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Role of ROK and PKC in Permeabilized Rabbit Femoral Artery

Lyndsay Jacquelyn Clelland
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ROLE OF ROK AND PKC IN PERMEABILIZED FEMORAL ARTERIAL SMOOTH MUSCLE

A Thesis submitted in partial fulfillment of the requirements for the degree of Masters of Science at Virginia Commonwealth University.

by

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Discoveries made with KCl-induced contractions have elucidated the more complex reactions involved in GPCRs signaling; once the mechanisms of smooth muscle Ca\(^{2+}\) sensitization and desensitization are fully understood, then the development of advanced treatments for vascular disorders such as hypertension, cerebral and coronary vasospasm, and vascular hyporeactivity following hemorrhagic shock may be possible. Studies have shown that KCl-induced contractions induce Ca\(^{2+}\)-sensitization. Therefore, we tested the hypothesis that KCl induced Ca\(^{2+}\)-sensitization is due to ROK activation by the increase in \([\text{Ca}^{2+}]_i\). To test this hypothesis, rabbit femoral arteries were permeabilized
with 20µg/ml α-toxin and 1% Triton X-100 and subjected to different calcium concentrations in the presence or absence of various ROK inhibitors. For a comparison we also used various PKC and MLCK inhibitors and repeated these experiments in intact tissues. We found that either \([\text{Ca}^{2+}]_i\), alone does not directly activate ROK or the permeabilization technique itself disrupts the normal ROK signaling system. Secondary findings revealed that α-toxin activates PKC pathways; in both chemically permeabilized preparations proteases also appear to be activated and MLCK is the primary kinase responsible for contraction.
1.1 Basic Smooth Muscle Physiology

The development of force in smooth muscle, similar to cardiac and skeletal muscles, involves the hydrolysis of adenosine triphosphate (ATP), actin-myosin cross-bridge cycling, and the transient elevation of intracellular calcium ([Ca\textsuperscript{2+}]). Although the three types of muscle possess similar properties, there are several structural and functional differences unique to smooth muscles.

Smooth muscle is typically found lining hollow organs such as blood vessels, the urinary bladder, airways and the gastrointestinal tract. In these and other systems, the smooth muscle functions in a highly coordinated fashion to develop force either to provide motility or to alter organ dimensions. For this reason, smooth muscle can be classified into two broad categories based on the type of contraction it exhibits, phasic or tonic. Phasic smooth muscles can cycle through relaxed and contracted phases, and are responsible for processes such as peristalsis. On the other hand, tonic smooth muscles are capable of sustaining contractions for long periods of time, allowing the tissue to withstand imposed loads such as those found in the vascular system.

Tonic arterial smooth muscle must tolerate sustained contractions against imposed loads for long periods of time without fatigue. This is possible, in part, because of a
highly developed and efficient economy of ATP use. Despite more than two decades of work, the precise molecular mechanisms involved in the regulation of tonic force maintenance remain to be determined. One model developed to explain high force maintenance at a high energy economy is the latch bridge model (Figure 1), in which dephosphorylation of attached crossbridges produce an attached crossbridge that detaches much more slowly than a phosphorylated crossbridge (Hai and Murphy 1988; Ratz, Hai et al. 1989). This proposed dephosphorylated, slowly detaching crossbridge was termed a latch-bridge (Dillon, Aksoy et al. 1981). Recent evidence that dephosphorylated crossbridges release ADP very slowly supports the latch-bridge model (Somlyo, Khromov et al. 2004). Evidence from our laboratory indicates that tonic force maintenance can be selectively attenuated by inhibition of rhoA kinase (ROK), and that such attenuation is independent of changes in cellular free calcium levels ([Ca$^{2+}$]i) (Urban, Berg et al. 2003). Thus, tonic force maintenance is highly dependent on ROK activity.
Another distinguishable attribute of smooth muscle physiology, as compared to skeletal and cardiac muscle, is the absence of troponin. In cardiac and skeletal muscle the transient increase in $[\text{Ca}^{2+}]$, binds directly to the protein troponin causing it to undergo a conformational change. This change exposes the globular actin molecules of the thin filament to the myosin heads of the thick filament. In the presence of ATP, crossbridge cycling may then occur, developing force. This process is different in smooth muscle.
In smooth muscle, the globular actin binding sites of the thin filament are not blocked by troponin, but may be blocked by the thin filament binding proteins, calponin and caldesmon (Earley, Su et al. 1998; Gusev 2001). Regardless, smooth muscle crossbridges, unlike striated muscle crossbridges, must be phosphorylated on the 20 kDa light chain of myosin (MLC) to be activated (Kamm and Stull 1985). That is, in order for actin and myosin to bind and go through the crossbridge cycling to developing force, MLC must be phosphorylated (Trybus 1989). Therefore, the MLC acts as a switch; when the MLC is phosphorylated, the muscle is capable of undergoing contraction, and when the MLC is not phosphorylated, it may not undergo contraction.

1.2 Phosphorylated Myosin Light Chain ratio

The level of MLC phosphorylation, as the fundamental determinate of tonic smooth muscle contraction, is regulated by a complex series of events leading to either its phosphorylation or dephosphorylation. Myosin light chain kinase (MLCK) is responsible for phosphorylating MLC, turning it “on”; and myosin light chain phosphatase (MLCP) is responsible for dephosphorylating MLC, turning it “off” (Kamm and Stull 1985; Somlyo, Wu et al. 1999). The balance between these two proteins is important in the regulation of smooth muscle contraction and can be described as the MLCK/MLCP ratio; whereas a ratio greater than one would indicate a contraction and a ratio less than one would indicate a less contracted state.
Smooth muscle MLCK is activated by an increase in \([\text{Ca}^{2+}]_i\), and this is why \([\text{Ca}^{2+}]_i\) has been attributed as the fundamental regulator of smooth muscle cells (Kamm and Stull 1985). As \([\text{Ca}^{2+}]_i\) levels rise, calcium binds to free intracellular calmodulin (CaM) forming a calcium-calmodulin complex (Ca-CaM); Ca-CaM binds to and activates MLCK. Other kinases may serve the same role, because a recent study using MLC kinase knockout (-/-) mice shows that arteries still contract (Somlyo, Wang et al. 2004). However, MLCK is considered to be the main kinase involved in the phosphorylation of the 20 kDa MLC.

Smooth muscle MLCP consists of three subunits. The 37 kDa catalytic subunit falls in the type 1 class of protein phosphatases (PP1c-delta) (Alessi, MacDougall et al. 1992; Gong, Cohen et al. 1992; Mitsui, Inagaki et al. 1992; Johnson, Cohen et al. 1997) and is responsible for dephosphorylating MLC. The catalytic subunit is activated and targeted to the substrate by myosin phosphatase targeting subunit (MYPT) (Hartshorne, Ito et al. 1998), and has a molecular weight of 110-120 kDa (Johnson, Cohen et al. 1997). The third subunit 21 kDa (Johnson, Cohen et al. 1997), (M20) binds to MYPT and has no defined function (Hartshorne, Ito et al. 1998). MLCP activity can be negatively regulated by its phosphorylation and by association with inhibitor proteins.

1.3 Calcium Regulation

It has long been known that calcium is responsible for the contraction of muscles. Although the transient increase in \([\text{Ca}^{2+}]_i\) has different effectors in the different types of
muscle, its necessity for contraction remains constant. For this reason, the study of calcium has been critical to the understanding of arterial smooth muscle physiology (Morgan and Morgan 1982; van Breemen, Chen et al. 1995; Karaki 2004).

