Summer 2003

K+-Induced Smooth Muscle Calcium Sensitization Requires RhoA Kinase (ROK) Translocation to Caveolae Which Is Inhibited in Non-Neuronal Cell Memory

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by

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A Dissertation Submitted to the Faculty of
Old Dominion University and Eastern Virginia Medical School
in Partial Fulfillment of the Requirement for the Degree of

DOCTOR OF PHILOSOPHY

BIOMEDICAL SCIENCES

OLD DOMINION UNIVERSITY
EASTERN VIRGINIA MEDICAL SCHOOL
August 2003

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ABSTRACT

K^+-INDUCED SMOOTH MUSCLE CALCIUM SENSITIZATION REQUIRES RHOA KINASE (ROK) TRANSLOCATION TO CAVEOLAE WHICH IS INHIBITED IN NON-NEURONAL CELL MEMORY

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KCl causes smooth muscle contraction by elevating intracellular free calcium ($[Ca^{2+}]_i$), while receptor stimulation activates an additional mechanism termed Ca^{2+}-sensitization that can involve activation of ROK and PKC. However, recent studies support the hypothesis that KCl may also increase Ca^{2+}-sensitivity (36). Our data showed that the PKC inhibitor, GF-109203X, did not, while the ROK inhibitor, Y-27632, did inhibit KCl-induced tonic (5') force and myosin light chain (MLC) phosphorylation in rabbit artery. Y-27632 also inhibited Bay K-8644- and ionomycin-induced MLC phosphorylation and force, but did not inhibit KCl-induced calcium entry or peak (~15") force. Moreover, KCl and Bay K-8644 nearly doubled the amount of ROK colocalized to caveolae at 30", a time that preceded inhibition of force by Y-27632. Colocalization was not inhibited by Y-27632, but was abolished by nifedipine and the calmodulin blocker, trifluoperazine. Since, ~30% of RhoA is colocalized with caveolin basally, these data suggest a novel model for Ca^{2+}-activated Ca^{2+}-sensitization, elicited by KCl contraction, that involves Ca^{2+}/cam dependent ROK translocation to caveolae and activation by RhoA.

Our laboratory previously showed that the degree of force produced for a given KCl-induced increase in $[Ca^{2+}]_i$ can be substantially altered by the history of receptor stimulation termed "non-neuronal cell memory", because the vascular smooth muscle cells stored information temporarily about the degree of prior activation (75). PE pretreatment inhibited of ROK translocation to caveolae in the subsequent KCl contraction and caused an increase in inactive RhoA. PKG activation also caused a similar inhibition of the RhoA/ROK signaling pathway. Ca^{2+}-desensitization elicited by PP1 inhibition caused caveolar internalization and inhibited of ROK translocation. PKG was also found to cause caveolar internalization, supporting the hypothesis that caveolar maintenance is kinase-mediated. These data support the hypothesis that the information storage method utilized in non-neuronal cell memory involves an inhibition of the RhoA/ROK pathway. Moreover, these data support the hypothesis that caveolae are loci for signal transduction pathways, and suggest that the membranous location of caveolae is an important factor in RhoA/ROK-mediated Ca^{2+}-sensitization.

This thesis is dedicated to my loving husband LT Timothy Thomas Urban. His support and encouragement have made the achievement of this degree possible. SOS 3:4.

ACKNOWLEDGMENTS

I extend a special thanks to my advisor Dr. Paul Ratz. His patience and encouragement amaze me. His endless creativity and the passion with which he approaches scientific investigation have motivated me to continue learning, reaching and trying. I would also like to thank all of the members of my committee for the time they have invested in my professional development and for their guidance. I thank the faculty and students at EVMS and ODU who have shown an interest in my education and those who have shared with me their knowledge and expertise. I am indebted to my lab mates who have been both friends and critics throughout this process.

I thank my parents for their excitement and enthusiasm. They have encouraged and supported me as long as I can remember. I thank my sister for setting the bar high and always reaching down to help me at every step of the journey. I thank my in-laws for trying to learn about my life and endeavors and for their constant praise, and my husband for sacrificing his needs so that I could fulfill this dream.

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LIST OF TERMS AND ABBREVIATIONS

TERM OR ABREV.	DEFINITION
Ca^{2+}	calcium
$[Ca^{2+}]_i$	intracellular free calcium
MLC	myosin light chain
RLC	regulatory light chain of myosin II
PKC	protein kinase C
PLC	phospholipase C
PIP_2	phosphatidylinositol-4,5-bis-phosphate
IP_3	inositol-1,4,5-trisphosphate
DAG	1,2-diacylglycerol
CPI-17	protein kinase C-potentiated inhibitory protein
MYPT1	myosin phosphatase targeting subunit
RHOA	small GTPase involved in Ca^{2+}-sensitization
RHOGDI	guanine nucleotide dissociation inhibitor of RhoA
GEF	guanine nucleotide nucleotide exchange factor
GAP	GTPase accelerating protein
ROK	RhoA kinase, effector of RhoA
cAMP	cyclic adenosine monophosphate
PKA	protein kinase A
cGMP	cyclic guanosine monophosphate
PKG	protein kinase G
NO	nitric oxide

EGF	epidermal growth factor
eNOS	endothelial nitric oxide synthase
LSM	laser scanning (confocal) microscope
PMT	photomultiplier tube
CAM	calmodulin
KCl	potassium chloride, depolarizes and contracts smooth muscle
PE	phenylephrine, α-adrenergic agonist
PT	phentolamine, α-adrenergic antagonist
Y-27632	ROK inhibitor
HA-1077	ROK inhibitor
GF-109203X	PKC inhibitor
NIFEDIPINE	L-type calcium channel inhibitor
BAY K-8644	L-type calcium channel agonist
IONOMYCIN	calcium ionophore
TFP	trifluoperazine, calcium/calmodulin inhibitor
8BR-cGMP	cell permeable activator of PKG
PP1	protein phosphatase 1
OA	okadaic acid, PP1 inhibitor
NAF	sodium fluoride, PP1 inhibitor

CHAPTER I

INTRODUCTION

An abnormal state of increased contraction of vascular smooth muscle is thought to play a role in hypertension, coronary and cerebral vasospasm and vascular smooth muscle cell proliferation (1). Recent studies suggest that the small GTPase RhoA and its effector RhoA kinase (ROK) play a major role in the regulation of vascular smooth muscle contraction by modulating the Ca^{2+}-sensitivity of myosin II. Ca^{2+}-sensitization, via the RhoA/ROK signaling pathway involves the inhibition of myosin light chain (MLC) phosphatase, the protein responsible for dephosphorylating the regulatory light chain of myosin II, which leads to the tonic (sustained contraction) phase of agonist-induced contractions (1). A further understanding of the mechanisms leading to Ca^{2+}-sensitization of smooth muscle is an important step in the development of treatments for vascular diseases.

This dissertation follows the style of *Circulation Research*.

CHAPTER II

ORGANIZATION AND CONTRACTION IN MUSCLE

II.1 Skeletal Muscle

The organization and contractile process of smooth muscle is quite different than that of skeletal muscle. Skeletal muscle is composed of bundles of multinucleated cells organized into fibers comprised of successively smaller subunits. Skeletal muscle fibers have a bandlike pattern due to a highly organized subcellular structure which led to the term *striated* muscle. The striated pattern of skeletal muscle is due to the repeating pattern of two sets of filaments in the bundles. The sarcomere is the basic unit of skeletal muscle structure which extends from one Z-line to the next and is composed of myosin fibers called thick filaments and actin fibers called thin filaments. The thin filaments attach to Z-lines and partially interdigitate into the myosin filaments giving the myofibril its alternating dark and light bands (2).

The thin filament is composed of two actin filaments wound in an α-helix. One strand of tropomyosin is loosely associated with the actin backbone and is thought to cover the active sites on actin. Troponin is a complex of three proteins that participates in the control of skeletal muscle contraction. The complex is composed of one subunit of each troponin I, which has a strong affinity for actin; troponin T, which has an affinity for tropomyosin; and troponin C which binds calcium (Ca^{2+}) ions.

Each myosin molecule, or thick filament, is composed of one set of two heavy chains (~200kDA each) and two sets of light chains (~20kDa each). The C-terminal of each heavy chain forms a long α-helical *tail*, and the two tails associate to form a left-handed coiled coil. The N-terminal half of each heavy chain has a globular tertiary

structure, called the *head* region, which contains the actin binding domain and the ATP-binding site. A flexible *neck* region is located between the head and tail regions and contains two calmodulin-like light chains. Several hundred myosin molecules aggregate to form the thick filament. The tails are packed in a bipolar staggered array where the heads protrude from each end and the center is a bare zone. The protruding head regions of the filaments, called *crossbridges*, link adjacent thin and thick filaments.

In skeletal muscle, an action potential in the cell membrane causes the Ca^{2+} concentration in the intracellular space to rise. Ca^{2+} binding to troponin causes a thin filament conformational change revealing the binding site for myosin on actin and allows the proteins to interact. As Ca^{2+} is removed from the intracellular space and dissociates from troponin, the thin filament returns to its original conformation and contraction ceases (3). The myosin heavy chain is an ATPase that hydrolyzes ATP to ADP and P_i to power muscle contraction. Briefly, the binding of ATP to myosin results in the dissociation of actin and myosin. The ATP is rapidly hydrolyzed to form myosin-ADP-P_i, which is the "high energy" conformation of myosin wherein the myosin head undergoes a conformational change and becomes "cocked." Next myosin-ADP- P_i weakly binds actin, and a release of P_i causes a stronger binding of myosin-ADP to actin. Subsequent release of ADP is the "power stroke" because myosin returns to its original conformation while still bound to actin. Myosin can then undergo another round of ATP hydrolysis. This describes the "walking" of myosin up the actin filament, and the sequential binding and release of actin by myosin results in muscle contraction. Therefore, in skeletal muscle, unlike smooth muscle, the activation of troponin by Ca^{2+} is necessary and, in the presence of ATP, sufficient to cause contraction.

II.2 Smooth Muscle

Smooth muscle cells are usually 20 to 500 μm in length and two to five μm in diameter and lack the striated appearance of skeletal muscle cells. There are two types of smooth muscle; multi-unit and unitary smooth muscle. Multi-unit smooth muscle is similar to skeletal muscle in that each cell can act independently and can be innervated by a single nerve ending. Multi-unit smooth muscle exists in the ciliary muscle of the eye and in the nictitating membrane in some lower animals, and is mainly controlled by nervous signals. In unitary smooth muscle, the fibers are aggregated into bundles or sheets and hundreds to thousands of muscle fibers contract together. Unitary smooth muscle, also called syncytial or visceral smooth muscle, is found in the vasculature and in the walls of most viscera of the body such as urinary bladder, gut, ureters and uterus. Gap junctions join adjacent cells so that ions can flow from one cell to another. Intercellular protein bridges exist between the dense bodies, which serve the same function as the Z lines in skeletal muscle, to allow for force transmission from one cell to another. Actin filaments are attached to dense bodies, some of which anchor the cytoskeleton to the cell membrane. The interaction of actin and myosin governs smooth muscle contraction, but the troponin complex requisite for skeletal muscle contraction is absent in smooth muscle (3).

Whereas skeletal muscle contraction which is governed by membrane potential control and the binding of calcium to a troponin, smooth muscle is not only regulated by a similar electromechanical coupling, but also by pharmomechanical coupling (4). Contractile stimuli cause an increase in intracellular free calcium ($[Ca^{2+}]_i$) from both extracellular stores, via non-specific cation channels and L-type voltage operated

channels, and intracellular stores (sarcoplasmic reticulum). This increase in $[Ca^{2+}]_i$ causes an increase in Ca^{2+} binding to calmodulin which results in activation of myosin light chain (MLC) kinase. MLC kinase phosphorylates the myosin 20kDa regulatory light chain (RLC). Smooth muscle MLC kinase is composed of a kinase catalytic core and a regulatory segment containing both a Ca^{2+}/calmodulin-binding sequence and an autoinhibitory segment. The autoinhibitory sequence prevents the binding of RLCs but not ATP. When activated by Ca^{2+}, calmodulin binds to MLC kinase at the Ca^{2+}/calmodulin-binding sequence, and the autoinhibitory segment is displaced from the catalytic core. This allows binding of the RLC and transfer of the phosphate from ATP to RLC on Ser^{19} which results in cross-bridge cycling and the production of contractile force (reviewed by 4,5,6,7). Smooth muscle MLC kinase has a different catalytic specificity than the skeletal muscle form, and in smooth muscle, MLC phosphorylation is sufficient to initiate contraction (8), but studies show that the regulation of contraction can not be fully explained by Ca^{2+}-activation of MLC kinase in all smooth muscle types (9, 10 and reviewed by 4).

A second smooth muscle regulatory system involving sensitization of contractile proteins to Ca^{2+} is now known to be responsible for an elevation in the degree of contractile force without further increases in $[Ca^{2+}]_i$, and is termed Ca^{2+}-sensitization (6, 5, 11). Proof of the existence of Ca^{2+}-sensitization was found in studies utilizing permeabilized smooth muscle cells, which allow the $[Ca^{2+}]_i$ to be held at a constant level. Studies demonstrated that in the presence of GTP, contractions could be elicited by agonists at a constant $[Ca^{2+}]_i$ (5,12, 13). Therefore, smooth muscle contraction is dependent not only on $[Ca^{2+}]_i$ and the Ca^{2+}/calmodulin-dependent activation of MLC

kinase, but also the Ca^{2+}-sensitivity (elevated degree of contractile force per unit of $[Ca^{2+}]_i$ of the contractile apparatus) (6, 5, 11,14).

II.3 Calcium Sensitization in Smooth Muscle

There are two generally accepted mechanisms proposed to govern Ca^{2+}-sensitization; regulation of the thin filament-associated proteins, called thin filament regulation, and regulation of MLC phosphorylation primarily via alterations in MLC phosphatase activity. Thin filament regulation suggests that the cross-bridges between actin and myosin are regulated by thin filament-associated proteins, such as calponin and caldesmon. Many of these studies investigate the inhibition of actin and myosin interactions and the effects of thin filament-associated proteins on cross-bridge cycling rates. Data that support this hypothesis include studies that indicate cross-bridge cycling rates can vary without concomitant changes in MLC phosphorylation, and studies showing a dissociation between tension (force) and MLC phosphorylation (15,16 and reviewed by 17; 18, 19). Data suggest that caldesmon tethers and aligns actin and myosin, but interferes with their interaction until contraction is elicited by receptor agonists. Other studies show that calponin participates in the signaling pathway of protein kinase C (PKC) activation (see Fig 1, page 7 for pathway). A complicating factor in thin filament regulation research is the issue of significant variances in results from different species and tissues. Studies also find opposing results with different agonists (reviewed by 20).

Although the precise signaling mechanisms which govern Ca^{2+}-sensitization are not known, many proposed mechanisms converge on the regulation of MLC phosphatase rather than on the regulation of thin filaments. Furthermore, some of the signaling

molecules involved in Ca^{2+}-sensitization due to MLC phosphatase inhibition have been linked to pathophysiological conditions such as high blood pressure and cerebral vasospasm and could be targets for therapeutic strategies (reviewed by 21).

Ca^{2+}-sensitization due to MLC phosphatase inhibition is produced by agents that activate G-protein-coupled receptors, phorbol esters, and arachidonic acid. Activation of G protein-coupled receptors, by Ca^{2+}-sensitizing agonists, results in the activation of phospholipase Cβ (PLC). PLC mediates the hydrolysis of phosphatidylinositol-4,5-bis-phosphate (PIP_2) into inositol-1,4,5-trisphosphate (IP_3) and 1,2-diacylglycerol (DAG). IP_3 causes the release of calcium from the sarcoplasmic reticulum (SR) which results in an increase in $[Ca^{2+}]_i$ and activation of MLC kinase. DAG and Ca^{2+} activate protein kinase C (PKC) (Fig 1).

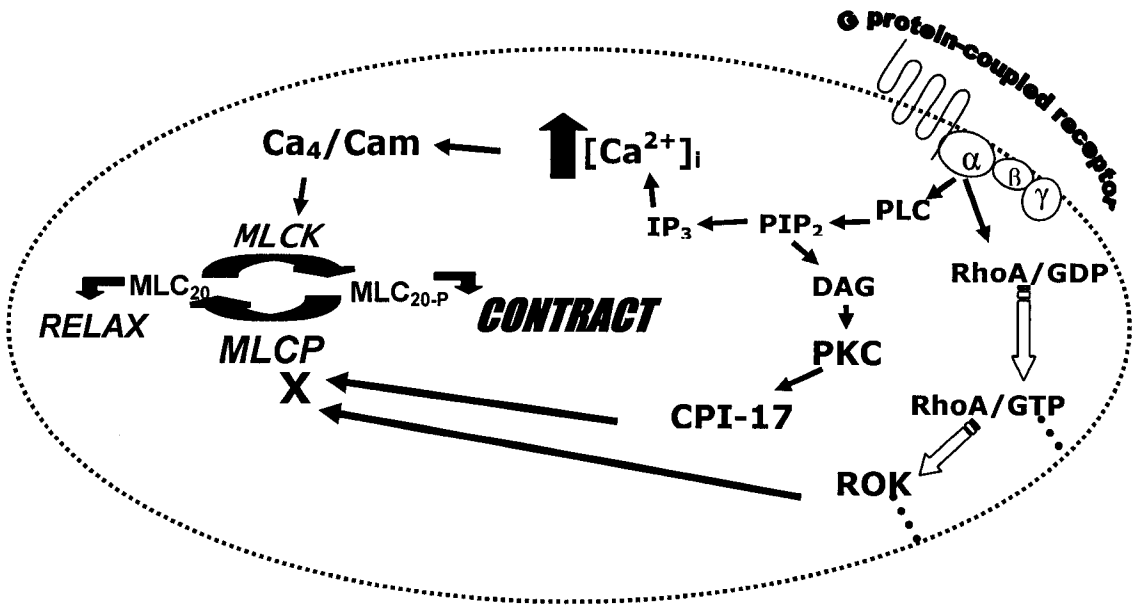

Figure 1. Model of G protein-activated Ca^{2+}-sensitization.

