similarly, it is possible that treatment with PKC inhibitors could produce contractile effects similar to those seen with calponin up-regulation, by blocking cytoskeletal remodeling and cellular contraction. Additionally, disinhibition with PKC inhibitors in cells with down-regulated calponin levels could affirm our findings that the observed effects are due to direct action of calponin and not a downstream consequence of PKC signaling through another pathway.
Appendix

From: Ava Caudill Dykes <caudillD@marshall.edu>
To: <pripka@the-aps.org>
Date: 4/17/2006 6:14:47 PM
Subject: пер missions

Dear Dr. Ripka,
I am writing to ask permission to reproduce an article published in Cell Physiology, on which I was first author, "Microtubule-dependent PKC-alpha localization in A7r5 smooth muscle cells (Am J Physiol Cell Physiol 285: C76-C87, 2003)." The article will appear as a chapter in my dissertation, as part of my graduation requirements at The Joan C. Edwards School of Medicine at Marshall University. All dissertation submissions at Marshall are electronic, and will be accessible through our website, www.marshall.edu, unless otherwise requested.
Thank you,
Ava C. Dykes

THE AMERICAN PHYSIOLOGICAL SOCIETY
9650 Rockville Pike-Bethesda, MD 20814-0991

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EDUCATION
Ph.D., Biomedical Sciences, Joan C. Edwards School of Medicine, Marshall University, Huntington, WV. Degree expected Spring 2006. Dissertation: Diverse Roles of PKC\(\alpha\) in Vascular Smooth Muscle Contraction. Advisor: Dr. Gary Wright


Bachelor of Science, Chemistry, Marshall University, Huntington, WV, December 1998.

AREAS OF SPECIALIZATION
Laser Scanning Confocal Microscopy
FRET Microscopy
Atomic Force Microscopy
Fluorescence Microscopy
Fluorescence Spectroscopy
Electron Microscopy
Cell Culture

PUBLICATIONS


PROFESSIONAL ORGANIZATIONS
American Physiological Society
Microscopy Society of America
American Chemical Society
American Society for Cell Biology
American Medical Writers Association
Alpha Chi Sigma – Chemistry Fraternity
National Postdoctoral Association
American Association for the Advancement of Science

DOCTORAL RESEARCH
Joan C. Edwards School of Medicine Department of Physiology, August 2001 – Present.
A7r5 smooth muscle cell culture was maintained for use in laser scanning confocal and atomic force microscopy studies of smooth muscle protein localization and interaction. Tissue characterization of rat aorta medial layer smooth muscle was also routinely performed. Dr. Gary Wright, Department of Physiology.

MASTER OF SCIENCE RESEARCH
Produced and characterized the assembly of three-dimensional DNA lattices employing UV-visible and fluorescence spectroscopy methods and atomic force microscopy. Dr. Michael Norton, Department of Chemistry.

SELECTED PRESENTATIONS


“Physiology of Blood Pressure and Electrocardiogram Measurements,” West Virginia Governor’s Honors Academy, Summer 2004.