Studies have shown that in tonic arteries the resting \([\text{Ca}^{2+}]_i\) is approximately 100nM in the rat femoral artery (Van Heijst, Blange et al. 2000) and 106+8nM in the rat pulmonary artery (Somlyo and Himpens 1989). Whereas the outside resting calcium level is approximately 1.5mM in the rat femoral artery (Van Heijst 2000). This balance of calcium concentrations is maintained by multiple membrane-based \(\text{Ca}^{2+}\) channels, pumps, compartments and intracellular buffers (Ratz 2005).
**Figure 2:** Schematic diagram of calcium entry into the cell. Modified figure (see review Ca\(^{2+}\) {Ratz, 2005}). Intracellular calcium is highly regulated by multiple membrane based channels, pumps, and compartments. Calcium is pumped from the intracellular matrix into the Sarcoplasmic Reticulum (SR) or out of the cell. Upon stimulation, channels open allowing for an influx of calcium. VOCC, Voltage operated calcium channel; SOCC, Store operated calcium channel; ROCC, Receptor operated calcium channel; SR, Sarcoplasmic reticulum. IP\(_3\), Inositol tri-phosphate.
1.4 Calcium Sensitization

In addition to the dependency on increases in [Ca$^{2+}$], for activation of contraction, studies as early as 1984 recognized that activation of smooth muscle G protein-coupled receptors (GPCR) by stimuli such as norepinephrine can produce greater increases in force for a given increase in [Ca$^{2+}$] than does activation of muscle by KCl (Morgan and Morgan 1984; DeFeo and Morgan 1985; Bradley and Morgan 1987; Karaki, Sato et al. 1988; Rembold and Murphy 1988; Sato, Ozaki et al. 1988; Gerthoffer, Murphey et al. 1989; Ozaki, Sato et al. 1989; Himpens, Kitazawa et al. 1990). These results are supported by data obtained from Ca$^{2+}$-clamped (permeabilized) tissues showing that, at constant Ca$^{2+}$ concentrations, cyclic nucleotides can induce relaxation (Ruegg and Paul 1982; Ruegg and Pfitzer 1985), and stimuli activating contractile GPCRs can elevate force (Kitazawa 1988; Nishimura, Kolber et al. 1988; Kobayashi, Kitazawa et al. 1989; Himpens, Kitazawa et al. 1990) concomitant with increases in MLC phosphorylation (Kitazawa, Gaylinn et al. 1991). Thus, the degree of sensitivity of smooth muscle contractile proteins to increases in [Ca$^{2+}$] is a regulated parameter. Importantly, Ca$^{2+}$ sensitivity appears to play as important a role in regulation of smooth muscle contraction as does increases in [Ca$^{2+}$], alone (reviewed by (Savineau and Marthan 1997; Hori and Karaki 1998; Somlyo and Somlyo 1998; Pfitzer 2001; Kawano, Yoshimura et al. 2002; Somlyo 2003)).
1.5 GPCR and their Role in Calcium Sensitivity

Several mechanisms are involved in regulating calcium sensitivity, such as the modulation of MLCK activity, mechanisms involving thin filament regulation, and possible regulation by heat shock proteins. However, calcium sensitivity is primarily regulated at the level of MLC phosphorylation by modulation of MLCP activity (Kitazawa, Gaylinn et al. 1991; Kitazawa, Masuo et al. 1991; Kubota, Nomura et al. 1992).

MLCP activity is largely regulated by G protein coupled receptors linked to one of two identified trimeric G proteins, $G_q$ and $G_{12/13}$ (Somlyo 2003). The activation of these G proteins leads to the generation of multiple second messengers which result in the downstream inhibition of MLCP (Figure 3). Therefore, most of the upstream signals generated upon GPCR stimulation, including ROK and PKC, are regulated at the plasma membrane although the motor proteins responsible for generation of force reside in the cell interior (Ratz 2005).

The GPCR associated with the trimeric $G_q$ protein are stimulated by many activators such as: acetylcholine [muscarinic M1], $\alpha$1-adrenergic agonists, angiotensin II, ATP [P2x and P2y], histamine [H1], platelet-derived growth factor (PDGF), serotonin [5-HT-1c], thyrotropin-releasing hormone (TRH) and vasopressin. Once stimulated, the $G_q$ protein triggers a cascade of activity beginning with the activation of phospholipase C beta (PLC$_{\beta}$) followed by the activation of phosphatidylinositol di-phosphate (PIP$_2$). PIP$_2$ is broken into two components, inositol 1,4,5-triphosphate (IP$_3$) stimulating calcium release from the SR and diacylglycerol (DAG) activating, with the aid of intracellular
calcium, protein kinase C (PKC). PKC phosphorylates a 17-kDa PKC-activated phosphatase inhibitor (CPI-17) on the Thr38 site. Phosphorylated CPI-17 binds to and inhibits MLCP.

As with the trimeric G\text{q} protein, the GPCR associated with trimeric G_{12/13} protein, also triggers a cascade of events upon stimulation. Some of those factors responsible for activation include: Ca\textsuperscript{2+} calmodulin-dependent protein kinases (CaMKI to IV), cAMP phosphodiesterase, MLCK, NAD\textsuperscript{+} kinase, nitric oxide synthase, phosphoinositide 3-kinase and plasma membrane Ca\textsuperscript{2+} ATPase (Ca\textsuperscript{2+} pump). Once stimulated, the trimeric G_{12/13} protein activates the low molecular weight GTPase, rhoA. RhoA recruits rhoA Kinase (ROK) to the plasma membrane and activates it. ROK may then phosphorylate either one of two myosin phosphatase targeting subunits (MYPT1-Thr696 or MYPT1-Thr653) associated with MLCP, resulting in inhibition.
Figure 3: Schematic diagram of G-protein coupled receptors. MLCP is inhibited by CPI-17 and ROK. Two identified trimeric G proteins are responsible: $G_q$ activates CPI-17 and $G_{12/13}$ activates ROK. $\alpha, \beta, \gamma$, subunits of the trimeric G protein; GTP, guanosine triphosphate; GDP, guanosine diphosphate; GDI-RhoA-GDP, inactive rhoA GTPase; RhoA-GTP, active rhoA GTPase; ROK, RhoA Kinase; PLC$\beta$, phospholipase C beta; PIP$_2$, phosphatidylinositol di-phosphate; IP$_3$, inositol 1,4,5-triphosphate; DAG, diacylglycerol; PKC, protein kinase C; CPI-17, 17-kDa PKC-activated phosphatase inhibitor.
1.6 Plasma Membrane

The smooth muscle plasma membrane contains receptors, channels and pumps that are necessary for the downstream regulation of contraction. Not only does the plasma membrane contain the GPCRs, $G_q$ and $G_{12/13}$, regulating MLCP, it is also hypothesized that the ROK is activated at the plasma membrane (Ratz 2005).

The smooth muscle plasma membrane contains several domains. One these domains include caveolae, which have been recently called into interest for their role in the regulation of smooth muscle contraction. Caveolae are small 50-100 nm cave-like invaginations of the plasma membrane housing a unique lipid domain that contains high concentrations of cholesterol and sphingolipids (Smart, Graf et al. 1999) and display an alternating pattern when viewed using immunohistochemical fluorescence-labeling of selected proteins (North, Galazkiewicz et al. 1993; Tanaka, Hijikata et al. 2001; Hagiwara, Nishina et al. 2002). Several studies support the hypothesis that GPCR stimulation increases translocation of inactive cytosolic rhoA (bound to rhoA-GDI) to peripheral, plasma membrane sites such as caveolae, where rhoA-GTP activates ROK (Fujihara, Walker et al. 1997; Gong, Fujihara et al. 1997; Taggart, Lee et al. 1999), supporting a caveolar role in regulation of $Ca^{2+}$ sensitization (Taggart 2001). Moreover, the hypothesis that caveolae play an important role in GPCR-induced contraction is supported by data showing that disruption of the caveolar structure with the cholesterol-depleting agent, methyl-$\beta$-cyclodextrin, or by caveolin-1 knockout, reduces the ability of some GPCR stimuli, but not KCl, to produce contraction (Drab, Verkade et al. 2001; Dreja, Voldstedlund et al. 2002; Je, Gallant et al. 2004)(see Ratz review ((Ratz 2005))).
1.7 Membrane Depolarization with KCl and KCl-induced Ca\(^{2+}\) Sensitization

High potassium physiological saline solutions are frequently used in the laboratory to depolarize smooth muscles cells, opening VOCCS, allowing for an influx of calcium, an increase in [Ca\(^{2+}\)], and force development. This technique is based on the well accepted premise that increases in [Ca\(^{2+}\)], is the fundamental regulator of smooth muscle cell contraction (Karaki 2004).

There are several benefits to using this technique to elicit a contraction in smooth muscles. The use of high K\(^{+}\) bypasses the complex mechanisms involved in GPCR activation of smooth muscles. For example, KCl, unlike stimuli that activate G\(_{\alpha}\), does not activate PLC and cause an increase in inositol triphosphate levels (Ratz 1990). As noted, the GPCR pathways involve many second messengers to activate series of reactions. The direct increase in [Ca\(^{2+}\)], by membrane depolarization provides simplicity to an otherwise complex mechanism. The ease of this technique also makes it highly reproducible.