PKC plays a role in the Ca^{2+}-sensitization elicited by G-protein coupled receptor activation by decreasing MLC phosphatase activity (reviewed by 22). PKC inhibits

MLC phosphatase by activating the 17 kDa protein kinase C-potentiated inhibitory protein (CPI-17) by phosphorylating it on threonine[38]. MLC phosphatase is composed of three subunits; a 37 kDa catalytic subunit of protein phosphatase type 1δ (PP1c), a large 130 kDa myosin binding or targeting subunit (MYPT1), and a 20 kDa noncatalytic subunit of unknown function. Activated CPI-17 phosphorylates the PP1 subunit of MLCP which results in dissociation of the MLC phosphatase subunits and inactivation of the enzyme (23, 24, 25, 26 and reviewed by 21). Studies indicate that PKC may play a lesser role than the protein RhoA in agonist-induced Ca^{2+}-sensitization (reviewed by 1).

II.4 RhoA Function and Regulation

RhoA is a member of the Ras superfamily of small monomeric GTPases. RhoA functions as a molecular switch as it cycles between an inactive, GDP-bound state and an active GTP-bound state. RhoA is cyclically regulated such that in resting cells RhoA is bound to Rho guanosine nucleotide dissociation inhibitor (RhoGDI). RhoGDI inhibits the exchange of GTP for GDP thereby inhibiting RhoA from becoming active.

RhoA can be activated by several G-proteins such as $G\alpha_{12,13}$, $G\alpha_q$ and $G\alpha_{i-2}$ depending on the cell type and agonist involved (27, 28 and reviewed by 21). When a cell is stimulated, guanine nucleotide exchange factors (GEFs) stimulate the exchange of GTP for GDP on RhoA resulting in the targeting of GTP-RhoA to the membrane. When RhoA has activated its target and is in the GDP-RhoA conformation, RhoGDI binds GDP-RhoA and extracts it from the cell membrane. Therefore, RhoA cycles from an inactive, cytosolic RhoGDI-bound form and an activated membrane-bound form. In the cytosol, the geranyl-geranylated (hydrophibic) tail of RhoA inserts into a hydrophiboc cavity of RhoGDI. This binding is reinforced by protein-protein interactions between the

highly conserved GDI-binding site on RhoA and the N-terminus of RhoGDI (21). RhoA activity is also regulated by GTPase-activating proteins (GAPs). GAPs accelerate the intrinsic GTPase of RhoA, speeding the conversion of active GTP-RhoA to inactive GDP-RhoA (1).

RhoA is involved in the regulation of a number of cell processes which regulate cell morphology, cell aggregation, membrane ruffling, stress-fiber formation and focal-adhesion formation. In smooth muscle cells, RhoA participates in the regulation of cell contraction as a major player in G protein-coupled receptor elicited Ca^{2+}-sensitization. The effects of RhoA are thought to be mediated in large part through its effector ROK (reviewed by 21).

II.5 ROK Function and Regulation

Ttwo isoforms of ROKhave been identified; ROKα/ROCK II and ROKβ/ROCK I which are 150-160 kDa. ROK isoforms have an N-terminal kinase domain, a central coiled-coil domain, and a C-terminal pleckstrin homology (PH) domain which has a cysteine-rich zinc finger insert. The Rho-binding (RB) domain is located in the coiled-coil domain (29). Data suggests that for ROK to become activated by RhoA, GTP-bound RhoA binds to the Rho binding domain of ROK. Studies suggest that RhoA binds to the Rho binding domain of ROK results in a conformational change in ROK that disrupts the autoinhibitory interaction between the kinase domain and the catalytic domain (30).

Whereas PKC (via the actions of CPI-17) inhibits MLC phosphatase by phosphorylating the PP1 subunit resulting in dissociation of the MLC phosphatase enzyme, ROK phosphorylates the MYPT1 subunit of MLC phosphatase inhibiting its phosphatase activity. This results in an increase in MLC phosphorylation and thus

contractile force in smooth muscle (Fig 1, page 7) (31,32,33). ROK phosphorylates MYPT1 at threonine^{695}, serine^{849} and threonine^{850} (chicken isoform 133kDa) (1). Studies indicate that phosphorylation of both threonine^{695} and threonine^{850} can cause inhibition of MYPT1 (34 and reviewed by 1). It is therefore unclear which site is responsible for the Ca^{2+}-sensitizing actions of ROK.

Furthermore, there is cross talk between RhoA/ROK mediated Ca^{2+}-sensitization and PKC mediated Ca^{2+}-sensitization. ROK has been shown to activate CPI-17 (the mediator of PKC activity). Moreover, ROK can directly phosphorylate MLC at the same site as MLC kinase. However, studies suggest that ROK mediated inhibition of MLC phosphatase is responsible for its Ca^{2+}-sensitizing actions (reviewed by 1).

ROK (α/ II) has been shown to contribute to vascular tone, is upregulated in hypertension, and plays a role in cytoskeletal reorganization such as stress fiber formation (35 reviewed by 1). ROK is known to play a major role in the Ca^{2+}-sensitization elicited by a variety of receptor agonists. Interestingly, a recent study suggests that ROK inhibition by HA-1077 and Y27632 results in the inhibition K^+-*induced* force (36).

II.6 KCl-induced Calcium Sensitizaton

K^+-stimulation (KCl) of smooth muscle causes depolarization of the cell membrane. This results in opening of voltage-gated Ca^{2+} channels and an increase in $[Ca^{2+}]_i$ and muscle contraction. Therefore, KCl is often used to induce contractile protein activation independently of G protein-coupled receptor activation. Moreover, an increase in $[Ca^{2+}]_i$ is therefore a component of both K^+-depolarization-induced contraction and G protein-coupled receptor-elicited contraction.

The degree of Ca^{2+}-sensitization produced by a particular stimulus is based on the steepness of the resulting force-$[Ca^{2+}]_i$ relationship as compared to that produced upon stimulation with KCl (reviewed by 37). However, Yangisawa and Okada proposed that *KCl itself* can produce an increase in the Ca^{2+}-sensitivity of arteries (38). A very recent study also suggests that ROK plays a role in the sustained phase of KCl-induced contractions (36).

This hypothesis is supported by work from our laboratory showing that KCl-induced contraction can be desensitized following an episode of maximal receptor activation, termed non-neuronal cell memory (39,40). This means that if arteries are contracted with a high concentration of an α-adrenergic agonist, the force (but not calcium) produced in a subsequent KCl contraction is inhibited when compared to KCl contractions in control arteries (that had not been previously contracted). This Ca^{2+}-desensitization suggests that the arteries retain a "memory" of the previous receptor activation, hence the name non-neuronal cell memory. Though the signaling pathway responsibly for non-neuronal call memory remains to be determined, our laboratory also showed that the degree of Ca^{2+}-sensitivity for both KCl and receptor agonists (the degree of force produced for a given $[Ca^{2+}]_i$), is dependent on the history of receptor activation (41). The finding that both G protein-coupled receptor-elicited contractions and KCl contractions can be desensitized could suggest a common pathway for Ca^{2+}-sensitization.

Furthermore, treatment of arteries with selective ROK inhibitors results in a reduction of KCl-induced force and MLC phosphorylation without a reduction in $[Ca^{2+}]_i$ that mimics the desensitized KCl response produced with non-neuronal cell memory.

These data support a role for ROK activation in KCl-induced contraction and suggest that inhibition of ROK activation could play a role in non-neuronal cell memory.

KCl contractions consist of a rapid rise in force peaking in ~15 seconds, termed the peak phase, and a slower rise to plateau (~3.5 min.), termed the tonic phase. Data from our laboratory indicate that the tonic phase of KCl-induced force is inhibited by the ROK inhibitor Y-27632 without causing an inhibition of intracellular Ca^{2+}, suggesting that ROK-induced Ca^{2+}-sensitization plays a role in the maintenance of force elicited by KCl in vascular smooth muscle. The precise signaling mechanisms involved in KCl-induced stimulation of vascular smooth muscle have not been fully resolved.

Ca^{2+}-sensitization involves the coordination of complex signal transduction events from the plasma membrane to the contractile apparatus. Receptor agonist stimulation that causes Ca^{2+}-sensitization has been shown to result in a redistribution of signaling molecules to the cell membrane (42). Although KCl appears to cause Ca^{2+}-sensitization, whether KCl-induced contractions result in a recruitment of signaling molecules to the plasma membrane of smooth muscle cells remains to be determined.

II.7 Cross talk involving Cyclic Nucleotides

Recent studies suggest that there is cross talk between the cyclic nucleotides, their respective protein kinases and the signaling pathways governing Ca^{2+}-sensitization and Ca^{2+}-desensitization (reviewed by 43). Smooth muscle relaxation can be achieved in two ways; one way is by removal of the contractile stimulus which is called passive relaxation. Active relaxation, however is due to the actions of cAMP-dependent protein kinase (PKA) and cGMP-dependent protein kinase (PKG). Studies indicate that cGMP mediates the relaxing effects of atrial natriuretic peptide and nitric oxide (NO). PKG

relaxation involves an inhibition of PLC activation, IP_3 production and intracellular calcium mobilization (reviewed by 43).

Furthermore, PKG is known to cause Ca^{2+} desensitization by dephosphorylation of myosin light chains (44,45,46,47). Studies suggest that G protein-coupled receptors, PLC and MLC kinase are substrates for PKA and PKG. Additionally, PKG acting downstream of cGMP has been shown to inhibit RhoA-dependent Ca^{2+}-sensitization (48). One study suggests that PKG could participate in Ca^{2+}-desensitization by phosphorylating active RhoA. PKG phosphorylation of RhoA results in its removal from the membrane, increasing its interaction with RhoGDI and stabilizing it in the inactive form (49). Another study suggests that ROK is a substrate for PKG (22). Though the role of cyclic nucleotides in smooth muscle contraction is complex and not fully understood there is evidence of cross talk between cyclic nucleotide dependent protein kinases and Ca^{2+}-sensitization.

CHAPTER III

CAVEOLAE

III.1 Defining Caveolae

Caveolae are invaginations of the plasma membrane found in many cell types, including smooth muscle cells, hypothesized to participate in clatherin-independent endocytosis, cholesterol transport and calcium signaling (50,51,52,53 and reviewed by 54). Though historically studied as transporters of molecules across endothelial cell membranes, caveolae now are being viewed as a multifunctional membrane system. The name caveolae intracellularis (little caves) was proposed by Yamada in 1995 to describe "a small pocket ... communicating with the outside of the cell" in gallbladder cells. Palade described similar structures in endothelial cells, but because he observed them shuttling molecules in the cells, he referred to them as plasmalemmal vesicles (55). Other studies supported Palade's hypothesis, that these were endocytic vesicles, but little work was done to determine the role of these structures in other cell types. The recent discovery of the integral caveolar proteins, caveolin, have renewed interest in caveolar function (56). Current caveolae research focuses on the compartmentalization of signal transduction molecules, and the idea that caveolin acts as a scaffold for signaling pathways by coordinating extracellular stimuli and intracellular effectors (57).

Caveolae can have a variety of morphological characteristics including a tubular, vesicular or flat shape and can be either open or closed at the cell surface. In smooth muscle, caveolae are relatively static structures and usually appear as singular invaginations in the cell membrane. Caveolae are 50-90 nm concavities along the longitudinal axis of smooth muscle cells interspersed with dense bodies (57).

Biochemical characterization includes resistance to Triton X-100 solubilization at 4°C, a light buoyant density and enrichment of cholesterol, glycosphingolipids, sphingomyelin, and glycoslyphosphatidlyinositol-anchored protein (54).

III.2 Caveolins

At least four mammalian isoforms of caveolin, caveolin-1α, -1β, -2 and -3 (22kDa) have been identified. Studies suggest that there is ubiquitous expression of each caveolin isoform in smooth muscle, specifically caveolin-1 and -3 are found through the arterial vasculature. Moreover, caveolin assumes a primarily plasma membrane distribution in smooth muscle cells (57). Caveolin isoforms interact to form homo- and hetero-oligomers that are stabilized by and require cholesterol for insertion into lipid membranes (reviewed by 58). A hydrophobic domain of 33-amino acids (residues 102-134) anchors caveolin to the cell membrane such that both the amino and carboxy terminals of caveolin are free in the cytosol (53). An N-terminal cytoplasmic region of caveolin-1 (residues 61-101) directs the interaction of caveolins to form hetero- and homo-oligomers (58). Within this region there is a "scaffolding domain" (residues 82-101) that is critical for the regulatory interactions of caveolin (59). The C-terminal domain (residues 135-178) acts as a bridge and allows oligomers of caveolin to interact with one another (58).

III.3 Caveolae in Signal Transduction

The caveolin scaffolding domain is necessary for the regulatory interaction of caveolin with many signal transduction molecules. Studies using the caveolin scaffolding domain have led to the identification of amino acid motifs common to many signaling molecules. Motifs containing ΨXΨXXXXΨ and ΨXXXXΨXXΨ where Ψ = is an

aromatic amino acid have been found in many molecules suggested to interact with or bind to caveolin (57). These motifs have been found in the switch 1 region of RhoA (residues 34-42), the catalytic domain of ROK (residues 151-159 and 164-181) and the catalytic domain of PKC-α (residues 522-528) (57).

Recent studies have shown caveolae to contain multiple receptors including the β-adrenergic, inositol trisphosphate (IP_3), muscarinic (M_2), endothelin and epidermal growth factor (EGF) receptors. Caveolae also contain numerous signal transduction molecules including G-protein α and β subunits, Src-family tyrosine kinases, protein kinase C (PKC) isoforms, Ras, calmodulin, and effectors such as mitogen activated protein (MAP) kinase, endothelial nitric oxide synthase (eNOS), and adenylyl cyclase (53). L-type Ca^{2+} channels, calreticulin and calsequestrin were found in caveolae-enriched membranes which suggests a role for caveolae in smooth muscle Ca^{2+} handling (60).

Studies involving the cytoplasmic delivery of a peptide encoding the scaffolding domain result in functional inactivation of G-protein α subunits, EGFR and PKC, thereby suggesting a role for caveolin as a general kinase inhibitor. Other studies indicate that caveolin does not inactivate Ha-Ras which suggests a role for caveolae as a site for the coordination of activated proteins in some signaling cascades. Some comparisons have been made of the caveolin-1 scaffolding domain to other modular protein domains, such as the plekstrin-homology domain. Therefore, there is a general hypothesis that caveolin acts as "molecular Velcro to nucleate the formation of signal transduction complexes" (58).

Receptor agonist stimulation that causes Ca^{2+}-sensitization has been shown to result in a redistribution of receptors for contractile agonists to caveolae (61). Specifically, muscarinic stimulation of isolated smooth muscle cells resulted in significant redistribution of ROK, RhoA and PKC-α to the cell periphery (62). Furthermore, the introduction of a peptide corresponding to the scaffolding domain of caveolin-1, into the cytosol of smooth muscle cells, inhibits the translocation of RhoA and PKC elicited by receptor agonist stimulation (62). These data suggest that caveolae, via the scaffolding actions of caveolin-1, could play a role in coordinating the signals leading from receptor agonist stimulation to Ca^{2+}-sensitization.

III.4 Caveolae Disruption

It has been shown that cholesterol is necessary for invagination and maintenance of caveolae, but a recent study shows that both microtubules and actin filaments also play a role in regulating the caveolar membrane system (63). The caveolae of cells incubated with cytochalasin D, which partially disassembles actin filaments, moved from a membranous distribution to an accumulation in the center of the cell. Interestingly, cells incubated with nocodazole, which depolymerizes microtubules, showed linear arrays of caveolae at the cell surface (63). These data could suggest different roles for actin filaments and microtubules in caveolar maintenance.

Furthermore okadaic acid, a protein phosphatase inhibitor, causes internalization of caveolae as determined by a decease in the number of invaginations and an intracellular accumulation of vesicles (64,54). This internalization was inhibited by staurosporine, a kinase inhibitor, suggesting that kinase activity may also be necessary to maintain the population of caveolae at the cell membrane (64). Studies suggest that the

membranous location of caveolae is necessary for it to coordinate signals between the extracellular space and the intracellular environment (62), but the specific effects of caveolar internalization on ROK-mediated Ca^{2+}-sensitization are not known.

CHAPTER IV

CONFOCAL MICROSCOPY

IV.1 Theory and Uses of Confocal Microscopy

An increase in the use of fluorescent microscopy in contemporary biology has revealed the limitations of wide-field (conventional) light microscopy. Light emitted or scattered by objects out of the plane of focus blur the image. One advance that can partially overcome the problems of conventional fluorescence microscopy is digital deconvolution software which can mathematically remove this out of plane light to sharpen an image. An alternative solution involves the use of confocal microscopy.

The light from a point source, which, in the case of laser scanning confocal microscopy (LSM) is a laser, is projected onto an object in the plane of interest. Light (fluorescence) from the focal plane is projected onto a variable pinhole diaphragm (which, for these studies, was set at 0.5μm). This light passes through the same objective used for focusing and then through a tube lens. Therefore, the focused spot on the specimen and the pinhole are at optically conjugate points. Essentially no light, except that coming from the plane of interest in the specimen, can pass through the pinhole and reach the detector. This virtually eliminates the blurring effect of out of plane light seen in conventional light microscopy.

Two galvanometer mirrors steer the illumination spot over the specimen. The fluorescence emitted at the focal plane, and light emitted at planes above and below it, is directed onto a main dichroic mirror that separates the emission fluorescence from the excitation light. The emission fluorescence is further separated by a series of dichroic beam splitters and directed to photomultiplier tubes (PMTs).

The resolution of the scanned area is variable (but for this project was set at 512 X 512) and the Z-resolution (0.3 μm), termed the optical section thickness, is determined by the microscope software and is based on the numerical aperture of the objective and the pinhole. In the line scanning mode, the lasers scan one line of the desired focal area, one at a time, and the analog signal from the PMTs is converted to a digital signal. This results in an almost simultaneous image from multiple laser excitations. Control of the focus movement (Z axis) allows for a series of images to be taken and projected as a three dimensional image, or analyzed as a stack of images.

CHAPTER V

SPECIFIC AIMS

The goals of this project are to investigate the signaling mechanisms leading from KCl stimulation to Ca^{2+}-sensitization in vascular smooth muscle and the mechanisms involved in the desensitization of KCl contractions following an episode of maximal receptor activation, termed non-neuronal cell memory. I hypothesize that calcium entry through L-type calcium channels and Ca^{2+}/calmodulin activation elicited by KCl stimulation leads to translocation of ROK to caveolae and subsequent activation by RhoA. I further hypothesize that Ca^{2+}-desensitization in non-neuronal cell memory of vascular smooth muscle involves two aspects of the RhoA/ROK signaling pathway; an inhibition in ROK translocation to caveolae and an increase in inactive, GDI-bound, RhoA possibly due to RhoA phosphorylation and removal from the cell membrane. Thus, the specific aims of this project are: (1) Determine the mechanism of tonic force maintenance in KCl- induced ROK-mediated Ca^{2+}-sensitization of arterial muscle; (2) Determine the mechanism by which force maintenance is down regulated in non-neuronal cell memory.

CHAPTER VI

MATERIALS AND METHODS

VI.1 Tissue Preparation

Tissues were prepared as previously described (65). Femoral and renal arteries from adult New Zealand white rabbits were cleaned of adhering tissue and stored in cold (0-4°C) physiological saline solution (PSS). The PSS was composed of, in mM, NaCl 140, KCl 4.7, $MgSO_4$ 1.2, $CaCl_2$ 1.6, $NaHPO_4$ 1.2, morpholino-propanesulfonic acid (MOPS) 2.0 (adjusted to pH 7.4), Na_2ethylenediamine tetraacetic acid (EDTA, to chelate heavy metals) 0.02, and D-glucose 5.6. To produce K^+-depolarization, KCl (110 mM) was substituted isosmotically for NaCl. High purity water, 17 megaΩ distilled and deionized, was used throughout. The endothelium of each artery was removed by gently rubbing the intimal surface with a metal rod. Tissues were cut into 3-4 mm wide artery rings.

Each muscle ring was secured in a tissue bath (Radnoti Glass Technology, Inc., Monrovia, CA) between stainless steel wire stirrups attached to a micrometer and isometric force transducer for, respectively, length adjustments and isometric force measurements (Model 52, Harvard Apparatus, South Natick, MA). Isometric contractions were measured as previously described (65). Voltage signals from force transducers were digitized (CIO-DAS16F, Computer Boards, Middleboro, MA), and visualized on a computer screen as force (g). Data acquisition and analyses were accomplished using DasyLab (DasyTec, Amherst, NH) and Microsoft Excel (Microsoft Corp.)

VI.2 Isometric Force

Contractile force (F) was measured as previously described (65). Tissues were allowed to equilibrate for 1 hour at 37°C in PSS with aeration. The muscle length for which active force was maximum (L_o) was determined for each tissue using an abbreviated length-tension curve and KCl (110 mM, substituted isosmotically for NaCl) as the stimulus (66,67). For each preloaded tissue, the degree of steady-state force (F) produced at L_o by incubation in KCl for 5-10 minutes was equal to the optimal force for muscle contraction (F_o), and subsequent contractions were calculated as F/F_o. Bay K-8644 (0.56-1 μM), a nifedipine analog that increases the open-state probability of L-type voltage-operated Ca^{2+} channels, was utilized as a contractile agent in some experiments (68,69). To produce contraction with Bay K-8644, it was necessary to pre-incubate femoral arteries with 7.5 mM KCl, a concentration of KCl which, alone, did not cause contraction. Ionomycin (10 μM), a calcium ionophore, and phenylephrine (PE), an α-adrenoreceptor agonist, were also used as contractile agents in some experiments. No further length changes were imposed once L_o was established. All tissues, except those contracted using PE, were incubated with 1 μM phentolamine (PT) to block potential α-adrenergic receptor activation caused by the release of norepinephrine from peri-arterial nerves that may occur upon stimulation with KCl.

VI.3 Myosin Light Chain (MLC) Phosphorylation

The degree of MLC phoshorylation was measured as previously described (65). Arteries were quick-frozen in an acetone-dry ice slurry, slowly warmed to room temperature, dried, weighed, and homogenized in 8M urea, 2% Triton X-100 and 20 mM dithiothreitol. Isoelectric variants of the 20 kDa myosin light chains (MLCs) were

separated by 2-D (IEF/SDS) PAGE followed by Western Blot with visualization using colloidal gold staining. The relative amounts of phosphorylated and non-phosphorylated MLCs were quantified by digital image analysis (Scion Image, NIH).

VI.4 Intracellular Free Calcium

$[Ca^{2+}]_i$ was measured in renal arteries as previously described (65). Arterial rings positioned inside a quartz cuvette housed in a fluorometer (Photon Technology International, Lawrenceville, NJ) and maintained at 37°C were loaded for 2.5 hours with Fura-2 (PE3)/AM (~7.5mM) and Pluronic F-127 (0.01% w/v) (TefLabs, Austin, TX) to enhance solubility. Tissues were then washed for 30 minutes in 3 changes of PSS. The fluorescence emission at a wavelength of 510 nm was collected for alternating excitations at 340 and 380 nm. The $[Ca^{2+}]_i$ signals produced during KCl-induced steady-state contraction in the absence and presence of Y-27632 were quantified as the fraction of the steady-state value produced during the tonic phase of the KCl contraction used for the L_o determination, and the minimum signal produced in a Ca^{2+}-free solution containing 5 mM EGTA plus 10 μM ionomycin, corrected for background fluorescence. The background fluorescence was recorded by incubating tissue in a 4 mM $MnCl_2$ quench solution containing 10 μM ionomycin.

VI.5 Tissue and Slide Preparation for Colocalization

The extent of ROK co-localization with caveolin at the plasma membrane of femoral artery smooth muscle cells was investigated. Femoral artery rings 3-4 mm long were treated as described in "Tissue Preparation". No difference in ROK colocalization to caveolin was identified in stretched (L_o) versus un-stretched (L_z) tissues under basal conditions (L_o: 24 ± 2%, L_z: 18 ± 2%, n=3, P>0.05) and when stimulated with KCl for 30

sec (L_o: 34 ± 2%, L_z: 34 ± 2%, n=3, P>0.05). Un-stretched artery rings, which were technically easier to fix than stretched tissues, were used for all subsequent histological studies. Tissues stimulated with KCl were fixed at 30 sec and 5 min. Colocalization of ROK and RhoA with caveolin, RhoA with caveolin, and caveolin with the nucleus in KCl-stimulated tissues was compared to that found in tissues not contracted with KCl (basal). Tissues stimulated with PE were fixed at 30 sec, 5 and 30 min, following 10 min PE washout, and at 30 sec and 5 min of the subsequent KCl contraction. Colocalization, of ROK and RhoA with caveolin, RhoA with caveolin, and caveolin with the nucleus in PE-stimulated tissues was compared to that found in tissues not contracted with PE (basal).

Tissues were fixed in 100% methanol at -20°C for 5 minutes and then placed in a solution containing 5% sucrose for 1 hour. Tissues were rapidly frozen in Optimal Cutting Temperature (OCT) frozen tissue embedding medium (VWR Scientific) using a histobath (Histobath2 Low Temperature Freezing Bath, Thermo Shandon Inc., Pittsburg, PA). Frozen tissues were cut into ~18 μm thick sections on a cryostat (Microm HM505E, Richard Allan Scientific, Kalamazoo, MI), and each section was placed onto charged slides (SuperFrost Plus, Fisher Scientific). Slides were dried for a minimum of 1 hour prior to storage at -80°C.

VI.6 Immunohistochemistry

Slides were thawed at room temperature for 30 minutes and washed twice in PSS. Small wells were created on each slide using an ImmEdge Pen (Vector Labs, Burlingame, CA). Tissues were permeabilized and blocked in one step using a solution of 0.1% Triton X-100 and 3% normal goat serum (Vector Labs, Burlingame, CA) in PSS.

The slides were washed twice in PSS and incubated with primary antibodies to ROK (anti-rock-2, goat polyclonal, Santa Cruz Biosciences, Inc., Santa Cruz, CA), caveolin (anti-caveolin, rabbit polyclonal, BD Biosciences), RhoA (anti-RhoA, mouse monoclonal, Santa Cruz Biosciences, Inc., Santa Cruz, CA), or RhoGDI (anti-RhoGDI, rabbit polyclonal, Santa Cruz Biosciences, Inc., Santa Cruz, CA) for one hour at an optimized dilution of 1:50. The anti-caveolin antibody used in this study does not discriminate between the 3 isoforms of caveolin. Slides were washed twice in PSS and incubated with fluorophore-conjugated secondary antibodies (Alexa Fluor 488 donkey anti-goat, for ROK; Alexa Fluor 594 chicken anti-rabbit, for caveolin and RhoGDI; and Alexa Flour 488 donkey anti-mouse, for RhoA; Molecular Probes, Inc., Eugene Oregon) for one hour at an optimized dilution of 1:200. For nuclear staining, slides were washed twice in PSS and incubated with a nuclear stain (TO-PRO-1 iodide monomeric cyanine nucleic acid stain iodide, Molecular Probes, Inc., Eugene Oregon) for 10 minutes at a dilution of 1:80,000. Tissue sections were washed a final time and coverslips were applied with an anti-fading medium (VectaShield Anti-fading Medium, Vector Labs, Burlingame, CA). Slides were viewed and images recorded using a laser scanning confocal microscope (Zeiss LSM 510, Carl Zeiss Microscopy, Germany). Each tissue section was excited with two lasers, Krypton/Argon (488 nm) and Helium/Neon1 (543 nm), using a rapid line-scanning protocol for excitation, and emission signals were collected through, respectively, 505-550 nm band pass and 560 nm long pass emission filters and quantified using a photomultiplier tube and proprietary software. For display purposes, a composite image was constructed in which, for example, ROK was green, caveolin was red and signal overlap (colocalization) was yellow. For RhoA

colocalization to caveolin, RhoA was green and caveolin was red. For RhoA colocalization with RhoAGDI, RhoA was green and RhoGDI was red, and for caveolin colocalization to the nucleus, caveolin was red and the nucleus was green.

Approximately 11 images were recorded in the z-dimension, which constituted one z-series. Images were taken 0.5 μm apart with a zoom of 2X on a 100X oil immersion objective. The pinhole was 0.5 airy units. One slide was randomly selected for viewing and analyses from each sectioned artery ring. A minimum of 2 z-series were recorded for each slide from different areas of the tissue. Images were exported in an uncompressed tagged-image format (tif) file and image analysis was conducted using MetaMorph software (Version 4.6r9, Universal Imaging Corporation).

VI.7 Image Analysis and Data Collection

Five consecutive images selected from two different z-series per slide permitted 10 images to be averaged for each artery ring. MetaMorph software (V4.6r9 Universal Imaging Corp.) separated each composite image into two images, one for each laser. The software compared the images, pixel by pixel, and determined the percentage overlap of the fluorescent signals. For example, in specific aim 1 we tested the hypothesis that KCl increased the degree of ROK translocation to caveolae at the plasma membrane, so colocalization of the two fluorophores was determined as the percentage overlap of the ROK signal onto the caveolin signal. RhoA colocalization with caveolin, RhoA colocalization with RhoGDI, and caveolin colocalization with the nucleus were determined in the same manner. Colocalization measured for all 10 images was averaged using a spreadsheet (Excel, Microsoft Corp.) and considered an *n* of 1.

VI.8 Drugs

Nifedipine and trifluoperazine (TFP), okadic acid, phenylephrine and 8br-cGMP were from Sigma Corporation. Bay K-8644, Y-27632 and ionomycin were from Calbiochem Corporation. HA-1077 and GF-109203X were from Alexis Corporation. Nifedipine, Bay K-8644, 8br-cGMP and ionomycin were dissolved in ethanol. Y-27632, phenylephrine and okadaic acid were dissolved in water. Ethanol was added at a final concentration no greater than 0.1%, a concentration that had no effect on KCl- or Bay K-8644-induced contractions or on the degree of ROK co-localization with caveolin.

VI.9 Statistics

The null hypothesis was examined using Students' t-test (when 2 groups were compared) or using a one-way analysis of variance (ANOVA). To determine differences between groups following ANOVA, the Student-Neuman-Keuls post-hoc test was used. In all cases, the null hypothesis was rejected at $P<0.05$. For each study described, the *n* value was equal to the number of rabbits from which arteries were taken.

CHAPTER VII

RESULTS

VII.1 Effect of ROK, PKC, and L-type calcium channel inhibitors on KCl-induced force

A KCl-induced contraction (110 mM KCl substituted isosmotically for NaCl) of rabbit femoral and renal artery consisted of a rapid rise in force that reached a maximum value within ~15 sec (Peak, Fig 2A) followed by a slower rise to a sustained level within ~3-5 minutes (Tonic; Fig 2A). To determine the effect of ROK inhibition on KCl-induced contraction, tissues were exposed to two different ROK inhibitors; Y-27632 (1 and 3 μM) or HA-1077 (10 μM), then contracted with KCl. Although peak force was not affected (Fig 2B), both Y-27632 and HA-1077 strongly inhibited tonic force by over 50% (Fig 2C).

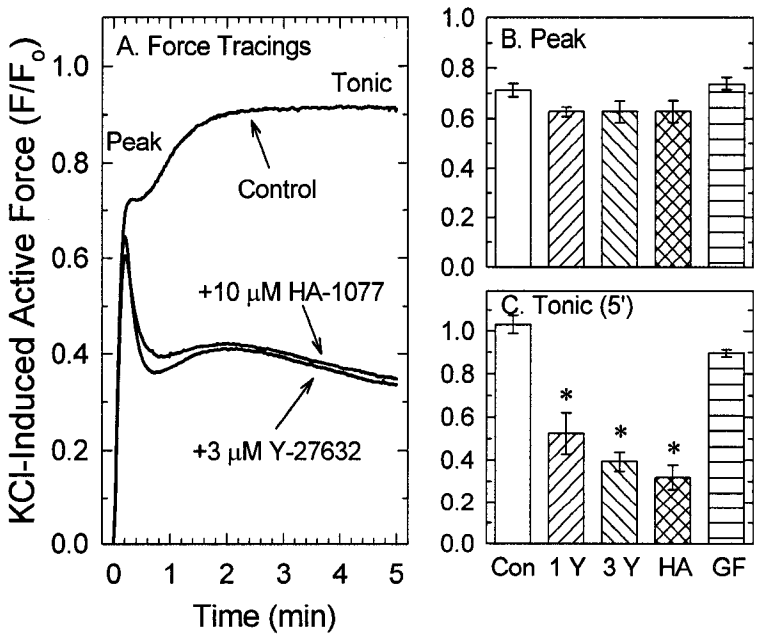

Figure 2. Effects of ROK inhibitors, Y-27632 (Y; 1 and 3 μM) and HA-1077 (HA; 10 μM), and the $PKC_{n,c}$ inhibitor, GF-109203X (GF; 1 μM), on peak (B) and tonic (C) KCl-induced force. Control (Con) tissues were not exposed to a drug. Typical force tracings are shown in panel A. Data in B and C are mean ± SE; n = 3-5; * $p < 0.05$ compared to control.

PKC has been shown to play a role in receptor agonist-induced Ca^{2+} sensitization, so we also investigated the role of PKC in KCl-induced contraction. GF-109203X, when used at 1 μM, inhibits conventional and novel isoforms ($PKC_{c,n}$) of PKC (32,70,71). GF-109203X did not inhibit KCl-induced peak or tonic force (Figs 2B and 2C). These data suggest that ROK does, but $PKC_{c,n}$ does not, play a role in the maintenance of KCl-induced tonic force in rabbit large arteries.