In addition to simplicity and reproducibility, the benefits of high K\(^{+}\) in the study of smooth muscle cells are extensive. High K\(^{+}\) has been used in comparative studies to help understand the complex mechanisms of GPCR activation, to understand the relationship between calcium and force, and has shed a great deal of light onto the subject of calcium sensitization (Karaki, Sato et al. 1988). Because KCl was originally thought to act solely by causing elevations in [Ca\(^{2+}\)], the discovery by several laboratories, including our own, that KCl can cause Ca\(^{2+}\) sensitization, was somewhat surprising. There is strong evidence for participation of ROK in the regulation of KCl-induced calcium sensitization. Whether or not PKC plays a role remains to be determined. Also,
precisely how KCl causes Ca\textsuperscript{2+}-sensitization remains to be determined. The two primary possible mechanisms include Ca\textsuperscript{2+}-induced Ca\textsuperscript{2+}-sensitization, and depolarization-induced Ca\textsuperscript{2+}-sensitization.

### 1.8 Permeabilization

Permeabilization of smooth muscles has been a valuable technique for the direct control of cytoplasmic solute composition and, consequently, for our understanding of contractile regulation (Kitazawa, Kobayashi et al. 1989). Additionally, researchers have been using this technique of chemically skinning smooth muscle in order to clamp calcium to better understand Ca\textsuperscript{2+}-sensitization (Anabuki, Hori et al. 2000; Sward, Dreja et al. 2000; Takeuchi, Kushida et al. 2004; Durlu-Kandilci and Brading 2006).

There are currently four different detergents used today to chemically permeabilize tissues: saponin, \(\beta\)-escin, \(\alpha\)-toxin and Triton X-100. Saponin is known to complex with cholesterol molecules in the surface membranes of cells to form pores approximately 9 nm in diameter (Bangham, Horne et al. 1962) and found to uncouple the receptors involved in physiological activation of smooth muscle (Kitazawa, Kobayashi et al. 1989). \(\beta\)-escin retains surface receptor coupling (Kobayashi, Kitazawa et al. 1989), and compared to saponin, has been found to have a milder action on the surface membrane and is a more potent modifier of SR function (Launikonis and Stephenson 1999). In contrast, \(\alpha\)-toxin is much milder than these two agents forming pores permeable to molecules of up to 3kDa; and has been found to develop maximal force upon \(\alpha\)-
adrenergic stimulation, retain receptor activation (Kitazawa, Kobayashi et al. 1989) and retain SR coupling. Finally, Triton X-100 leads to an extensively permeabilized cell in which all membrane functions are eliminated including the loss of receptor and SR coupling; and loses responsiveness to PKC activators (Kitazawa, Takizawa et al. 1999).

1.9 Objective

As demonstrated, the use of KCl to induce smooth muscle contraction in the laboratory is very common and effective and it has been shown that KCl-induced contraction induces Ca\(^{2+}\)- sensitization. Once the cell signaling and contractile protein regulatory systems induced by KCl are completely mapped, responses to KCl can quite accurately be applied for comparison to more fully understand the vastly more complex GPCR-induced regulation of smooth muscle contraction (Ratz 2005). Therefore, the objective of this project was to test the hypothesis that KCl-induced calcium sensitization reflects Ca\(^{2+}\)-induced calcium sensitization by measuring the effect of selective ROK inhibition on Ca\(^{2+}\)-dependent contractions induced in chemically permeabilized isolated femoral artery rings.
MATERIALS AND METHODS

2.1 Tissue Preparation

Tissues were prepared as previously described (Ratz 1993). Adolescent male and female New Zealand White rabbits were anesthetized and then injected with pentobarbital as approved by the Medical College of Virginia at Virginia Commonwealth University Institutional Animal Care and Use Committee protocol #0305-3208l. Femoral arteries were immediately removed and placed in cold (4°C) normal physiological saline solution (NPSS; in mM: 140 NaCl, 4.7 KCl, 2.0 morpholino-propanesulfonic acid (MOPS), 0.02 Na₂ethylenediamine tetraacetic acid (EDTA) -to chelate trace heavy metals, 1.2 Na₂HP0₄•7H₂O, adjusted to pH 7.4 with 5M NaOH at 37°C, 1.2 MgCl₂, 1.6 CaCl₂ and 5.6 α-D-glucose)). High-purity (18.2 MΩ) deionized water was used throughout the study. Fat and adventitia were removed mechanically under a binocular dissecting microscope (Olympus SZX12 or Zeiss 10093), and the endothelium was removed by gently rubbing of the intimal surface with a rough metal rode approximately the size of the arterial lumen diameter. The arteries were cut into 2-3 mm-wide rings. Tissues were stored for no more than four days, covered at 4°C in NPSS.
2.2 Isometric Force

Contractile force (F) was measured as previously described (Ratz 1993). Each artery ring was suspended in an aerated 5ml tissue bath (Figure 4)(Myograph System-610M -Danish Myo Technology, Denmark) between two stainless steel pins, attached to a micrometer and isometric force transducer for length adjustments and force measurements, respectively. The bath contained cold (4°C) NPSS. Voltage signals from the Myograph were digitized and visualized on a computer screen as F (in g). Data were acquired through an analog-digital converter board (National Instruments) and analyzed using DASYLab (DasyTech, Amherst, NH)(Figure 5). Following a warm-up period and \( L_0 \) determination, the tissues were ready to undergo permeabilization without any further length changes. All solutions were kept at 37°C prior to permeabilization.

All tissues underwent the standard “wake-up” and \( L_0 \) determination before experimentation occurred.

2.2.1. Raw Data

All raw data is presented in grams of force and the graphical models mimic actual force tracing (Figures 6 & 7). These include the “wake-up” and \( L_0 \) contractions in the intact tissue, as well as the “wake-up” contraction in permeabilized tissues. Statistics were not performed on raw data.
Figure 4: Diagram from the Myograph System-610M owners manual. This system allows for temperature and aeration control, length adjustments, isometric force measurements and easy solution changes. Following mounting and equilibration, the passive length-tension relationships of the vessels are determined, in the normalization procedure. This ensures reproducibility among the segments and between experiments. During the actual experiments, the circumference of the vessel is kept constant, i.e. the vessels are examined under isometric conditions. Compounds are added directly to the chamber and vessel tension is measured for contractile or relaxing effects of the tested compound.
2.2.2. Standard Warm-up & Tissue “Wake-Up”

The tissues warmed for 45 minutes to 1 hour at 37°C in aerated NPSS until the bath temperature reached 37°C. The myograph system was calibrated with the DASYLab software (Force Calibration Kit -Danish Myo Technology, Denmark) following warm-up.

The tissues were then stretched to 0.5 grams of force and allowed to relax for 5 minutes and the process was repeated. Following the second stretch and relaxation, NPSS was replaced with a high potassium solution (KPSS; NPSS in which 110 mM KCl was substituted isosmotically for NaCl) for 5 minutes to elicit a contraction, washed twice with NPSS and allowed to relax for 10 minutes. This process was repeated two more times. Following the third KPSS contraction, the last 30 seconds of the contraction was measured for the average maximal force ($F_T$) (Figure 6).
2.2.3. L₀ Determination

The muscle length for which active force was maximum (L₀) was determined for each tissue using an abbreviated length-tension curve (Herlihy and Murphy 1973; Ratz and Murphy 1987). Previous studies in Dr. Ratz’s lab have found that a passive (Fₚ) to active (Fₐ = Fₜ - Fₚ) ratio of 0.13 ± 0.03, is the standard L₀ for the rabbit femoral artery.

This process involved stretching the tissues by 0.5 gram force increments every five minutes until they reached 2 grams of force, releasing the tissue down to the calculated passive force value and immediately contracting with KPSS for five minutes. Stretching to 2 grams of force and rapidly releasing the length allows for the breaking of attached latch bridges and an accurate passive force measurement. The calculated passive force value is estimated by the following formula, \((Fₕ * 0.13)/1.13\), where 1.13 is an adjustment factor due to the fact that Fₜ and not Fₐ was used in the calculation. Following contraction the artery rings were washed twice with NPSS, and relaxing for ten minutes in the NPSS. The Fₜ was measured and the process was repeated once more or until the Fₚ/Fₐ values were in range (Figure 6).