VII.2 Effect of Y-27632 and nifedipine on KCl-induced force and $[Ca^{2+}]_i$

Inhibition of KCl-induced tonic force by Y-27632 could be caused by inhibition of ROK, or by inhibition of Ca^{2+} entry caused by a non-specific effect of the drug on Ca^{2+}channels. We therefore measured the effect of Y-27632 on steady-state increases in force and $[Ca^{2+}]_i$ produced by KCl. Tissues were loaded with the Ca^{2+} indicator, fura-2 (see "Methods") and contracted with KCl. Y-27632 (1 μM) was added and force and $[Ca^{2+}]_i$ were allowed to reach a new steady-state. The L-type calcium channel inhibitor, nifedipine (10 nM), was subsequently added to completely relax tissues by causing Ca^{2+} channel blockade (Figs 3A and 3C). Y-27632 reduced tonic KCl-induced force by nearly 40% (Figs 3A and 3B) but did not alter tonic $[Ca^{2+}]_i$ (Figs 3C and 3D). Nifedipine further reduced tonic force to the basal level (Figs 3A and 3B) and nearly abolished $[Ca^{2+}]_i$ (Fig 3C and 3D). These data indicate that 1 μM Y-27632 does not block KCl-induced calcium entry, and suggest that KCl induces the activation of ROK.

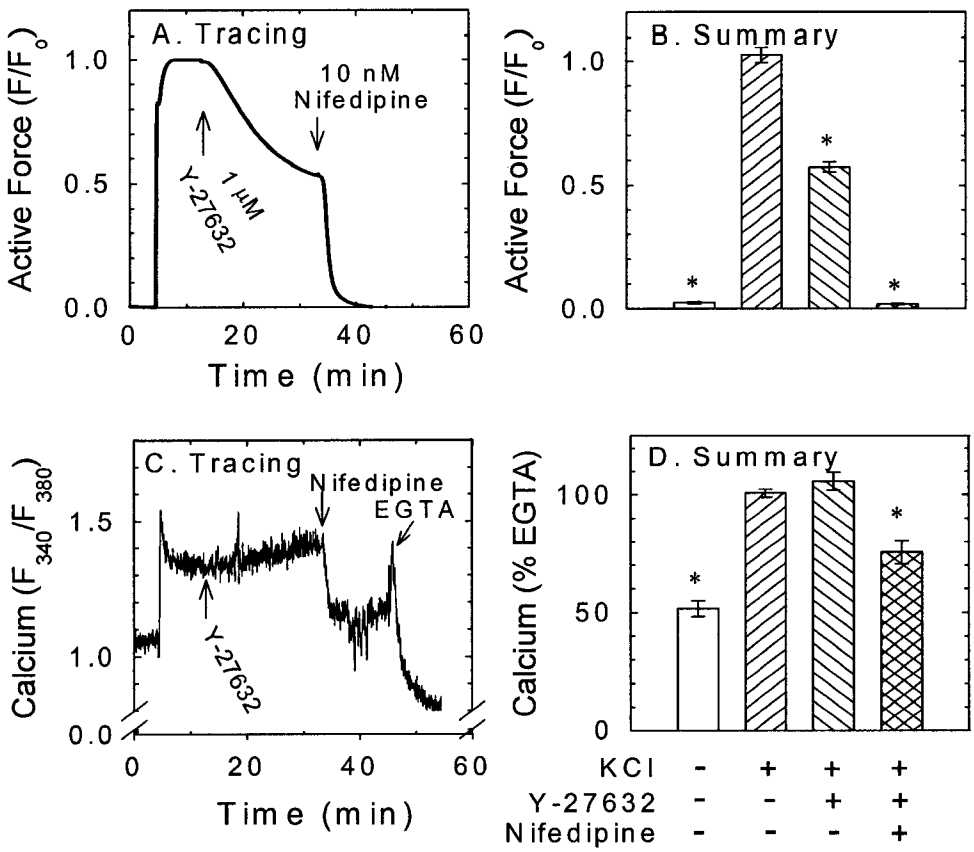

Figure 3. Effect of Y-27632 (1 μM) and nifedipine (10 nM) on KCl-induced force (A & B) and $[Ca^{2+}]_i$ (C & D). Typical force and calcium tracings are shown in, respectively, A & C. Data in B and D are mean ± SE; *n*=3; * $p< 0.05$ compared to KCl-induced responses.

VII.3 Effect of Y-27632 on KCl-, Bay K-8644-, and ionomycin-induced MLC phosphorylation and force

The level of MLC phosphorylation basally was 9% ± 1%, and KCl-induced contraction resulted in an increase in MLC phosphorylation to ~37%. Y-27632 (1 μM) nearly abolished this increase (Fig 4A). We also investigated the effect of Y-27632 on responses produced by an agent that selectively activates L-type Ca^{2+}channels to cause Ca^{2+} entry and contraction. Bay K-8644 (0.56 μM), a nifedipine analog but Ca^{2+}channel agonist, produced a slow rise in force that reached a plateau in ~20 min. Y-27632 (1 μM)

nearly abolished Bay K-8644-induced steady-state MLC phosphorylation (Fig 4A) and reduced steady-state force by over 70% (Fig 4B). We next examined the ability of Y-27632 to inhibit responses produced by the Ca^{2+} ionophore, ionomycin, an agent that can bypass L-type calcium channels to produce contraction by Ca^{2+} entry or release of intracellular Ca^{2+}. Ionomycin (10 μM) produced a rise in force that reached a plateau in ~6.5 min. Y-27632 (3 μM) nearly abolished ionomycin-induced MLC phosphorylation (Fig 4A) and force (Fig 4C). The ionophore-dependent transfer of Ca^{2+} ions into smooth muscle cells elicited by ionomycin has been shown to cause membrane depolarization and thus force development (72). As seen in Fig 4C, we found that 1 μM nifedipine did not inhibit 10 μM ionomycin-induced contraction in rabbit femoral artery.

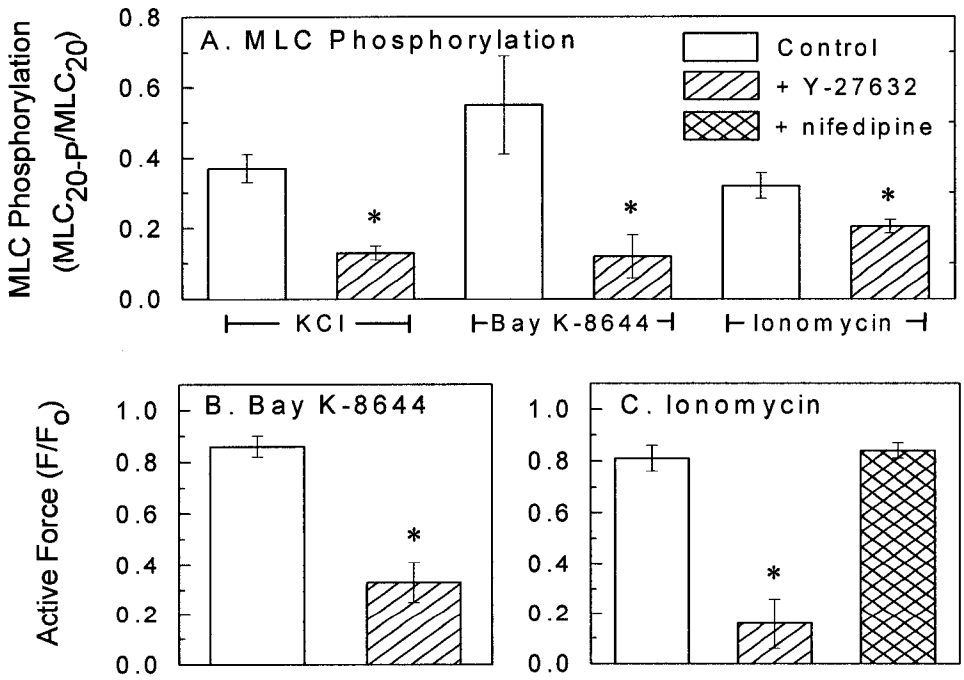

Figure 4. Effect of the ROK inhibitor, Y-27632 (1 μM) on KCl-induced, 0.56 μM Bay K-8644-induced and 10 μM ionomycin-induced MLC phosphorylation (A) and Bay K-8644- and ionomycin-induced force (B and C, respectively). Effect of 1 μM nifedipine on ionomycin-induced force (C). Control tissues were not exposed to a drug. Data are mean ± SE; n=3-5; * $p < 0.05$ compared to control.

VII.4 Time dependent effects of Y27632 and nifedipine on KCl-induced force and MLC phosphorylation

Basal force and MLC phosphorylation were not affected by either 1 μM Y27632 or 1 μM nifedipine (Fig 5). Whereas nifedipine nearly abolished the KCl-induced increase in force and phosphorylation at 10 seconds and 1 and 5 minutes of contraction, Y27632 did not decrease KCl-induced force and phosphorylation at 10 seconds, but did inhibit both force and phosphorylation at 1 and 5 minutes of contraction (Fig 5).

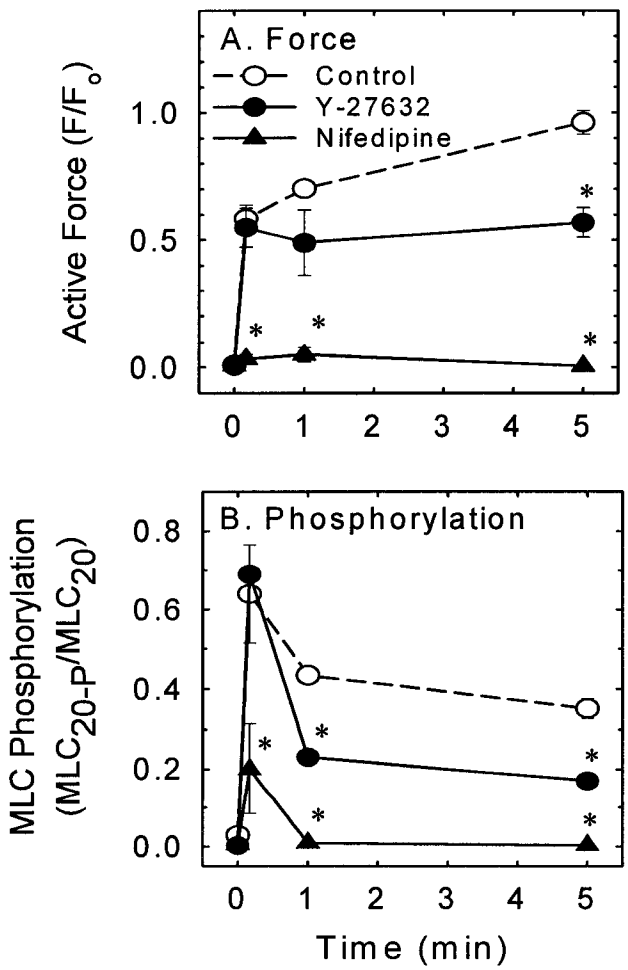

Figure 5. Effect of 1 μM Y-27632 and 1 μM nifedipine compared to control (no drug addition) on KCl-induced increases in force (A) and MLC phosphorylation (B). Data are mean ± SE; *n*=3-6; * $p < 0.05$ compared to control.

VII.5 Effect of KCl and Bay K-8644 on ROK colocalization with caveolin at the cell periphery

In an image of an unstained femoral artery, captured using a 40X oil immersion objective of a laser scanning confocal microscope (see "Methods"), auto-fluorescence was evident in the adventitia and intima, shown, respectively, on the upper left and lower right corners of the image of Fig 6A. The media, however, displayed very little auto-fluorescence, although elastic lamina between layers of smooth muscle cells could be seen (Fig 6A). Images at 200X used for fluorescence co-localization measurements in this study were taken from areas of the media between elastic lamina rich in vascular smooth muscle cells and nearly devoid of auto-fluorescence.

The secondary antibodies used in this study (see "Methods"), when applied alone displayed minimal fluorescence when a section of rabbit femoral artery media was viewed at 200X between layers of elastic lamina (Fig 6B). However, vascular smooth muscle cells were clearly evident in fluorescence confocal images taken from tissues labeled with ROK and caveolin primary antibodies such that caveolin appeared red (Fig 6C) and ROK appeared green (Fig 6D). Caveolin was localized at the cell periphery, as expected (Fig 6C), while ROK was distributed throughout the cytosol and at the cell periphery (Fig 6D). Figures 6C-E are representative images of our data showing ROK and caveolin staining. Due the secondary antibodies used, in images showing RhoA colocalization with caveolin, RhoA appeared as green and caveolin appeared as red. In images showing colocalization of RhoA to RhoGDI, RhoA appeared as green and RhoGDI appeared as red, and in images showing caveolin colocalization with the nucleus, caveolin appeared as red and the nucleus appeared as green.

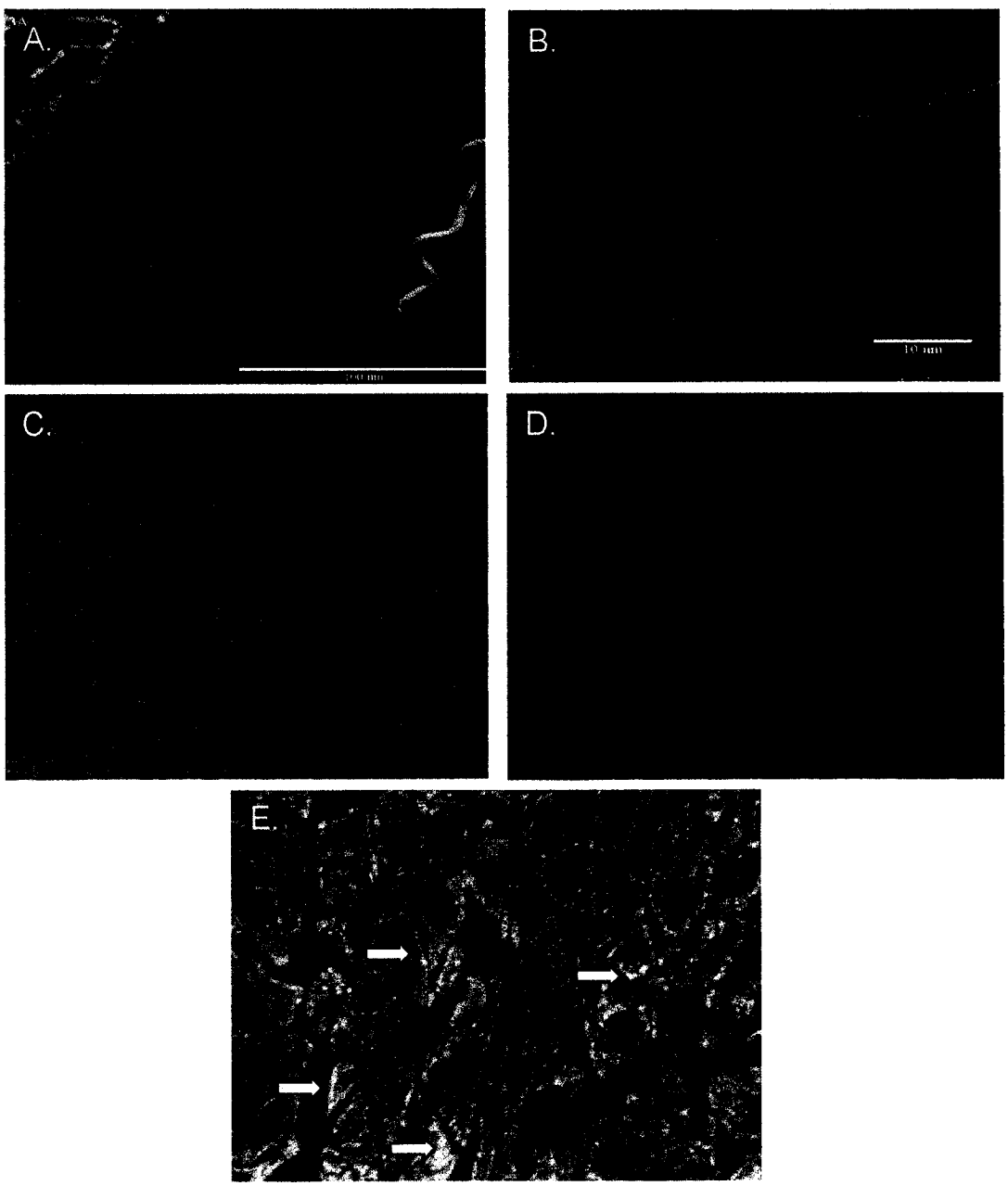

Figure 6. Laser scanning confocal images of rabbit femoral artery. A) unstained 40X image showing auto-fluorescence in adventitia (upper left) and intima (lower right). B) 200X image labeled with Alexa Fluor 488 donkey anti-goat, and Alexa Fluor 594 chicken anti -rabbit 2° antibodies, showing minimal non-specific labeling. C-E) 200X image of an artery stimulated for 30" with KCl double-labeled with anti-ROKα goat polyclonal antibody (plus Alexa Fluor 488 donkey anti-goat 2°) and anti -caveolin rabbit polyclonal antibody (plus Alexa Fluor 594 chicken anti-rabbit 2°), as described in "Methods". Image E) is a composite of images C) showing caveolin (red) staining only and D) showing ROK (green) staining only. The yellow color in "E" represents ROK colocalization with caveolin.

In un-contracted (basal) tissues ~18% of ROK was colocalized with caveolin at the cell periphery (quantified using MetaMorph software as described in "Methods", and visualized graphically as yellow in Fig 6E). The amount of ROK co-localized with caveolin at the cell periphery nearly doubled at 30" of KCl stimulation (Fig 6A). At 5 min of a KCl stimulus, the amount of ROK colocalized with caveolin at the cell periphery had returned to the basal level (Fig 6A).

Thus, ~16% of ROK translocated to caveolae at the smooth muscle cell membrane early during development of a KCl-induced contraction (30") and prior to development of the slower component of a KCl-induced contraction (tonic phase) that was inhibited by the ROK inhibitor, Y-27632 (see Figs 2 and 5). Moreover, an equal amount of ROK (~16%) returned to the cytosol during the tonic phase of contraction. These data together indicate that ROK translocation to the periphery of the cell preceded the tonic phase of a KCl-induced contraction but that sustained ROK translocation was not necessary for the maintenance of tonic contraction.

The calcium channel agonist, Bay K-8644, like KCl, produced a similar movement of ROK to the periphery of vascular smooth muscle cells. The addition of 7.5 mM KCl 10 min prior to addition of Bay K-8644, which was needed for Bay K-8644-induced contraction in rabbit femoral and renal arteries, did not produce a contraction, nor did it increase ROK translocation from the cytosol to periphery (basal, Fig 7B, compared to basal in the absence of 7.5 mM KCl, shown in Fig 7A). However, at 30 sec of stimulation with Bay K-8644, ~31% of ROK was colocalized with caveolin at the cell periphery (Fig 7B). At 5 min, the percentage of ROK colocalized with caveolin returned to the basal level (Fig 7B).

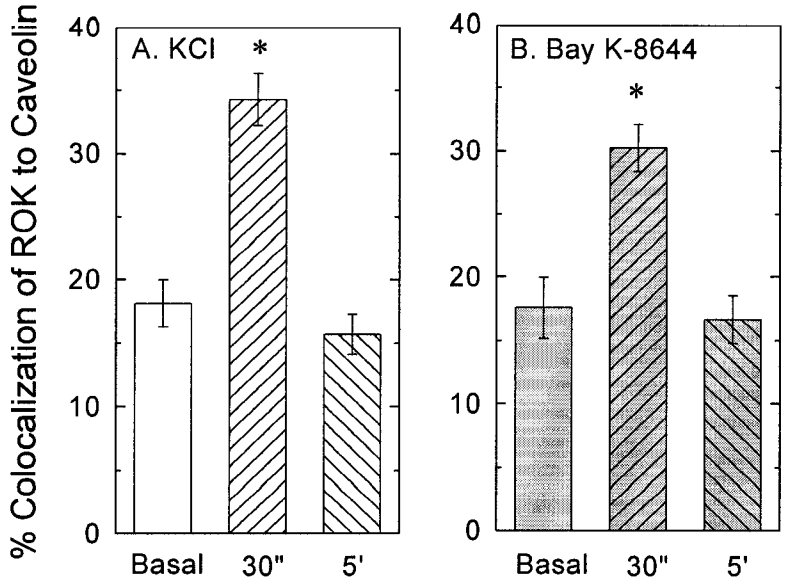

Figure 7. Percent colocalization of ROK with caveolin at the cell periphery in rabbit femoral artery at 30 sec (30") and 5 min (5') of stimulation with KCl (A) and 0.56 μM Bay K-8644 (B). Tissues stimulated with Bay K-8644 were exposed to 7.5 mM KCl, a concentration that alone, did not cause contraction. Basal tissues were not stimulated. Data are mean ± SE; n=3; * $p < 0.05$ compared to basal.

VII.6 Effect of nifedipine and trifluoperazine (TFP) on the ability of KCl, Bay K-8644 and ionomycin to cause increased ROK colocalization with caveolin at the cell periphery

Our data show that stimuli that increase $[Ca^{2+}]_i$ also cause an increase in the degree of ROK translocation to the cell periphery, suggesting that increased $[Ca^{2+}]_i$ causes ROK translocation to caveolin. Thus, we examined whether inhibition of L-type voltage-operated Ca^{2+} channels and Ca^{2+}/calmodulin would inhibit KCl-induced ROK translocation to the cell periphery. Nifedipine (Fig 8A) abolished the increase in colocalization of ROK with caveolin at the cell periphery at 30 sec of stimulation with KCl, suggesting that calcium entry through L-type calcium channels plays a role in KCl-

induced translocation of ROK. TFP, a Ca^{2+}/calmodulin inhibitor (Fig 8B), also abolished the increase in colocalization of ROK with caveolin at 30 sec of stimulation with KCl. Y-27632 did not inhibit ROK translocation (Fig 8C), suggesting that ROK activation was not necessary for KCl-induced ROK translocation to the cell periphery.

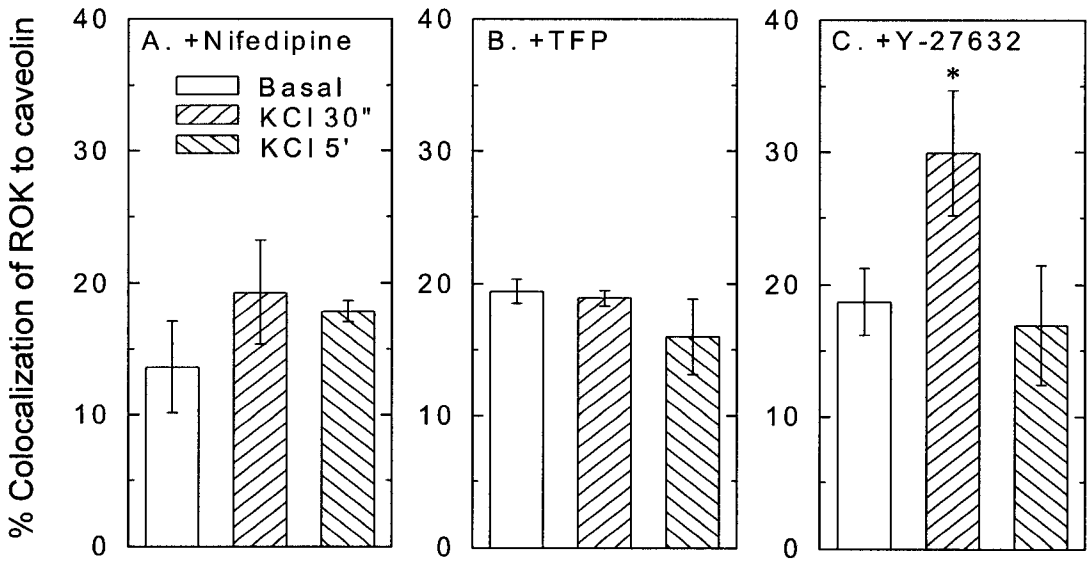

Figure 8. Effect of nifedipine (1 μM; A), trifluoperazine (TFP, 100 μM; B) and Y-27632 (1 μM; C) on percent colocalization of ROK with caveolin at the cell periphery in rabbit femoral artery stimulated for 30 sec (30") and 5 min (5') with KCl. Basal tissues were not stimulated. Data are mean ± SE; n=3; * $p < 0.05$ compared to basal.

Although 1 μM nifedipine inhibited both KCl-induced (Fig 8A) and Bay K-8644-induced (Fig 9) increases in ROK translocation to caveolin at 30 sec, nifedipine did not inhibit ionomycin-induced ROK translocation (Fig 9). These data, along with our finding that nifedipine did not inhibit ionomycin-induced early force development (Fig 4C) suggest that nifedipine-insensitive Ca^{2+} translocation or release of intracellular Ca^{2+} by ionomycin caused ROK translocation to caveolin. TFP inhibited Bay K-8644 and

ionomycin induced ROK translocation to caveolin (Fig 9), supporting a role for Ca^{2+}-calmodulin in regulation of ROK translocation.

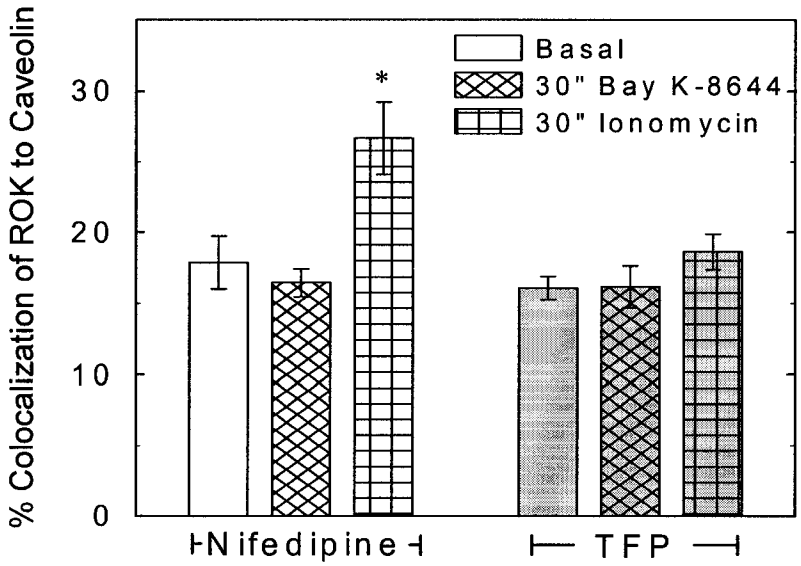

Figure 9. Effect of 1 μM nifedipine and 100 μM trifluoperazine (TFP) on the ability of 1 μM Bay K-8644 and 10 μM ionomycin to increase ROK colocalization to caveolae at 30 sec of stimulation. Data are mean ± SE; n=3; * $p< 0.05$ compared to basal.

VII.7 Nifedipine concentration-dependent inhibition of ROK translocation to caveolae

If 1 μM nifedipine inhibited KCl-induced ROK translocation to caveolin because of inhibition of Ca^{2+} entry, then lower nifedipine concentrations that partially inhibit Ca^{2+} entry should cause partial inhibition of ROK translocation. This was found to be the case. Whereas 1 μM abolished the increase in ROK translocation produced by KCl at 30 sec (Fig 10, and see Fig 8A), lower nifedipine concentrations known to cause partial

inhibition of calcium entry and contraction (69) caused only partial inhibition of KCl-induced ROK translocation to caveolin (Fig 10).

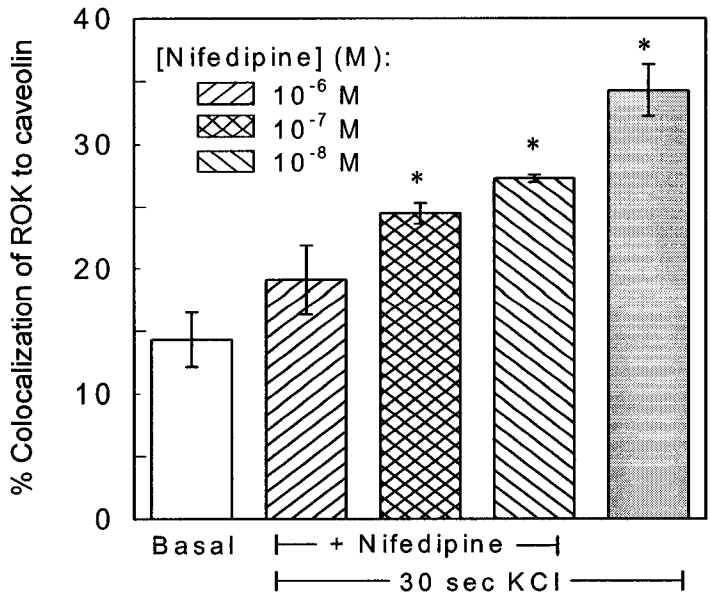

Figure.10. Concentration-dependent inhibition of ROK translocation to caveolae by the calcium channel blocker, nifedipine. Data are mean ± SE; n=4; * $p < 0.05$ compared to basal. 30 sec KCl data in the absence of nifedipine is taken from Fig 6A.

VII.8 Effect of KCl on RhoA translocation to caveolae

Others have shown that upon stimulation with receptor agonists there is a translocation of RhoA to the cell periphery. These studies did not quantify RhoA movement as colocalization with caveolae, but as increase in RhoA in the cortical region of the cell (42,21). We determined that in un-contracted (basal) tissues ~30% of RhoA was colocalized with caveolin at the cell periphery, and there was no increase in RhoA colocalization with caveolin at 30" of KCl stimulation (Fig 11). These data suggest a new model for interaction between RhoA and ROK in which the amount of RhoA at the

caveolae basally is sufficient to activate the ROK that translocates there during a KCl contraction.

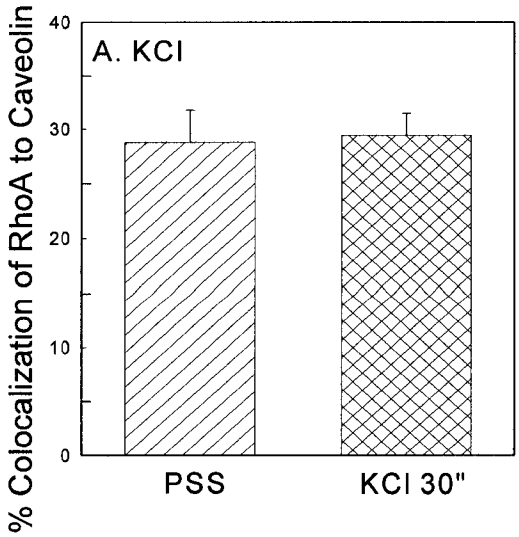

Figure 11. Percent colocalization of RhoA with caveolin at the cell periphery in rabbit femoral artery at 30 sec of stimulation with KCl. Basal tissues were not stimulated. Data are mean ± SE; *n*=3; * p< 0.05 compared to basal.

VII.9 Effect of non-neuronal cell memory on ROK and RhoA translocation to caveolae

Previous data from our laboratory shows that rabbit femoral artery muscle can retain a memory of previous receptor activation, called non-neuronal cell memory. Femoral arteries were contracted with a maximum concentration (10^{-5}M) of the α-adrenoreceptor agonist phenylephrine (PE) and relaxed by 10 minute PE washout, which is sufficient to completely relax the artery. Tonic force and MLC phosphorylation in a subsequent KCl contraction were reduced by 60 % when compared to tissues not previously contracted with PE, whereas $[Ca^{2+}]_i$ was unchanged (39,40).

In the present study, the colocalization of ROK and RhoA to caveolin was investigated during PE stimulation and the subsequent KCl contraction to investigate the postreceptor signaling mechanisms governing non-neuronal cell memory. ROK

colocalization to caveolin doubles, from basal levels, at 30 seconds of PE contraction (Fig 12A) and remains greater than the basal colocalization level at 5 and 30 minutes of PE contraction. Upon PE washout and muscle relaxation, the level of ROK colocalization to caveolin was reduced to the prestimulus basal level. However, unlike tissues that were not pretreated with PE that display robust increases in ROK translocation to caveolin at 30 sec of KCl stimulation, those pretreated with PE showed no increase in ROK translocation to caveolin when subsequently stimulated with KCl for 30 sec (Fig 12A).

These data support our hypothesis that tonic force maintenance requires ROK translocation to caveolin, and suggest that one reason PE-pretreatment inhibits KCl-induced force maintenance is that PE-pretreatment inhibits stimulus-induced increases in ROK translocation to caveolae.

We found that under basal conditions, a high percentage (~30%) of the principal cell activator of ROK, RhoA, is colocalized to caveolin at the cell periphery (Fig 11). Thus, we also investigated the effect of PE-pretreatment on RhoA colocalization to caveolin. Interestingly, RhoA colocalization to caveolin was decreased below the basal level at 30 minutes of PE contraction and remained below the basal level during the subsequent KCl contraction (Fig 12B). These data suggest that another mechanism of desensitization (reduced force maintenance without reduced increases in $[Ca^{2+}]_i$) in non-neuronal cell memory involves decrease in RhoA available for activation of ROK at caveolae.