In the Intact Tissue- KCl Induced Contraction (see section 3.1) tissue preparation, 1µM phentolamine was used to block α-receptor agonists during the L₀ determination. Phentolamine was not used in any other L₀ determination.
Figure 6: Wake-Up and L₀ determination. Following warm-up in normal physiological saline solution (NPSS) tissues were stretched to 0.5 grams of force two times, resting for five minutes in between each stretch. Following the second stretch, the tissues were contracted with high potassium saline solution (KPSS) for five minutes. Following the contraction, tissues were washed twice with NPSS and allowed to rest for ten minutes. This wake-up process was repeated two additional times. L₀ determination is similar to the wake-up except the tissues are stretched by 0.5 grams of force at five minute intervals until 2 grams of force is reached. Length is then released to a calculated value corresponding to the ideal passive force. Tissues are then contracted with KPSS for five minutes, washed twice with NPSS, then allowed to relax for ten minutes. This process is repeated at least one more time or until the passive to active force ratio is 0.13 ± 0.03.
2.3 KCl-induced Contraction in Intact Tissue

Following L0 determination, the femoral artery rings were washed twice with a NPSS and allowed to completely relax. The drug inhibitors were then added to three of the four chambers and incubated for 20 minutes, the fourth chamber acted as the control. After drug incubation, the solutions were switched to KPSS containing the same drug inhibitors and allowed to contract for minimum of ten minutes. Raw data were collected and normalized before statistics were run.

2.4 Calcium Response Curve- Intact Tissue

Following L0 determination, the femoral artery rings were washed twice with a nominally calcium-free physiological saline solution (0Ca-NPSS; NPSS without Ca²⁺ added), and allowed to rest for ten minutes. The artery rings were then treated with a high potassium nominally calcium-free physiological saline solution (0Ca-KPSS; KPSS without Ca²⁺ added) for five minutes and then washed with 0Ca-NPSS. The drug inhibitors were then added to three of the four chambers and incubated for 20 minutes, the fourth chamber acted as the control. After drug incubation, the solutions were switched to 0Ca-KPSS containing the same drug inhibitors and allowed to rest for five minutes. A calcium concentration-response curve (0.10mM – 2.00mM) was constructed by adding a specific amount of 0.1M Ca²⁺ -KPSS stock solution (0Ca-KPSS with 1M CaCl₂ stock solution added). Raw data were collected and normalized before statistics were run.
2.5 Tissue Permeabilization

Artery rings at L₀ were washed with a calcium-free solution, “relaxing solution” (RS; in mM: 74.1 potassium methanesulphonate, 4.0 magnesium methanesulphonate, 4.0 Na₂ATP, 4.0 EGTA, 5.0 creatine phosphate, and 30.0 PIPES (adjusted to pH 7.1 with 1N KOH and ionic strength was kept constant at 0.18 with additional 0.5M potassium methanesulfonate)), and then subject to chemical permeabilization by either 20 μg/ml α-toxin or 1% Triton X-100 (See 2.5.1 & 2.5.2).

2.5.1. Permeabilizing with Alpha Toxin & “Wake-Up”

RS was replaced with 20μg/ml α-toxin, enough to cover the tissue, for 30 minutes at 30°C and continued for 20 minutes at 25°C in RS with the addition of 10μM A23187 (to deplete SR calcium). Following permeabilization the tissues were washed thoroughly with RS to remove the α-toxin and held at 30°C. In order to “wake-up” the artery rings following permeabilization, the tissues were contracted three times with a free calcium concentration of pCa = 6.5 to 6.0 (depending on tissue responsiveness) in the presence of 10μM A23187 and allowed to relax in RS between contractions. The third contraction (F₁) was used to normalize experimental data by measuring the Fₚ and Fₜ values.
2.5.2. Permeabilizing with Triton X-100 & “Wake-Up”

RS was replaced with 1% Triton X-100, enough to cover the tissue, for 15 minutes at 30°C. Following permeabilization the tissues were washed thoroughly with RS to remove the Triton X-100 and held at 30°C.

In order to “wake-up” the artery rings following permeabilization, the tissues were contracted twice with a free calcium concentration of pCa = 6 and allowed to relax in RS after each contraction. The second contraction ($F_2$) was used to normalize experimental data by measuring the $F_P$ and $F_T$ values.
Figure 7: Post-permeabilization “Wake-Up.” Following permeabilization, the arteries are contracted with calcium in order to establish a maximal force curve to normalize the experimental data. The α-toxin “wakes-up” faster and also deteriorate faster; therefore, two contractions are performed. The Triton X-100 is slower to respond and requires three wake-up contractions.
2.6 Calcium Response Curve- Permeabilized Tissue

To deplete sarcoplasmic reticular calcium, 10µM A23187 (a calcium ionophore) was used in all permeabilized tissue. In Triton X-100 permeabilized tissue, 1unit/ml of calmodulin (Sigma Aldrich, St. Louis, MO) was added to compensate for loss during permeabilization. All tissues and solutions were held at 30°C throughout the calcium response curve as a change in temperature would alter the calculated free calcium level.

Inhibitors were then added to three of the four chambers and incubated for 15-20 minutes, on average, except when otherwise noted, the fourth chamber acted as the control. Following incubation, calcium was then added incrementally, allowing for each contraction to reach a maximal contraction (waiting until the contraction leveled out or began to drop) before the next dose of calcium was added. Raw data were collected and normalized before statistics were run.
2.7 Drugs & Solutions

All detergents, inhibitors and calcium ionophores were purchased from Calbiochem Corp, San Diego, CA.

2.7.1. Contracting Solution

To activate muscle contractions at specific free calcium levels, “contracting solutions” (CS) were made by the addition of the appropriate volume of 1M CaCl2 (Fluka Chemicals) stock to the RS bath as determined using WEBMAXC (Patton, Thompson et al. 2004).

2.7.2. MLCK Inhibitors

Wortmannin was dissolved in dimethylsulfoxide (DMSO) and MLCK Inhibitor Peptide 18 (MLCK-18) was dissolved in deionized water.

2.7.3. ROK Inhibitors

H-1152, Y-27632 and HA1077 were dissolved in deionized water.

2.7.4. PKC Inhibitors

Bisindolylmaleimide I (GF 109203X) and Gö 6976 were dissolved in DMSO, Protein Kinase C Inhibitor Peptide 19-31 (PCK 19-31, RFARKGALRQKNV) and Protein Kinase Cζ Pseudosubstrate inhibitor (PCKζ, SIYRRGARRWRKL) were both dissolved in 5% acetic acid.
2.7.5. Calcium Ionophore

A23187, Free Acid, *Streptomyces chartreusensis* (Calcimycin) was dissolved in methanol.

2.7.6. α-Adrenergic Receptor Agonist

Phentolamine was dissolved in deionized water (Sigma-Aldrich Co., St. Louis, MO)

2.8 Statistics

For each study described, the *n* value was equal to the number of rabbits from which arteries were taken. Raw data were recorded, normalized, entered into GraphPad Prism 3.02 (Graphpad Software, Inc., San Diego, CA) and analyzed using the “Sigmoidal dose-response (variable slope)” equation: \( Y = \text{Bottom} + \frac{\text{Top} - \text{Bottom}}{1 + 10^{((\log EC_{50} - X) \times \text{Hillslope})}} \), where \( X \) is the logarithm of concentration, \( Y \) is the response, \( Y \) starts at the Bottom (minimum) and goes to the Top (maximum) with a sigmoidal shape. This is identical to the “four parameter logistics equation.” Statistics were examined on these four parameters: Minimum, Maximum, \( \log EC_{50} \) and Hillslope. The null hypothesis was examined using Student’s t-test; the null hypothesis was rejected at \( P < 0.05 \). Graphical representations are based on curve-fit data to these four parameters. Statistical analyses and curve fitting were performed using Prism 3.02, Excel
2002 (Microsoft Corporation, Redmond, WA) and SigmaPlot 9.01 (Systat Software, Inc.).

2.8.1. Normalization of Intact Tissue

Experiments conducted in the intact tissues are normalized to the final $L_0$ contraction ($F_0$) because this is the last contraction prior to the manipulation of the tissue. Therefore, active force is represented as $F/F_0$, where $F$ is the experimental contraction. These include the standard KCl-induced contraction and calcium concentration-response curve in the intact tissue.