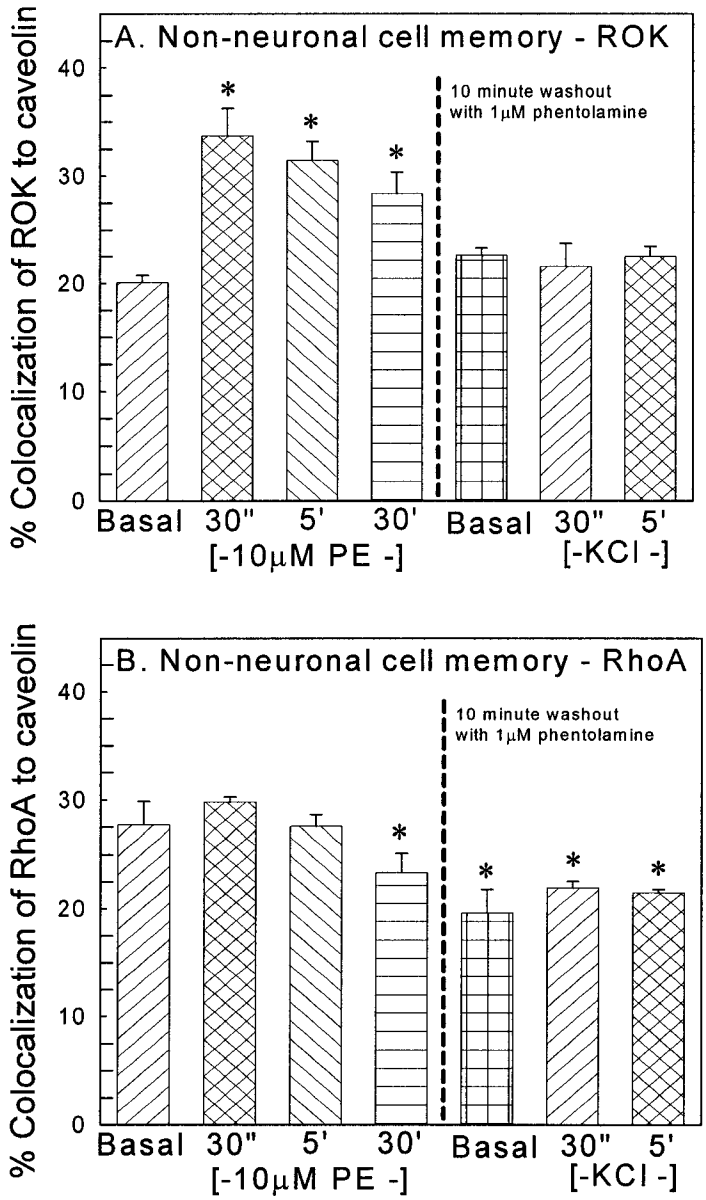

Figure 12. Percentage colocalization of ROK (A) and RhoA (B) to caveolin in non-neuronal cell memory. Arteries were contracted with 10 μM phenylephrine for 30 minutes, and the washed out for 10 minutes in the presence of the α-adrenoreceptor antagonist phentolamine (1 μM). The arteries were subsequently contracted in KCl for 5 minutes. Tissues were fixed each time point for analysis as described in ('Methods'). Data are mean ± SE; n=4; * p< 0.05 compared to basal.

VII.10 Effect of lower PE concentration and longer washout period on inhibition of ROK translocation in non-neuronal cell memory

Our laboratory has shown that the degree of downregulation in non-neuronal memory depends on the concentration of PE used during the pretreatment episode, the duration of the receptor activation and the duration of the washout period (41). Whereas

10μM PE pretreatment caused desensitization of both KCl- and histamine-induced contractions, an EC50 concentration of PE (0.1 μM) did not induce desensitization, non-neuronal cell memory, when tissues were subsequently stimulated to contract. The degree of desensitization produced after pretreatment with 10μM PE was significantly weaker in tissues following a PE washout of 2 hours than those washed for 10 minutes (41). I therefore tested the hypothesis that pretreatment with 0.1 μM PE or a 2 hour washout following 10 μM PE pretreatment would not inhibit the colocalization of ROK to caveolin in a subsequent KCl contraction. Following pretreatment with 0.1 μM PE, ROK colocalization to caveolin at 30 seconds of KCl contraction nearly doubled from basal levels (Fig 13A). Also, ROK colocalization to caveolin nearly doubled if the PE washout time is increased to 2 hours (Fig 13B).

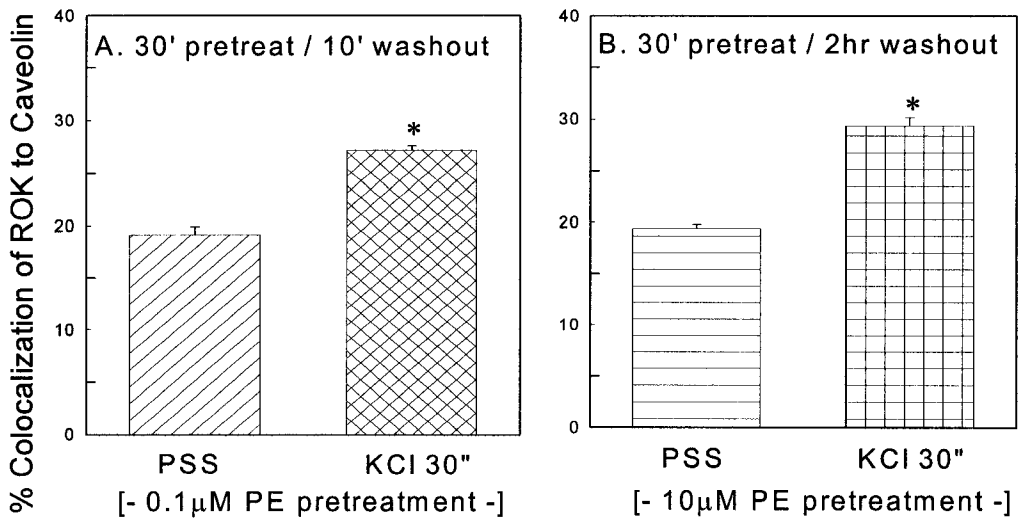

Figure 13. Percentage colocalization of ROK to caveolin in non-neuronal cell memory with lower concentration of PE (0.1 μM; A) and increased washout time (2 hours; B). Data are mean ± SE; n=3; * $p < 0.05$ compared to basal.

Therefore, KCl produced the same degree of ROK translocation to caveolin at 30 seconds when compared to arteries that were not pretreated if the concentration of the PE stimulus was decreased from 10 μM to 0.1 μM, and if the washout time was increased from 10 minutes to 2 hours. These data offer further evidence that non-neuronal cell memory involves the RhoA /ROK signaling pathway.

VII.11 Effect of 8br-cGMP treatment on ROK and RhoA translocation to caveolin

Our laboratory previously reported that there is a basal level of vasodilator-stimulated phosphoprotein [VASP(Ser^{293})] phosphorylation in intact femoral arteries, which is a measure of PKG activity. VASP phosphorylation is abolished by 1*H*-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ) and increased in the presence of a cell-permeable activator of PKG, 8br-cGMP (73). Treatment of arteries with 8br-cGMP also resulted in a decrease in active force but not $[Ca^{2+}]_i$ to subsequent histamine and Bay K-8644 contractions in a manner similar to non-neuronal cell memory. The effect of 100 μM 8br-cGMP treatment on ROK and RhoA colocalization to caveolin was determined to investigate the role of PKG activity in non-neuronal cell memory.

While not reducing the degree of basal ROK colocalized with caveolin, 8br-cGMP prevented the increase in ROK colocalization to caveolin produced at 30 seconds by KCl and PE (Fig 14A) compared to untreated arteries (Fig 7A). Moreover, the level of RhoA colocalization to caveolin basally was decreased from ~28% to ~18% for basal arteries (Fig 14B).

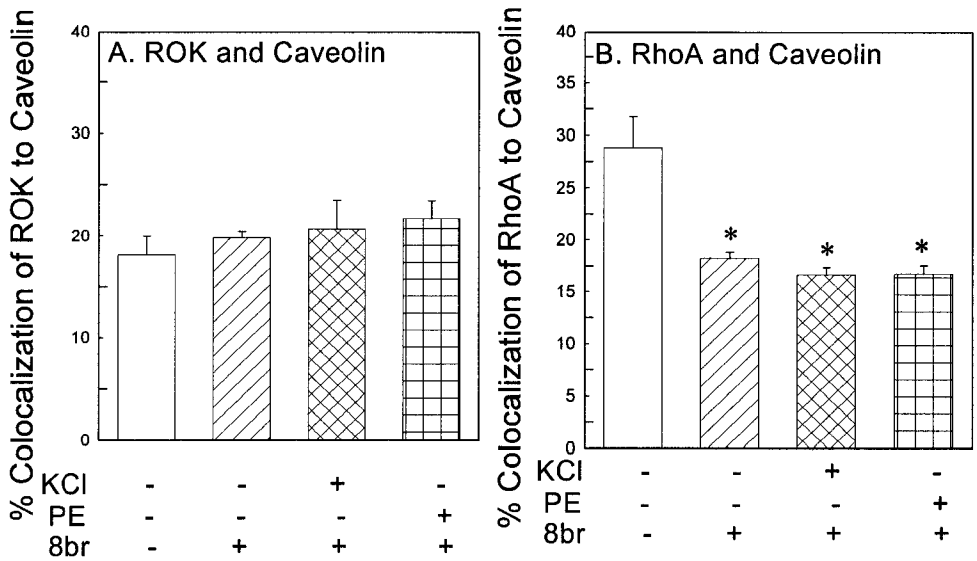

Figure 14. Colocalization of ROK (A) and RhoA (B) to caveolin in KCl- and PE-stimulated tissues pretreated with 100 μM 8br-cGMP. Data are mean ± SE; n=3-6; * p< 0.05 compared to basal.

We found that neither KCl nor PE produced an increase in RhoA colocalization to caveolin, from basal levels, at 30 sec of stimulation (Figs 11 and 12B), and in the presence of 8br-cGMP, neither KCl nor PE produced an increase from the low level induced by 8br-cGMP alone. These data are similar to that determined following pretreatment with 10 μM PE (Figs 12A&B), and could suggest either a common mechanism for desensitization of the KCl response through convergence of signaling pathways, or that PE-induced increases in PKG activity caused non-neuronal cell memory.

VII.12 Effect of non-neuronal cell memory on RhoA colocalization to RhoGDI

RhoA cycles between the inactive GDP-bound form and the active GTP-bound form via guanine nucleotide exchange and GTP hydrolysis. The protein RhoGDI retains RhoA in an inactive state by inhibiting guanine nucleotide exchange and hydrolysis.

Moreover, RhoGDI extracts Rho proteins from the cell membrane maintaining them as cytosolic and inactive proteins (74). We found that both treatment with 8br-cGMP and 30 minutes of 10 μM PE pretreatment reduced the basal amount of RhoA colocalized to caveolin (Fig 14B and 12B, respectively). A possible explanation for this reduction in basal RhoA at the cell periphery is that these stimuli increase the amount of RhoA bound to RhoGDI in the cytosol.

We tested this hypothesis and found that there was an increase in the colocalization of RhoA with RhoGDI at 30 minutes of PE contraction and at 30 seconds of a subsequent KCl contraction (Fig 15A). These data suggest that an increase in inactive, GDI-bound RhoA is involved in non-neuronal cell memory. Furthermore, there is an increase in the colocalization of RhoA with RhoGDI in tissues pretreated with 8BR-cGMP (Fig 15B).

Taken together these data suggest that a common component in the mechanism for downregulation in both non-neuronal cell memory (PE pretreatment) and arteries pretreated with 8br-cGMP is an inhibition of RhoA activity by the binding of RhoGDI to RhoA. Removal of active RhoA from the membrane, as determined by a decrease in RhoA colocalization to caveolin, in PE and 8br-cGMP pretreated arteries further supports this idea (Figs 12B and 14B).

Recent studies indicate that 8br-cGMP, via the actions of PKG, causes Ca^{2+}-desensitization by dephosphorylation of myosin light chains (44,45). Recent data also suggest that PKG phosphorylates RhoA on Ser^{188} resulting in its removal from the membrane and increasing its interaction with RhoGDI (49). Our data demonstrate a reduction in ROK translocation and reduced RhoA activity by increasing the proportion

of RhoA in the cytosolic, RhoGDI-bound form, and therefore support the hypothesis that PKG causes Ca^{2+}-desensitization.

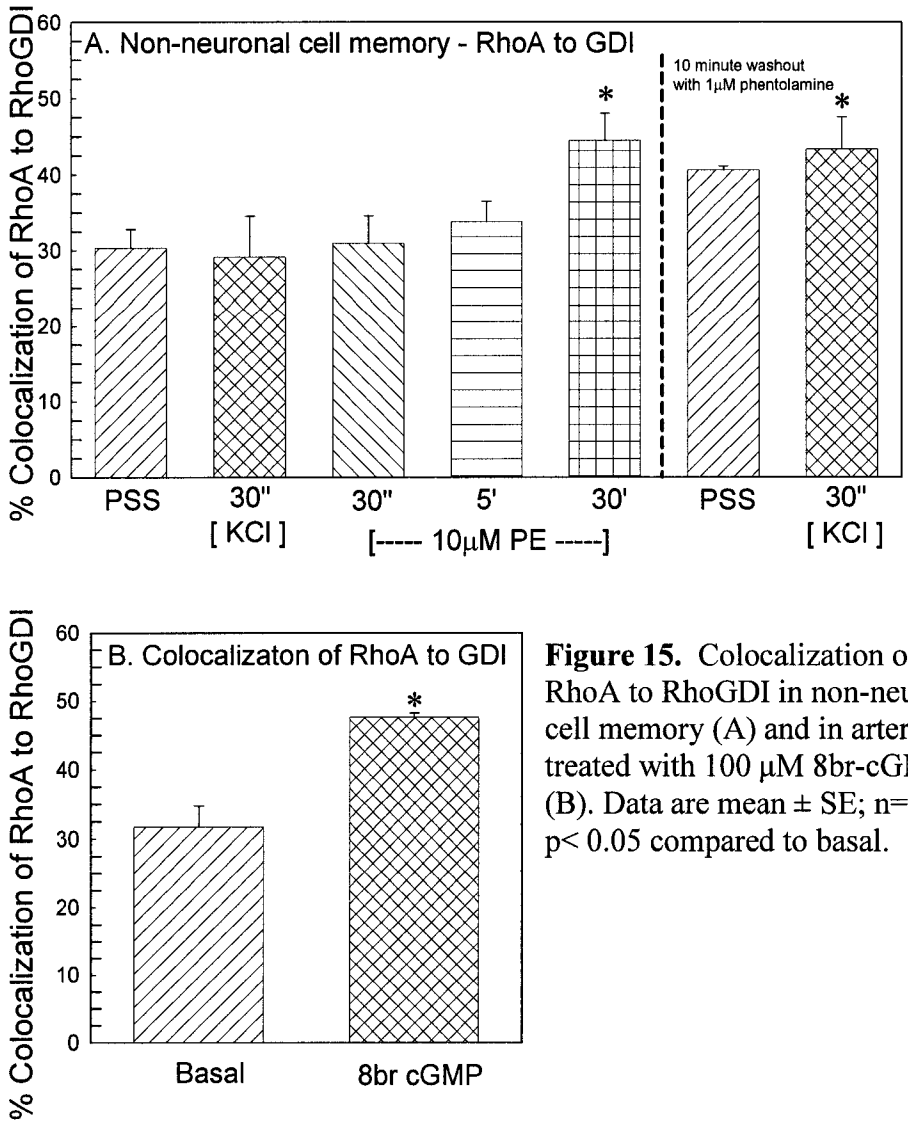

Figure 15. Colocalization of RhoA to RhoGDI in non-neuronal cell memory (A) and in arteries treated with 100 μM 8br-cGMP (B). Data are mean ± SE; n=3; * $p < 0.05$ compared to basal.

VII.13 Effect of okadaic acid on KCl-induced force and $[Ca^{2+}]_i$

Our laboratory has also shown desensitization of femoral arteries to KCl-induced increases in force and $[Ca^{2+}]_i$ by pretreatment with known PP1 inhibitors, NaF and okadaic acid (OA) (75). The mechanism of PP1 inhibitor desensitization is not known

but has been hypothesized to involve desensitization of L-type calcium channels or proteins associated with the channels (75,76). Others have shown that okadaic acid induces the removal of caveolae from the cell surface and that caveolae internalization is a kinase dependent phenomenon (64). It has been reported that translocation of ROK and RhoA to the cell membrane is necessary for their Ca^{2+} sensitizing actions which could suggest that an intact caveolar membrane at the cell membrane is also necessary for the coordination of RhoA/ROK signaling.

We found that by 45 minutes, 1 μM okadaic acid abolished the force elicited by KCl (Fig 16A) and greatly reduced the degree of KCl-induced $[Ca^{2+}]_i$ (16B). Given the report that okadaic acid leads to reductions in the amount of peripheral caveolae available for signalosome activity (64), these data suggest that intact caveolae are necessary for KCl-induced force maintenance. The data also suggest a role for kinase-mediated caveolar disruption in desensitization of the KCl response.

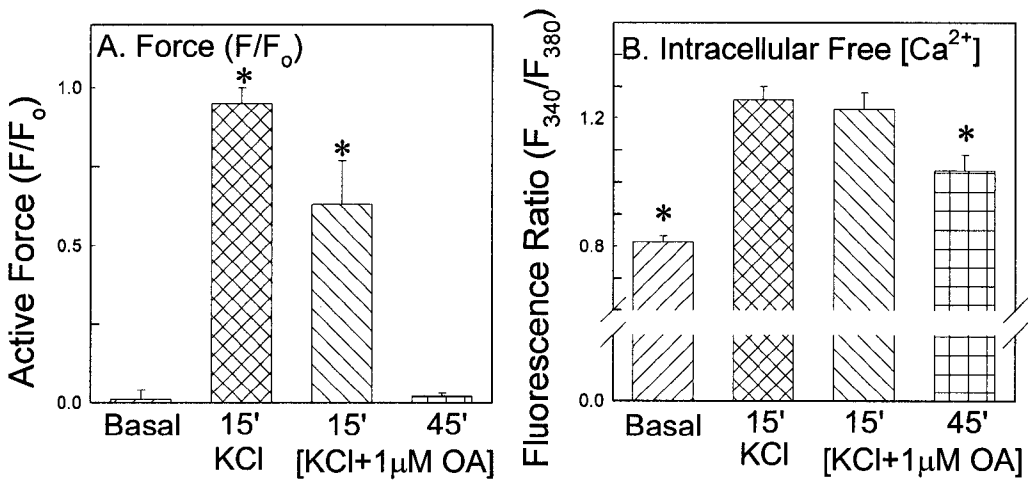

Figure 16. Effect of 1 μM okadaic acid on KCl-induced force (A) and $[Ca^{2+}]_i$ (B). Data are mean ± SE; n=3; * $p< 0.05$ compared to basal (A) and to 15'(B).