2.8.2. Normalization of Alpha Toxin Permeabilized Tissue

Experiments conducted in the $\alpha$-toxin permeabilized artery rings were normalized to the third “wake-up” contraction following permeabilization. The tissues permeabilized with $\alpha$-toxin had variable responses to the first “wake-up”; therefore, three “wake-ups” were performed where the second and third “wake-up” had to possess the same responsiveness to the same level of calcium. Experimental data were normalized to the third “wake-up” ($F_1$) because it is the last contraction prior to the manipulation of the tissue. Therefore, active force is represented as $F_2/F_1$, where $F_2$ is the experimental contraction. These include all tissues permeabilized with $\alpha$-toxin.
2.8.3. Normalization of Triton X-100 Permeabilized Tissue

Experiments conducted in the Triton X-100 permeabilized artery rings were normalized to the second “wake-up” contraction following permeabilization. The tissues permeabilized with α-toxin deteriorate more quickly than α-toxin and the first and second “wake-up” possess the same responsiveness to the same level of calcium. Experimental data were normalized to the second “wake-up” (F₁) because it is the last contraction prior to the manipulation of the tissue. Therefore, active force is represented as F₂/F₁, where F₂ is the experimental contraction. These include all tissues permeabilized with Triton X-100.

2.8.4. Permeabilization Detergents and Curve Fitting

The α-toxin was dissolved in relaxing solution to a concentration of 20µg/ml and a 10 % Triton X-100 was diluted with relaxing solution to a concentration of 1% Triton X-100. These solutions were reused and slowly lost potency over 1-2 months time. The curves shifted to the right and down. A control was used in each experiment and statistics were run on the experiment versus control for each day. In the graphical representations beginning with Figure 10, the sigmoidal force curves represented in panel (A) in those diagrams with multiple panels or in single panel sigmoidal force curves, these sigmoidal curves are based on a summarized data in order to show the relationship between the different inhibitors versus a control. The method used to represent these summarized sigmoidal force curves involved averaging all of the control data for each type of permeabilization technique. For each individual experiment the difference between
treatment and control was measured for each tissue; the difference for each type of inhibitor was then averaged and that difference was plot against the averaged control curve. This created a summarized curve that represents each inhibitor’s effect compared to the control. The remaining figures are based on actual data and are manipulated as described above in the statistics section.
RESULTS

3.1 Intact Tissue- KCl Induced Contraction

There are two phases of a KCl-induced (110mM) contraction in the femoral artery, an initial peak phase contraction followed by a sustained, tonic phase contraction. The initial peak phase reaches ~70% of the normalized contraction immediately following stimulation with a high potassium solution. Following the initial peak, force continues to develop at a slower rate until it reaches ~90% of the normalized contraction, at which time the contraction is sustained with only a very small decrease in force over time. The tonic phase is less than 100% because KCl-induced contractions produced in the presence of the α-adrenergic receptor antagonist, phentolamine are normalized to that produced by KCl in the absence of α-receptor blockade, and ~10% of a KCl-induced contraction without α-receptor blockade can be attributed to release of norepinephrine from periarterial nerves.

The ROK inhibitors, Y-27632 and HA-1077, selectively inhibited the tonic but not phasic phase of a KCl-induced contraction (Ratz 2005) in rabbit femoral artery; and Y-27632 does not inhibit KCl-induced increases in [Ca^{2+}], but does inhibit KCl-induced increases in MLC phosphorylation (Urban, Berg et al. 2003). Moreover, we showed that 1µM of the PKC inhibitor, GF-109203X, did not produce inhibition (Ratz 2005). These
data suggest calcium sensitization occurs during the tonic phase of contraction, and that ROK but not PKC plays a role in KCl-induced calcium sensitization. Data shown in Figure 8, in which the newer and highly selective ROK inhibitor, H-1152 was used, confirms these findings, and a more complete temporal analysis of the effect of GF-109203X suggests that PKC may play a minor role in KCl-induced early contraction. In particular, neither the PKC inhibitor, GF-109203X (1µM) nor the ROK inhibitor, H-1152 (0.3µM) significantly reduced the initial peak contraction. However, the ROK inhibitor reduced the sustained contraction at both the two and one-half minute and the ten minute mark, whereas the PKC inhibitor reduced only the two and one-half minute mark. These data suggest that during a KCl-induced contraction in intact tissue, ROK is largely responsible for sustained contraction, whereas PKC may play a small role only during the early phase of the contraction.
Figure 8. Examples of force tracings (A) and average force responses produced at the early, peak phase and at 2.5 and 10 min of the tonic phase (B) of contractions induced by 110mM KCl in intact femoral arteries in the absence of a drug (Control) and presence of GF-109203X (1µM, inhibitor of conventional PKC isotypes) and H-1152 (0.3µM, inhibitor of ROK). Data in (B) are averages ± SE. n = 3, * = P < 0.05 compared to control values.
3.2 Intact Tissue – Calcium Concentration-Response Curve in a KCl-Induced Contraction

To determine the role of calcium in a KCl-induced (110 mM) steady-state (tonic) contraction in the intact femoral artery, a calcium concentration-response curve was constructed using a nominally calcium-free physiological saline solution (0Ca-NPSS). The tissue did not produce force when treated with the high potassium 0Ca-KPSS solution alone (data not shown). The calcium concentration-response curve (pCa -4.0 to -2.75) in intact femoral artery incubated in 0Ca-KPSS did not produce an initial peak contraction. Instead, force developed monotonically to increases in extracellular calcium. These data support the hypothesis that during membrane depolarization, the level of extracellular calcium determines the level of \([\text{Ca}^{2+}]_i\), and there is a correlation between the amount of extracellular calcium and the strength of the contraction.

The study shown in Figure 8 was done using a single extracellular calcium concentration (1.6mM). To determine the degree to which KCl-induced ROK-dependent tonic contraction is contingent on the level of extracellular calcium, tissues were treated with the ROK inhibitor, H-1152 (0.3µM), and a calcium concentration-response curve was constructed and compared to the control response in the absence of the ROK inhibitor (Figure 9). For a comparison, the effect of the PKC inhibitor, GF-109203X (1µM) on a calcium concentration-response curve was also studied. A strong MLCK inhibitor was also used to test the hypothesis that MLCK plays the predominant role in regulation of KCl and Ca\(^{2+}\)-induced contraction. Tissues were treated with kinase antagonists 15 minutes prior to and during the calcium concentration-response curve. The
MLCK inhibitor nearly abolished the contraction (P < 0.001), indicating that a KCl-induced contraction is highly dependent on MLCK. The ROK inhibitor also significantly reduced contractions at all levels of the calcium concentration-response curve, (P < 0.001), whereas the PKC inhibitor did not significantly attenuate force at any calcium concentration. These data support previous studies indicating that during a KCl-induced contraction, ROK is activated, allowing for calcium sensitization to occur, whereas the role of conventional PKC isotypes is insignificant.

![Figure 9](image)

**Figure 9.** KCl-induced calcium concentration-response curve. Force tracing of contraction induced by increasing levels of calcium (pCa -4.0 to -2.75) in intact femoral arteries in a 110mM KCl solution in the absence of a drug (Control) and the presence of GF-109203X (1µM, inhibitor of conventional PKC isotypes), H-1152 (0.3µM, inhibitor of ROK) and wortmannin (1µM, inhibitor of MLCK). n = 3, * = P < 0.001.
3.3 Permeabilized Tissue – Alpha Toxin vs. Triton X-100

Two different detergents were used to permeabilize the femoral artery, 20μg/ml alpha toxin and 1% Triton X-100, and calcium concentration-response curves (pCa -7.5.0 to -5.5) were constructed. The chemically permeabilized tissue produced a maximal contraction of approximately 8-10% of KCl-induced intact tissue contraction.

Additionally, the α-toxin permeabilized tissue responded to lower doses of calcium and reached a higher maximum contraction then the Triton X-100 permeabilized tissue (Figure 10 (A)). The Triton X-100 preparation had a significant decrease in maximal force (Figure 10 (C)) and EC$_{50}$ (Figure 10 (B)) compared to the α-toxin preparation (P < 0.05). This shift to the right produced in Triton X-100 permeabilized muscle compared to α-toxin permeabilized muscle is supported by previous studies (Kitazawa, Takizawa et al. 1999; Adegunloye, Lamarre et al. 2003). However, one study found the opposite to be true; they found that Triton X-100 had higher calcium sensitivity than α-toxin (Van Heijst, Blange et al. 2000).