VII.14 Effect of okadaic acid and sodium fluoride on KCl-induced ROK colocalization with caveolin at the cell periphery

To investigate the effect of caveolar disruption on the RhoA/ROK signaling pathway, arteries were treated with either 10 mM NaF or 1 μM OA, and ROK colocalization to caveolin was determined. The colocalization of ROK to caveolin at 30 seconds of KCl contraction was abolished by treatment with either NaF or OA (Fig 17A).

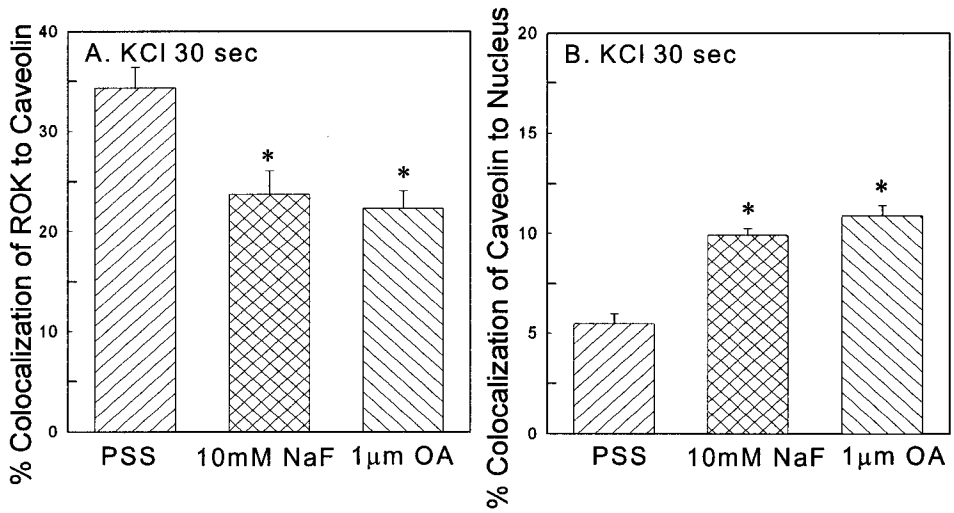

Figure 17. Effect of 1 μM okadaic acid and 10 mM NaF on KCl-induced ROK colocalization with caveolin at the cell periphery (A) and colocalization of caveolin with the nucleus (B). Data are mean ± SE; n=3; * $p < 0.05$ compared to basal.

To qualify the degree of caveolar movement away from the peripheral sites induced by treatment with either NaF or OA, we measured the degree of colocalization of caveolin with the nucleus. Under basal conditions, less that 5% of total cellular caveolin colocalizes with the nuclear compartment, and following exposure to the protein phosphatase inhibitors, OA and NaF, this percentage doubled (17B). This increase in

nuclear caveolin supports the contention that OA and NaF decrease the amount of peripheral caveolae by causing caveolar internalization (Fig 17B).

VII.15 Effect of actin filament and microtubule disruption on KCl-induced ROK colocalization with caveolin at the cell periphery

Recent studies suggest that depolymerization of microtubules causes non-receptor-dependent contraction of vascular smooth muscle (77), and others report that contractions caused by microtubule depolymerization involve ROK-dependent Ca^{2+}-sensitization (78,79). It has been hypothesized there is dual control of caveolae by actin filaments and microtubules such that microtubule disruption results in an increase in invagination depth of caveolae and actin filament disruption results in a decrease in transportation between the endoplasmic reticulum and caveolae (63). To determine the role of microtubules and actin filaments in KCl-induced ROK translocation, we determined ROK colocalization with caveolae in the presence of the microtubule depolymerizing agents nocodazole and colchicine and the actin filament disrupting agent cytochalasin D. Treatment with cytochalasin D (1 μM) reduced the KCl-induced increase in ROK colocalization to caveolin by ~50%. Treatment with colchicine (10 μM) did not affect ROK translocation, but nocodazole (10 μM) inhibited ROK colocalization with caveolae by ~25%. These data suggest that both actin filaments and microtubules play a role in ROK signaling, and these data support the hypotheses of others that caveolae and thus caveolar signaling is under the dual control of actin filaments and microtubules (63). Specifically, our results indicating that cytochalasin D causes a ~50% reduction in ROK translocation, support the hypothesis that transport to caveolae requires intact actin filaments.

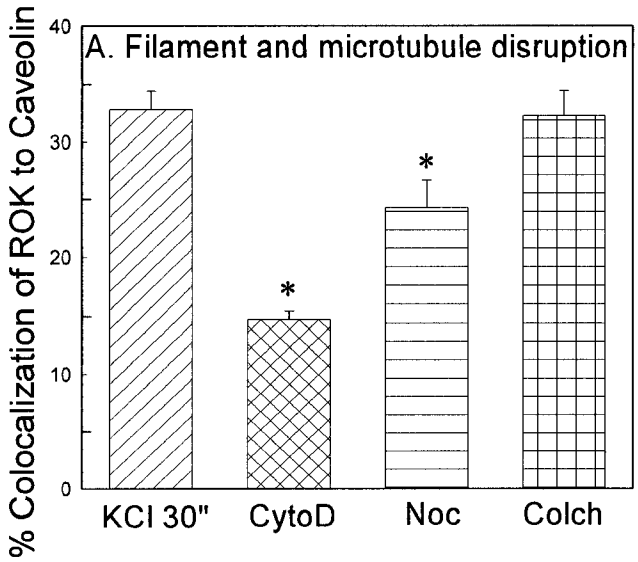

Figure 18. Colocalization of ROK with caveolin in arteries treated with cytochalasin D (cytoD; 1 μM), nocodazole (Noc; 10 μM), and colchicine (colch; 10 μM). Data are mean ± SE; n=3; * $p < 0.05$ compared to KCl at 30".

VII.16 Effect of 8br-cGMP and PE pretreatment on KCl-induced caveolin colocalization with the nucleus

To investigate the role of caveolar internalization in non-neuronal cell memory and 8br-cGMP-induced desensitization of the KCl responses, arteries were pretreated with either 100μM 8br-cGMP or 10μM PE for 30 minutes. The colocalization of caveolin to the nucleus more than doubled following pretreatment with 8br-cGMP, but PE pretreatment for 30 minutes did not cause a significant increase in caveolar colocalization with the nucleus.

These data suggest that non-neuronal cell memory (PE pretreatment) and 8br-cGMP pretreatment do not operate by entirely identical mechanisms to cause desensitization of a subsequent KCl contraction, but do share two common features. First is an inhibition of ROK colocalization to caveolae, and second is a decrease of RhoA at caveolae as determined by both a decrease in RhoA colocalization to caveolae and an increase in RhoA colocalization with RhoGDI .

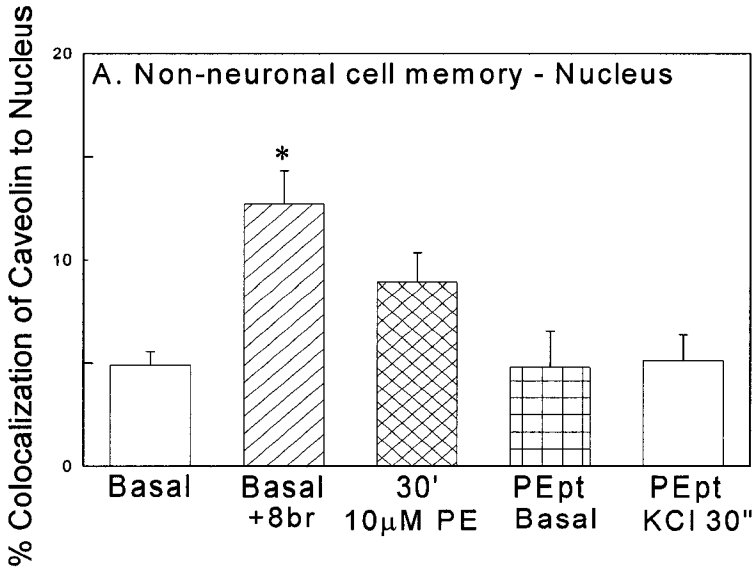

Figure 19. Colocalization of cavolin to nucleus in 100 μM 8br-cGMP and 10 μM PE pretreated arteries. Data are mean ± SE; n=3 * $p < 0.05$ compared to basal.

CHAPTER VIII

DISCUSSION

Vascular smooth muscle contraction is governed by an increase in $[Ca^{2+}]_i$ and by Ca^{2+}-sensitization (reviewed by 37). Whereas the Ca^{2+}/calmodulin-dependent activation of MLC kinase is a well understood mechanism for causing smooth muscle contraction, the precise signaling mechanisms involved in Ca^{2+}-sensitization have not been entirely elucidated. The clinical relevance of study in this field is that many pathophysiological conditions, including hypertension and vasospasm of the coronary and cerebral vessels, have been linked to an abnormal state of vascular smooth muscle contraction and an overactivity of pathways leading to Ca^{2+}-sensitization.

One hypothesis for Ca^{2+}-sensitization of smooth muscle involves regulation of the interaction of actin and myosin by thin filament associated proteins. Though there is evidence that supports this hypothesis in some tissues, the majority of proposed mechanisms for Ca^{2+}-sensitization involve regulation of MLC phosphatase (reviewed by 20, 22). Several studies indicate that the Ca^{2+}-sensitization elicited by receptor agonists involves inhibition of the myosin targeting subunit of MLC phosphatase (MYPT1) via the actions of PKC and RhoA. PKC activates PKC potentiated inhibitory protein (CPI-17) which phosphorylates the protein phosphatase (PP1) subunit of MLC phosphatase resulting in dissociation and inactivation of the enzyme. Recent studies suggest, however, that PKC does not play a major role in Ca^{2+}-sensitization. RhoA activates ROK which phosphorylates and inhibits MYPT1. Some studies suggest that ROK can also activate CPI-17 and directly phosphorylate myosin light chains at the same site as MLC

kinase. It is currently thought that direct phosphorylation of MYPT1 is the primary Ca^{2+}-sensitizing action of ROK (reviewed by 22).

K^+-depolarization (KCl) of smooth muscle, like receptor activation, causes an increase in $[Ca^{2+}]_i$, but KCl-stimulation does not cause activation of G protein-coupled receptors. Thus, KCl is used to induce contractile protein activation independently of plasma membrane receptor activation. Recent studies suggest that KCl can also produce an increase in the Ca^{2+}-sensitivity of arteries (38, 36). Our laboratory has previously shown that KCl-induced contraction can be desensitized. Upon pretreatment of arteries with receptor agonists, the degree of force produced for a given KCl-induced increase in $[Ca^{2+}]_i$ can be substantially altered (40,41). This is also true for receptor-induced Ca^{2+}-sensitivity (41). Since desensitization of KCl-induced contractions suggests that the smooth muscle cells stored information about the previous receptor activation, we termed this phenomenon non-neuronal cell memory. Moreover, we found that in naïve arteries, receptor agonists cause a greater degree of Ca^{2+}-sensitization than KCl (41).

Thus, receptor- and KCl-induced contractile mechanisms share at least one feature, namely, the degree of Ca^{2+}-sensitivity induced upon muscle stimulation is dependent on the history of receptor stimulation. We propose that this shared feature reflects a common subcellular mechanism regulating Ca^{2+}-sensitivity. The present study is an investigation of mechanisms regulating the Ca^{2+}-sensitization produced by KCl and the Ca^{2+}-desensitization of KCl-induced contractions in non-neuronal cell memory.

Y-27632 is known to be a highly selective inhibitor of ROK. The concentrations of Y-27632 required for 50% inhibition of phenylephrine-contracted aorta (IC_{50}) and 50% inhibition of isolated ROK (K_i) are, respectively, 0.7 μM and ~0.1-0.8 μM

(80,31,81,82). HA-1077, another potent inhibitor of ROK, has a K_i of 2-11 μM for ROK, and neither Y-27632 nor HA-1077 significantly inhibits MLC kinase or PKCα activities at the concentrations used in the present study (81,83). We found that tonic force produced by KCl in rabbit arteries was inhibited ~50% by 1 μM Y-27632 and more than 60% by 10 μM HA-1077, whereas early peak force was not affected by either ROK inhibitor. Likewise, early peak MLC phosphorylation was unaffected by 1 μM Y-27632, whereas 1 μM nifedipine nearly abolished both peak and tonic force and MLC phosphorylation.

Given the high selectivity of Y-27632 and HA-1077 for ROK inhibition, these data suggest that ROK did not participate in early force development, but played an essential role in the maintenance of KCl-induced steady-state force. Moreover, KCl-induced increases in $[Ca^{2+}]_i$ were not affected by 1 μM Y-27632, but MLC phosphorylation was. These data indicate that at this concentration, Y-27632 did not act as a Ca^{2+} channel blocker to reduce MLC phosphorylation and force. The Ca^{2+} channel blocker, nifedipine, inhibited both peak and tonic MLC phosphorylation and force, supporting a role for Ca^{2+} in early force development and delayed activation of ROK leading to tonic force maintenance.

Although Y-27632 is highly selective for inhibition of ROK, a recent study revealed that 1 μM Y-27632 can also inhibit PKCδ, a novel PKC isotype (32). However, PKCδ is also strongly inhibited by 1 μM GF-109203X, a staurosporine analog that is used to concentration-dependently inhibit conventional and novel isoforms of PKC (71,70,32). In the present study, 1 μM GF-109203X had no effect on KCl-induced contraction (see Figs 2B and 2C). Together, these data support the hypothesis that PKC

does not play a role in KCl-induced tonic force maintenance, but that a common mechanism shared by KCl and receptor agonists is activation of ROK.

To induce Ca^{2+}-sensitization, ROK must interact with the plasma membrane (reviewed by 21), and recent studies suggest that small plasma membrane invaginations, named caveolae, are major centers for coordination of cellular signal transduction. Caveolae contain multiple receptors, signal transduction molecules, voltage-gated ion channels, and numerous effectors (reviewed by 54,58,53,57). Caveolins, the structural proteins of caveolae, act as scaffolds for assembly and interaction of signaling molecules at caveolae (reviewed by 58).

That caveolae are loci for receptor agonist-induced Ca^{2+}-sensitization was proposed recently (57). Furthermore, it was shown that receptor agonist-stimulation of smooth muscle causes a redistribution of RhoA, ROK and PKCα from the cytosol to the cell periphery (42). The present study extends this model to include K^+-depolarization as a stimulus that also causes ROK translocation to caveolae at the plasma membrane of rabbit arterial smooth muscle.

In this study, we found that the amount of ROK colocalized with caveolin at the cell periphery doubles from basal levels upon contraction with either KCl or the L-type calcium channel agonist Bay K-8644. Moreover both receptor agonists and KCl cause increases in $[Ca^{2+}]_i$ and our data support the hypothesis that Ca^{2+} plays an essential role in KCl-induced ROK translocation. This is because the Ca^{2+} channel blocker, nifedipine, and the calmodulin blocker, trifluoperazine, when used at concentrations that are known to inhibit contraction (84), prevented KCl-induced ROK translocation to caveolae.

The fact that nifedipine concentration-dependently inhibited KCl-induced ROK translocation further supports a role for Ca^{2+} in the regulation of ROK activation and concomitant force maintenance. Moreover, 1 μM Y-27632 inhibited Bay K-8644- and calcium ionophore (ionomycin)-induced MLC phosphorylation and force. Nifedipine did not inhibit ionomycin-induced increases in ROK translocation and force which suggests that the contraction elicited by ionomycin involved Ca^{2+} entry that was independent of L-type calcium channels. Furthermore, the Ca^{2+} entry elicited by ionomycin was sufficient to cause ROK translocation. Though G proteins have been shown to cause Ca^{2+}-sensitization, these data strongly support a role for an increase in $[Ca^{2+}]_i$, a common feature of both receptor activation and KCl contraction, in the pathways leading to Ca^{2+}-sensitization.

Receptor agonists produce a greater degree of Ca^{2+}-sensitization than KCl, and these data could suggest a mechanism for the observed differences in Ca^{2+}-sensitization. The differences could involve the activation of PKC, which plays a role in receptor agonist-induced Ca^{2+}-sensitization, but which we *did not* find to participate in KCl-induced Ca^{2+}-sensitization. Furthermore, our data show that 30% of RhoA is colocalized with caveolin basally (Fig 11), whereas others suggest that the translocation of RhoA elicited by receptor agonists to the cell membrane is required for its Ca^{2+}-sensitizing actions and that receptor agonists cause an increase in RhoA at the plasma membrane (42, and reviewed by 1). Taken together these data suggest a novel model for Ca^{2+}-activated Ca^{2+}-sensitization, elicited by KCl contraction, that involves Ca^{2+}/cam dependent ROK translocation to caveolae to be activated by the RhoA already there (Fig 20).