These data support that of others who showed that there is either membrane bound processes that are being disrupted or the loss of several intracellular molecules from Triton X-100-permeabilized smooth muscle (Li, Eto et al. 1998; Kitazawa, Takizawa et al. 1999; Adegunloye, Lamarre et al. 2003), that are involved in calcium sensitization, that are not disrupted or lost in the α-toxin preparation.

The Hill Slope for the Triton X-100 preparation was higher than the α-toxin preparation, but it was not significant (Figure 10 (D)). This suggests that the cooperativity for calcium is similar in the two preparations.
Figure 10. Examples of force tracing (A) LogEC₅₀ (B), minimum and maximal contractions (C) and Hillslope (D) in chemical permeabilized femoral arteries, skinned with either 20µg/ml α-toxin (retains both SR and receptor coupling, forming pores permeable to molecules of up to 3kDa) or 1 % Triton X-100 (extensively permeabilized cell in which all membrane functions are eliminated including the loss of receptor and Sarcoplasmic Reticulum (SR) coupling) in the absence of any drug inhibitors (Controls), respectively C-Toxin or C-Triton. Data in (B, C, D) are averages ± SE. α-toxin n = 6, Triton X-100 n = 14, * = P < 0.05
3.4 Permeabilized Tissue- MLCK inhibitor

To determine if MLCK plays the predominant role in the regulation of Ca\(^{2+}\)-induced contractions in chemically permeabilized tissue, a calcium concentration-response curve (pCa -7.5.0 to -5.5) was performed in both α-toxin and Triton X-100 permeabilized femoral artery in the presence of MLCK inhibitors. Tissues were treated with kinase antagonists 20 minutes prior to and during the calcium concentration-response curve. Low doses of wortmannin, 1µM, significantly reduced the maximum peak contraction (Figures 11 (A)(C) & 12 (A)(C)) and the logEC\(_{50}\) (Figures 11 (B) & 12 (B)) in both the α-toxin and Triton X-100 preparations, whereas the Hill slope (Figures 11 (D) & 12 (D)) was not significantly different in either of the two preparations, (P < 0.005). High doses of wortmannin, 10µM, completely abolished the contraction except at the very highest doses of calcium. These data suggest that a Ca\(^{2+}\)-induced contraction in α-toxin and Triton X-100 chemically permeabilized tissue is highly dependent on MLCK.

Data was normalized and curve-fitted to a sigmoidal curve as described in the Materials & Methods section. Statistics were run on the 1µM wortmannin preparations, additional data is needed to run statistics on the 10µM preparations.

3.4.1. Alpha Toxin - 1µM Wortmannin

The minimum (Figures 11 (A)(C)) of the force curve produced by tissues exposed to 1µM wortmannin (0.002 ± 0.002 F\(_2\)/F\(_1\)) was not significantly different than that produced by control tissues (0.005 ± 0.004 F\(_2\)/F\(_1\)), (P = 0.47).
The maximum (Figures 11 (A)(C)) of the force curve was significantly different when comparing 1µM wortmannin-treated tissue (0.39 ± 0.14 F₂/F₁) and control tissues (1.04 ± 0.07 F₂/F₁), (P = 0.01).

Higher calcium levels were required to initiate force in 1µM wortmannin-treated tissues (pCa -6.5) compared to the control tissues (pCa -7.0); resulting in a significant difference in log (Figure 11 (B)) between the 1µM wortmannin treated tissue (pCa -6.39 ± 0.04) compared to the control (pCa -6.6 ± 0.09), (P = 0.005).

The Hill slope (Figure 11 (D)) of the force curve was not significantly different when comparing 1µM wortmannin treated tissues (8.53 ± 3.97) and control tissues (5.98 ± 1.26), (P = 0.57).
Figure 11. Examples of force tracing (A) LogEC$_{50}$ (B), minimum and maximal contractions (C) and Hill slope (D) in 20µg/ml α-toxin (retains both SR and receptor coupling, forming pores permeable to molecules of up to 3kDa) permeabilized femoral arteries, in the absence of a drug (Control) and presence of wortmannin (1µM & 10µM, inhibitor of MLCK). Data in (B, C, D) are averages ± SE. $n = 3$, * = P < 0.05 compared to control values.
3.4.2. Triton X-100- 1µM wortmannin

The minimum (Figure 12 (A)(C)) of the force curve produced by tissues exposed to 1µM wortmannin (0.001 ± 0.002 F₂/F₁) was not significantly different than that produced by control tissues (0.001 ± 0.001 F₂/F₁), (P = 0.91).

The maximum (Figure 12 (A)(C)) of the force curve was significantly different when comparing 1µM wortmannin-treated tissue (0.36 ± 0.05 F₂/F₁) and control tissues (0.79 ± 0.04 F₂/F₁), (P = 0.0005).

Higher calcium levels were required to initiate force in 1µM wortmannin treated tissues (pCa -6.25) compared to the control tissues (pCa -6.5); resulting in a significant difference in logEC₅₀ (Figure 12 (B)) between the 1µM wortmannin-treated tissues (pCa -6.18 ± 0.06) and control tissues (pCa -6.47 ± 0.05), (P = 0.01).

The Hill slope (Figure 12 (D)) of the force curve was not significantly different when comparing 1µM wortmannin treated tissues (7.54 ± 1.46) and control tissues (5.44 ± 1.45), (P = 0.35).
Figure 12. Examples of force tracing (A) LogEC$_{50}$ (B), minimum and maximal contractions (C) and Hillslope (D) in 1% Triton X-100 (extensively permeabilized cell in which all membrane functions are eliminated including the loss of receptor and Sarcoplasmic Reticulum (SR) coupling) permeabilized femoral arteries, in the absence of a drug (Control) and presence of wortmannin (1µM & 10µM, inhibitor of MLCK). Data in (B, C, D) are averages ± SE. $n = 4$, * = $P < 0.05$ compared to control values.
3.5 Permeabilized Tissue-Specific ROK Inhibitors

To determine the degree to which a Ca\(^{2+}\)-induced contraction is contingent on ROK in chemically permeabilized femoral artery, a calcium concentration-response curve (pCa -7.5.0 to -5.5) was performed in α-toxin and Triton X-100 permeabilized tissues in the presence of ROK inhibitors. Tissues were treated with ROK antagonists 15 minutes prior to and during the calcium concentration-response curve. Three doses, 0.3µM, 1µM and 3µM, of the specific ROK inhibitor H-1152 were used in both preparations. For a comparison, additional ROK inhibitors were used in both preparations: 10µM Y-27632 and 10µM HA-1077. Surprisingly, there was only one significant difference in all of these preparations, the Hill slope of the 3µM H1152 (Figure 16 (C)) in the α-toxin preparation, but not in the Triton X-100 preparation. These data suggest that a Ca\(^{2+}\)-induced contraction in α-toxin and Triton X-100 chemically permeabilized tissue is not dependent on ROK.

Data was normalized and curve-fitted to a sigmoidal curve as described in the Materials & Methods section. Statistics were run on all of these samples except for the 1µM H-1152, 10µM Y-27632 and 10µM HA-1077 α-toxin preparations.
3.5.1. Alpha Toxin - 0.3µM H1152

The minimum (Figure 13 (A)(C)) of the force curve produced by tissues exposed to 0.3µM H1152 (0.005 ± 0.004 F₂/F₁) was not significantly different than that produced by control tissues (0.01 ± 0.012 F₂/F₁), (P = 0.69).

The maximum (Figure 13 (A)(C )) of the force curve was not significantly different when comparing 0.3µM H1152-treated tissues (0.96 ± 0.10 F₂/F₁) and control tissues (1.07 ± 0.04 F₂/F₁), (P = 0.34).

The Log EC₅₀ (Figure 13 (B)) produced by tissues exposed to 0.3µM H1152 (pCa -6.69 ± 0.09) was not significantly different than that produced by control tissues (pCa -6.79 ± 0.12), (P = 0.53).

The Hill slope (Figure 13 (C)) of the force curve was not significantly different when comparing 0.3µM H1152-treated tissue (8.33 ± 1.27) and control tissues (5.90 ± 1.51), (P = 0.29).
Figure 13. Examples of force tracing (A) LogEC$_{50}$ (B), minimum and maximal contractions (C) and Hillslope (D) in 20µg/ml α-toxin (retains both SR and receptor coupling, forming pores permeable to molecules of up to 3kDa) permeabilized femoral arteries, in the absence of a drug (Control) and presence of H-1152 (0.3µM, 1µM & 10µM, inhibitor of ROK). Data in (B, C, D) are averages ± SE. $n = 3$. 