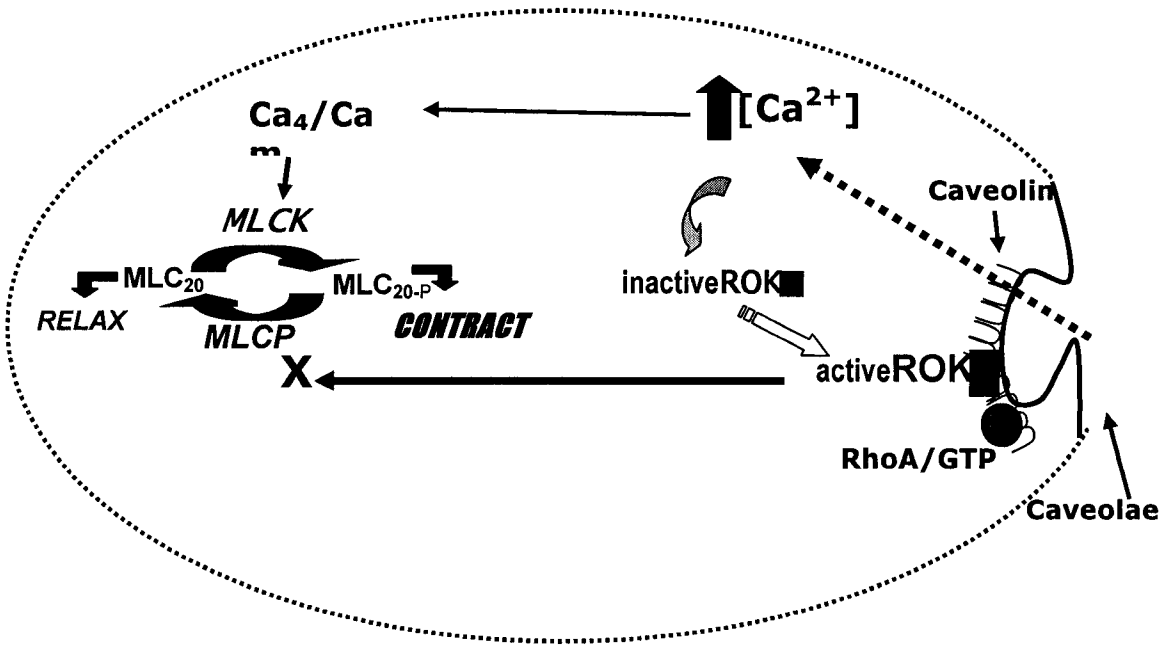

Figure 20. Model of Ca^{2+}-activated Ca^{2+}-sensitization.

Our laboratory has shown that the degree of force produced for a given KCl-induced increase in $[Ca^{2+}]_i$ can be substantially altered by the history of receptor stimulation. For example, femoral arteries were contracted with a maximum concentration (10^{-5}M) of the α-adrenoreceptor agonist phenylephrine (PE) and relaxed by 10 minute PE washout. During a subsequent KCl contraction, the tonic force and increases MLC phosphorylation, but not increases in$[Ca^{2+}]_i$ are reduced by 60 % when compared to naïve tissues not previously contracted with PE (40). We termed this phenomenon non-neuronal cell memory, because the vascular smooth muscle cells stored information temporarily about the degree of prior activation (85). Non-neuronal cell memory could play a role in maintaining vasodilation of arteries when factors normally responsible for relaxing the smooth muscle cells are not functioning. For example, if the

vascular endothelial cells are damaged and unable to produce nitric oxide to maintain the proper state of vasodilation, non-neuronal cell memory could protect the artery from excess contraction that could deprive the downstream organ or tissues of blood. Previous data from our laboratory shows that pretreatment of arteries with PE reduced the ability of histamine to cause a subsequent contraction (41). In this way non-neuronal cell memory could serve as a protective mechanism, or a redundant system in arteries if the endothelial-derived vasodilatory apparatus is working properly, so that proper blood flow is maintained. The precise subcellular mechanism causing non-neuronal cell memory remains to be elucidated, but this study was designed to investigate the possibility that Ca^{2+}-desensitization elicited in non-neuronal cell memory involves an inhibition of the signaling pathways involved in Ca^{2+}-sensitization.

We found that, unlike control tissues showing a robust increase in ROK translocation at 30 seconds of KCl-stimulation, tissues pretreated with PE showed no increase in ROK translocation to caveolin when subsequently stimulated with KCl for 30 seconds. Moreover, whereas ~30% of RhoA is colocalized to cavelin basally, in tissues not previously contracted with PE, RhoA colocalization to caveolin was decreased below the basal level at 30 minutes of PE contraction and remained below the basal level during the subsequent KCl contraction (Fig 12B). Together these data provide a molecular explanation for non-neuronal cell memory in vascular smooth muscle. The data suggest that the inhibition of KCl-induced force maintenance by PE-pretreatment involves both an inhibition of the stimulus-induced increases in ROK translocation to caveolae and a decrease in RhoA available for activation of ROK at caveolae. Moreover, these data suggest that inhibition of the RhoA/ROK system, which has been shown to play a major

role in Ca^{2+}-sensitization, is part of the information storage mechanism in non-neuronal cell memory.

Previously our laboratory reported that non-neuronal memory is reversible, such that the degree of downregulation to a subsequent contraction is dependent on the concentration of PE used during the pretreatment episode and the duration of the washout period (41). Our lab previously showed that a high PE concentration (10 μM) resulted in inhibition of histamine-induced force, but a low concentration (1 μM) did not reduce force when compared to tissues that were not pretreated. Additionally, a PE washout period longer that 90 minutes, reversed the desensitization induced by PE pretreatment (41).

In the present study, pretreatment with a sub-maximal concentration of PE (0.1 μM) and increasing the PE washout time to 2 hours resulted in a doubling of ROK colocalization to caveolin at 30 seconds of KCl contraction. Thus, the degree of KCl-induced ROK translocation to caveolin at 30 seconds in naïve (those not pretreated) was the same as pretreated arteries if the concentration of the PE stimulus was decreased from 10μM to 0.1μM, and if the washout time was increased from 10 minutes to 2 hours. These data demonstrate that non-neuronal cell memory is a reversible phenomenon and further support a role for the RhoA/ROK pathway in non-neuronal cell memory.

Endothelial nitric oxide (NO) causes relaxation of vascular smooth muscle and vasodilation, and its actions are mediated by an increase in cGMP. PKG is known to mediate the actions of cGMP (49). PKG causes Ca^{2+}-desensitization by dephosphorylation of myosin light chains (44,45,46,47), and recent data also suggest that PKG phosphorylation of active RhoA results in its removal from the membrane.

Phosphorylation of RhoA increases its interaction with RhoGDI and stabilizes it in the inactive form (49).

These data show that 8br-cGMP, a cell permeable activator of PKG, prevented the increase in ROK colocalization to caveolin produced at 30 seconds by KCl and PE. Furthermore increased PKG activity resulted in a decrease in the basal level of RhoA colocalized with caveolin from ~28% to ~18% (Fig 13B). In tissues treated with 8br-cGMP there was no increase in RhoA colocalization to caveolae upon stimulation with KCl or PE. Thus, increased PKG activity not only resulted in an inhibition of ROK translocation, but also caused a decrease in active RhoA at the membrane. The similarity of these data to that determined following PE (10μM) pretreatment could suggest that a common mechanism for desensitization of the KCl response, with both PKG activity and non-neuronal cell memory, is inhibition of the RhoA/ROK signaling pathway.

Furthermore, in arteries treated with 8br-cGMP there was an increase in the colocalization of RhoA with RhoGDI. An increase in RhoA and RhoGDI colocalization was also found in arteries at 30 minutes of PE contraction and at 30 seconds of a subsequent KCl contraction. These data support the work of others which showed that PKG stabilizes the inactive conformation of RhoA and suggest that RhoA is similarly inactivated during non-neuronal cell memory.

Desensitization to KCl-induced increases in force and $[Ca^{2+}]_i$ in femoral arteries is also caused by the PP1 inhibitors such as okadaic acid (OA). Others have shown that OA treatment results in kinase-dependent caveolae internalization (64). Recent studies propose caveolae as a site for coordination of signaling pathways, so it is possible that caveolae disruption could inhibit signaling pathways that utilize caveolae for signal

coordination. In this study, we found that treatment of arteries with OA and NaF abolished the colocalization or ROK to caveolin at 30 seconds of KCl contraction suggesting that PP1 inhibition caused desensitization of the KCl response in part through an alteration of the RhoA/ROK signaling pathway. These data support a role for intact caveolae in the RhoA/ROK pathway leading to Ca^{2+}-sensitization.

To determine whether PP1 inhibition with OA resulted in internalization of caveolae, we determined the amount of caveolin colocalized with the nucleus. The data revealed that treatment with OA and NaF doubled the amount of caveolin colocalized with the nucleus from basal levels.

We also found that increased PKG activity, elicited by 8br-cGMP, increased caveolin colocalization with the nucleus. Thus, we suggest a novel role for PKG in caveolar internalization which supports the work of others who propose that caveolar maintenance is kinase-dependent. PE pretreatment (non-neuronal cell memory) did not cause caveolar internalization. Thus, our data suggest that inhibition of the RhoA/ROK signaling pathway rather than a disruption of caveolae is the information storage method utilized in non-neuronal cell memory and not a disruption of caveolae.

A recent study demonstrated that caveolae are also under the dual control of actin filaments and microtubules (63). The authors show that microtubules are responsible for maintaining the depth of caveolar invagination. They further hypothesize that transport of proteins to caveolae is controlled by actin filaments (63). Because in the present study we found that intact caveolae are necessary for RhoA/ROK-mediated Ca^{2+}-sensitization, we investigated the role of cytoskeletal elements on the RhoA/ROK signaling pathway. The disruption of actin filaments with cytochalasin D decreased ROK translocation at 30

seconds of KCl contraction by ~50%. Depolymerization of microtubules with nocodazole also decreased ROK translocation. These data support the work of others that demonstrates a role for actin filaments in transporting proteins to caveolae, but our data also suggest that microtubules play a role in ROK mediated signaling in smooth muscle.

In summary, we propose a novel model for Ca^{2+}-sensitization. We found that ROK translocation to caveolae is elicited by KCl. Moreover we found that 30% of RhoA is colocalized to caveolin basally, and that PKC does not play a role in KCl-induced tonic force maintenance. These data when taken together suggest that an increase in $[Ca^{2+}]_i$, which is a component of receptor activation, causes Ca^{2+}-sensitization. Furthermore our novel model of Ca^{2+}-activated Ca^{2+}-sensitization, suggests two mechanisms for the difference in the degree of Ca^{2+}-sensitization produced by receptor agonists and KCl. First we determined that PKC does not play a role in KCl-induced Ca^{2+}-sensitization, and second we found there is not an increase in RhoA colocalization to caveolin above the 30% there basally.

We also suggest that the information storage method utilized in non-neuronal cell memory involves an inhibition of the RhoA/ROK pathway. Specifically, PE pretreatment causes an inhibition of ROK translocation to caveolae in the subsequent KCl contraction and also results in an increase in inactive RhoA. The finding, in this study, that PKG activation also leads to a similar inhibition of the RhoA/ROK signaling pathway suggests a common mechanism for Ca^{2+}-desensitization. The desensitization elicited by PP1 inhibition involved an internalization of caveolae and an inhibition of ROK translocation. These data support the hypothesis that caveolae are loci for signal

transduction pathways, but also suggests that the membranous location of caveolae is an important factor in RhoA/ROK-mediated Ca^{2+}-sensitization. The finding that PKG activation leads to caveolar internalization supports the hypothesis that caveolar maintenance is kinase mediated, but the precise signals involved in PKG-mediated caveolar internalization were not determined.

CHAPTER IX

CONCLUSION

These data suggest that increases in $[Ca^{2+}]_i$ rather than strong membrane depolarization, per se, caused translocation of ROK to caveolae. Y-27632 did not prevent KCl-induced ROK translocation at 30", but did inhibit KCl-induced tonic (i.e., 5') contraction, suggesting that ROK activation may not be required to cause ROK translocation. Moreover, KCl-induced ROK translocation to the plasma membrane preceded the ability of Y-27632 to inhibit force. Thus, we suggest that an increase in $[Ca^{2+}]_i$ can induce Ca^{2+} sensitization by causing Ca^{2+}-dependent ROK translocation to caveolae for activation by RhoA that is found there basally.

However, these data do not rule out additional signaling events that may be coordinated at the cell membrane, such as activation of ZIP-like kinase (86) which has been shown cause sustained MLC phosphorylation and force in the face of temporally falling $[Ca^{2+}]_i$. Thus, a future direction of this research could be to investigate the role of possible downstream effectors of ROK, and their precise effect on MLC phosphatase and Ca^{2+}-sensitization in smooth muscle.

Furthermore, receptor agonists produce a greater increase in Ca^{2+}-sensitivity than KCl (41). Data suggest that receptor agonists cause stronger increases in ROK activity through additional translocation of RhoA to caveolae (reviewed by (21)), or by activating additional Ca^{2+}-sensitizing mechanisms involving PKC (42, 32) that are independent of increases in $[Ca^{2+}]_i$ (34). Though these data suggest two mechanisms for differences in the degree of Ca^{2+}-sensitization elicited by KCl contractions and receptor agonists, it

remains to be determined whether these are the only mechanistic differences between these Ca^{2+}-sensitizing stimuli.

Our laboratory has previously shown that the degree of Ca^{2+}-sensitization elicited by both receptor agonists and KCl is dependent on the history of receptor activation (non-neuronal cell memory) (41). In this study, our data demonstrate an inhibition of ROK translocation to caveolae, and a decrease in RhoA colocalization with caveolin as mediators of Ca^{2+}-desensitization. These data suggest that an inhibition of the RhoA/ROK signaling pathway is a key to the information storage mechanism elicited in non-neuronal cell memory and desensitization of the KCl response.

Others have shown that PKG activation, can cause Ca^{2+}-desensitization by dephosphorylation of myosin light chains and inactivation of RhoA (44,45,49). Our data support a role for PKG in desensitization of the KCl response. The data also demonstrate that a decrease in RhoA at the caveolae and an increase in RhoA colocalization with RhoGDI is a common feature in both PKG-mediated desensitization and non-neuronal cell memory. It remains to be determined if PKG plays a role in non-neuronal cell memory, but our data do suggest that inhibition of the RhoA/ROK signaling which leads to desensitization of the KCl response is a convergent point in these two signaling pathways.

Desensitization to KCl-induced increases in force and $[Ca^{2+}]_i$ can also be elicited by the PP1 inhibitors okadaic acid and NaF. Our data suggests that OA also causes internalization of caveolar. These data support the work of others that indicate that caveolar maintenance is kinase-mediated. Moreover, these data demonstrate that intact caveolae are important in ROK-mediated Ca^{2+}-sensitization, which supports the role for

caveolar as major centers for coordination of signal transduction cascades in vascular smooth muscle.

The novel finding that PKG activation results in caveolar internalization supports the hypothesis that caveolar internalization is kinase-mediated. Also, these data demonstrates that both non-neuronal cell memory and PKG activation involve an inhibition of a common pathway (the RhoA/ROK signaling pathway) though the two methods of Ca^{2+}-desensitization vary. Thus, caveolar internalization, due to PKG activity, may play a role in Ca^{2+}-desensitization but it is only one facet of Ca^{2+}-desensitization.

Our data also demonstrate that an intact cytoskeleton is necessary for Ca^{2+}-sensitization. Since disruption of actin filaments abolished ROK movement, a future direction of this study could be to investigate the role of unconventional myosins moving along actin filaments in the process of protein translocation. Furthermore, the control of caveolar invagination, or depth, could be important to various signaling pathways as many receptors and channels are found there. In some cell types caveolae not only act at scaffolds at the cell membrane but also act as shuttles between organelles (reviewed by 58, 54). Smooth muscle caveolae are relatively static structures at the membrane, but there is currently little information regarding the effect of the maintenance of caveolar size and shape on the signaling pathways. Our data suggest that Ca^{2+} can induce Ca^{2+}-sensitization, and it is known that much of the Ca^{2+} handling machinery in smooth muscle cells is located in caveolae, including L-type Ca^{2+} channels (60). Thus, another future direction of this work could be to investigate the role of caveolar internalization or disruption on Ca^{2+} signaling and the Ca^{2+} channels in smooth muscle.

In summary, we propose a novel model for Ca^{2+}-sensitization. We conclude that Ca^{2+} can induce Ca^{2+}-sensitization by causing Ca^{2+}/cam-dependent ROK translocation to caveolae and activation of ROK at the caveolae by the RhoA there basally. We conclude that the desensitization elicited in non-neuronal cell memory involves both an inhibition of ROK translocation as well as a decrease in RhoA colocalization with caveolin. We conclude that PKG-mediated Ca^{2+}-desensitization, though also resulting in inhibition of the RhoA/ROK signaling in a manner similar to non-neuronal cell memory, involves internalization of caveolae which is not a component of non-neuronal cell memory. Furthermore, caveolar internalization elicited by PP1 inhibition and PKG activation Ca^{2+}-desensitizes smooth muscle by inhibiting RhoA/ROK signaling. These data support the hypothesis that caveolae are centers for signal transduction, and we conclude that caveolae play a major role in coordinating Ca^{2+}-activated, RhoA/ROK-mediated Ca^{2+}-sensitization in arterial smooth muscle.

CHAPTER X

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Publications:

Urban N.H., Berg K.M. and Ratz P. K^+-Depolarization (KCl) induces RhoA Kinase (ROK) Translocation to Caveolae and Ca^{2+}-sensitization of Arterial Muscle.*sub.5/03.*

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