(A) Active Force (F2/F1) vs. pCa for Control, 0.3µM H-1152, 1µM H-1152, and 3µM H-1152.

(B) LogEC$_{50}$ for Control and 0.3µM H-1152.

(C) Minimum & Maximum Active Force (F2/F1) for Control and 0.3µM H-1152.

(D) Hill Slope for Control and 0.3µM H-1152.
3.5.2. **Triton X-100 - 0.3µM H1152**

The minimum (Figures 14 (A)(C)) of the force curve produced by tissues exposed to 0.3µM H1152 (-0.002 ± 0.0007 F₂/F₁) was not significantly different than that produced by control tissues (0.0007 ± 0.0009 F₂/F₁), (P = 0.09).

The maximum (Figures 14 (A)(C)) of the force curve was not significantly different when comparing 0.3µM H1152-treated tissues (0.82 ± 0.05 F₂/F₁) and control tissues (0.76 ± 0.04 F₂/F₁), (P = 0.08).

The Log EC₅₀ (Figures 14 (B)) produced by tissues exposed to 0.3µM H1152 (pCa -6.25 ± 0.05) was not significantly different than that produced by control tissues (pCa -6.42 ± 0.07), (P = 0.08).

The Hill slope (Figures 14 (D)) of the force curve was not significantly different when comparing 0.3µM H1152-treated tissue (5.47 ± 0.96) and control tissues (6.98 ± 1.26), (P = 0.37).
Figure 14. Examples of force tracing (A) LogEC$_{50}$ (B), minimum and maximal contractions (C) and Hillslope (D) in 1% Triton X-100 (extensively permeabilized cell in which all membrane functions are eliminated including the loss of receptor and Sarcoplasmic Reticulum (SR) coupling) permeabilized femoral arteries, in the absence of a drug (Control) and presence of H-1152 (0.3µM, inhibitor of ROK). Data in (B, C, D) are averages ± SE. $n = 5$. 
3.5.3. Triton X-100 - 1µM H1152

The minimum (Figures 15 (A)(C)) of the force curve produced by tissues exposed to 1µM H1152 (-0.0013 ± 0.001 F₂/F₁) was not significantly different than that produced by control tissues (0.006 ± 0.005 F₂/F₁), (P = 0.20).

The maximum (Figures 15 (A)(C)) of the force curve was not significantly different when comparing 1µM H1152-treated tissues (0.75 ± 0.03 F₂/F₁) and control tissues (0.79 ± 0.04 F₂/F₁), (P = 0.20).

The Log EC₅₀ (Figure 15 (B)) produced by tissues exposed to 1µM H1152 (pCa - 6.23 ± 0.04) was not significantly different than that produced by control tissues (pCa - 6.32 ± 0.09), (P = 0.08).

The Hill slope (Figure 15 (D)) the force curve produced by tissues exposed to 1µM H1152 (5.47 ± 0.96) and control tissues (6.98 ± 1.26), (P = 0.39).
Figure 15. Examples of force tracing (A) LogEC$_{50}$ (B), minimum and maximal contractions (C) and Hillslope (D) in 1% Triton X-100 (extensively permeabilized cell in which all membrane functions are eliminated including the loss of receptor and Sarcoplasmic Reticulum (SR) coupling) permeabilized femoral arteries, in the absence of a drug (Control) and presence of H-1152 (1µM, inhibitor of ROK). Data in (B, C, D) are averages ± SE. $n = 4$. 
3.5.4. **Alpha Toxin - 3µM H1152**

The minimum (Figures 16 (A)(C)) of the force curve produced by tissues exposed to 3µM H1152 (0.004 ± 0.0008 F₂/F₁) was not significantly different than that produced by control tissues (0.01 ± 0.005 F₂/F₁), (P = 0.19).

The maximum (Figures 16 (A)(C) of the force curve was not significantly different when comparing 3µM H1152-treated tissues (1.31 ± 0.23 F₂/F₁) and control tissues (1.43 ± 0.25 F₂/F₁), (P = 0.75).

The LogEC₅₀ (Figures 16 (B)) produced by tissues exposed to 3µM H1152 (pCa - 6.39 ± 0.03) was not significantly different than that produced by control tissues (pCa - 6.40 ± 0.04), (P = 0.91).

The Hill slope (Figures 16 (D)) the force curve was significantly different when comparing 3µM H1152-treated tissues (9.11 ± 0.14) and control tissues (7.26 ± 0.65), (P = 0.05).
Figure 16. Examples of force tracing (A) $\text{LogEC}_{50}$ (B), minimum and maximal contractions (C) and Hillslope (D) in 20$\mu$g/ml $\alpha$-toxin (retains both SR and receptor coupling, forming pores permeable to molecules of up to 3kDa) permeabilized femoral arteries, in the absence of a drug (Control) and presence of H-1152 (3$\mu$M, inhibitor of ROK). Data in (B, C, D) are averages ± SE. $n = 3$, * = $P < 0.05$ compared to control values.
3.5.5. **Triton X-100 - 3µM H1152**

The minimum (Figures 17 (A)(C)) of the force curve produced by tissues exposed to 3µM H1152 (-0.0005 ± 0.002 F₂/F₁) was not significantly different than that produced by control tissues (0.001 ± 0.001 F₂/F₁), (P = 0.47).

The maximum (Figures 17 (A)(C)) of the force curve was not significantly different when comparing 3µM H1152-treated tissues (0.80 ± 0.04 F₂/F₁) and control tissues (0.78 ± 0.04 F₂/F₁), (P = 0.71).

The LogEC₅₀ (Figure 17 (B)) produced by tissues exposed to 3µM H1152 (pCa - 6.24 ± 0.04) was not significantly different than that produced by control tissues (pCa - 6.29 ± 0.09), (P = 0.63).

The Hill slope (Figure 17 (D)) of the force curve was not significantly different when comparing 3µM H1152-treated tissue (5.94 ± 0.52) and control tissues (5.99 ± 0.56), (P = 0.94).
**Figure 17.** Examples of force tracing (A) LogEC<sub>50</sub> (B), minimum and maximal contractions (C) and Hillslope (D) in 1% Triton X-100 (extensively permeabilized cell in which all membrane functions are eliminated including the loss of receptor and Sarcoplasmic Reticulum (SR) coupling) permeabilized femoral arteries, in the absence of a drug (Control) and presence of H-1152 (3µM, inhibitor of ROK). Data in (B, C, D) are averages ± SE. n = 4.
3.5.6. Alpha Toxin - 10µM Y27632 & 10µM HA1077

Our protocol requires that a minimum of an $n = 3$ are run, in order to perform statistical analysis. However, these two ROK inhibitors, 10µM Y-27632 and 10µM HA-1077, exhibited the same characteristics as our previous ROK studies, as there was no inhibition. Therefore, additional experiments were not performed with these inhibitors as it suggested that additional ROK inhibitor studies would yield similar results.

![Graph showing active force vs. pCa for Alpha Toxin with Y27632 and HA1077](image)

**Figure 18.** Examples of force tracing in 20µg/ml α-toxin (retains both SR and receptor coupling, forming pores permeable to molecules of up to 3kDa) permeabilized femoral arteries, in the absence of a drug (Control) and presence of 10µM Y-27632 and 10µM HA-1077 (both are inhibitors of ROK). $n = 2$. 
3.5.7. **Triton X-100 - 10µM Y27632**

The minimum (Figures 19 (A)(C)) of the force curve produced by tissues exposed to 10µM Y27632 (0.002 ± 0.005 F₂/F₁) was not significantly different than that produced by control tissues (0.004 ± 0.006 F₂/F₁), (P = 0.8).

The maximum (Figures 19 (A)(C)) of the force curve was not significantly different when comparing 10µM Y27632-treated tissue (0.73 ± 0.03 F₂/F₁) and control tissues (0.77 ± 0.06 F₂/F₁), (P = 0.58).

The LogEC₅₀ (Figure 19 (B)) produced by tissues exposed to 10µM Y27632 (pCa -6.25 ± 0.03) was not significantly different than that produced by control tissues (pCa -6.28 ± 0.04), (P = 0.56).

The Hill slope (Figure 19 (D)) of the force curve was not significantly different when comparing 10µM Y27632-treated tissue (8.67 ± 2.87) and control tissues (7.46 ± 1.94), (P = 0.74).
Figure 19. Examples of force tracing (A) LogEC_{50} (B), minimum and maximal contractions (C) and Hillslope (D) in 1% Triton X-100 (extensively permeabilized cell in which all membrane functions are eliminated including the loss of receptor and Sarcoplasmic Reticulum (SR) coupling) permeabilized femoral arteries, in the absence of a drug (Control) and presence of Y-27632 (10µM, inhibitor of ROK). Data in (B, C, D) are averages ± SE. n = 3.
3.5.8. Triton X-100 - 10µM HA1077

The minimum (Figures 20 (A)(C)) of the force curve produced by tissues exposed to 10µM HA1077 (0.002 ± 0.004 F₂/F₁) was not significantly different than that produced by control tissues (0.004 ± 0.006 F₂/F₁), (P = 0.81).

The maximum (Figures 20 (A)(C)) of the force curve was not significantly different when comparing 10µM HA1077-treated tissues (0.76 ± 0.04 F₂/F₁) and control tissues (0.77 ± 0.06 F₂/F₁), (P = 0.94).

The LogEC₅₀ (Figure 20 (B)) produced by tissues exposed to 10µM HA1077 (pCa -6.26 ± 0.03) was not significantly different than that produced by control tissues (pCa -6.28 ± 0.04), (P = 0.82).

The Hill slope (Figure 20 (D)) of the force curve was not significantly different when comparing 10µM HA1077-treated tissues (7.68 ± 2.28) and control tissues (7.46 ± 1.94), (P = 0.94).
Figure 20. Examples of force tracing (A) LogEC$_{50}$ (B), minimum and maximal contractions (C) and Hillslope (D) in 1% Triton X-100 (extensively permeabilized cell in which all membrane functions are eliminated including the loss of receptor and Sarcoplasmic Reticulum (SR) coupling) permeabilized femoral arteries, in the absence of a drug (Control) and presence of HA-1077 (10µM, inhibitor of ROK). Data in (B, C, D) are averages ± SE. $n = 3$. 
3.6 Permeabilized Tissue- PKC inhibitors

To determine the degree to which a \( \text{Ca}^{2+} \)-induced contraction is contingent on PKC in chemically permeabilized femoral artery, a calcium concentration-response curve (pCa -7.5.0 to -5.5) was performed in \( \alpha \)-toxin and Triton X-100 permeabilized tissue in the presence of PKC inhibitors. Tissues were treated with PKC antagonists for 15-45 minutes prior to and during the calcium concentration-response curve. Two doses, 1 \( \mu \text{M} \) and 3 \( \mu \text{M} \), of the PKC inhibitor GF 109203X were used in the \( \alpha \)-toxin preparation, and three doses, 1 \( \mu \text{M} \), 3 \( \mu \text{M} \) and 3 \( \mu \text{M} \), were used in the Triton X-100 preparation. For a comparison, additional PKC inhibitors were used in the \( \alpha \)-toxin preparation 1 \( \mu \text{M} \) Gö 6976 (n = 1), 10 \( \mu \text{M} \) (n = 1) and 20 \( \mu \text{M} \) (n = 1) PKC 19-31 (a pseudosubstrate peptide inhibitor of PKC\( \alpha \)), and 10 \( \mu \text{M} \) pseudosubstrate peptide inhibitor of PKC\( \zeta \) (n = 2). Statistics were not run on these data, instead it provided a dosage guideline for the Triton X-100 preparations. In the Triton X-100 preparations the following PKC inhibitors were used, 20\( \mu \text{M} \) PKC 19-31 (n = 3) and 10\( \mu \text{M} \) pseudosubstrate peptide inhibitor of PKC\( \zeta \) (n = 3). The different antagonists and dosages of antagonist targeted different PKC isotypes. As a result, these inhibitors produced varied results.

Data were normalized and curve-fitted to a sigmoidal curve as described in the Materials & Methods section.
3.6.1. **Alpha Toxin - 1µM GF 109203X**

The minimum (Figures 21 (A)(C)) of the force curve produced by tissues exposed to 1µM GF 109203X (-0.12 ± 0.02 F_2/F_1) was not significantly different than that produced by control tissues (0.01 ± 0.02 F_2/F_1), (P = 0.23).

The maximum (Figures 21 (A)(C)) of the force curve was significantly different when comparing 1µM GF 109203X-treated tissues (0.69 ± 0.06 F_2/F_1) and control tissues (1.08 ± 0.04 F_2/F_1), (P = 0.01).

The LogEC_{50} (Figure 21 (B)) produced by tissues exposed to 1µM GF 109203X (pCa -6.76 ± 0.13) was not significantly different than that produced by control tissues (pCa -6.79 ± 0.12), (P = 0.86).

The Hill slope (Figure 21 (D)) of the force curve was not significantly different when comparing 1µM GF 109203X- treated tissue (3.50 ± 1.01) and control tissues (5.90 ± 1.51), (P = 0.26).

3.6.2. **Alpha Toxin - 3µM GF 109203X**

The minimum (Figures 21 (A)(C)) of the force curve produced by tissues exposed to 3µM GF 109203X (0.0003 ± 0.005 F_2/F_1) was not significantly different than that produced by control tissues (0.01 ± 0.005 F_2/F_1), (P = 0.17).

The maximum (Figures 21 (A)(C)) of the force curve was not significantly different when comparing 3µM GF 109203X-treated tissues (0.70 ± 0.13 F_2/F_1) and control tissues (1.43 ± 0.25 F_2/F_1), (P = 0.06).
The LogEC$_{50}$ (Figure 21 (B)) produced by tissues exposed to 3µM GF 109203X (pCa -6.23 ± 0.05) was not significantly different than that produced by control tissues (pCa -6.40 ± 0.04), (P = 0.052).

The Hill slope (Figure 21 (D)) of the force curve was significantly different when comparing 3µM GF 109203X-treated tissue (3.31 ± 0.51) and control tissues (7.25 ± 0.65), (P = 0.01).
**Figure 21.** Examples of force tracing (A) LogEC$_{50}$ (B1 & B2), minimum and maximal contractions (C1 & C2) and Hillslope (D1 & D2) in 20µg/ml α-toxin (retains both SR and receptor coupling, forming pores permeable to molecules of up to 3kDa) permeabilized femoral arteries, in the absence of a drug (Control) and presence of GF-109203X (1µM, a potent inhibitor of conventional PKC isotypes; 3µM, inhibitor of atypical PKC isotypes). Data in (B1, B2, C1, C2, D1, D2) are averages ± SE. $n = 3$, * = $P < 0.05$ compared to control values.
3.6.3. **Triton X-100- 1µM GF 109203X**

The minimum (Figures 22 (A)(C)) of the force curve produced by tissues exposed to 1µM GF 109203X (-0.002 ± 0.0007 F₂/F₁) was not significantly different than that produced by control tissues (0.0004 ± 0.0009 F₂/F₁), (P = 0.07).

The maximum (Figures 22 (A)(C)) of the force curve was not significantly different when comparing 1µM GF 109203X-treated tissues (0.76 ± 0.06 F₂/F₁) and control tissues (0.74 ± 0.04 F₂/F₁), (P = 0.77).

The LogEC₅₀ (Figure 22 (B)) produced by tissues exposed to 1µM GF 109203X (pCa -6.29 ± 0.04) was not significantly different than that produced by control tissues (pCa -6.37 ± 0.05), (P = 0.27).

The Hill slope (Figure 22 (D)) of the force curve was not significantly different when comparing 1µM GF 109203X treated tissues (6.64 ± 0.95) and control tissues (7.36 ± 1.16), (P = 0.64).

3.6.4. **Triton X-100- 3µM GF 109203X**

The minimum (Figures 22 (A)(C)) of the force curve produced by tissues exposed to 3µM GF 109203X (-0.002 ± 0.0005 F₂/F₁) was not significantly different than that produced by control tissues (0.008 ± 0.007 F₂/F₁), (P = 0.21).

The maximum (Figures 22 (A)(C)) of the force curve was not significantly different when comparing 3µM GF 109203X-treated tissues (0.68 ± 0.05 F₂/F₁) and control tissues (0.78 ± 0.04 F₂/F₁), (P = 0.18).
The LogEC$_{50}$ (Figure 22 (B)) produced by tissues exposed to 3µM GF 109203X (pCa -6.27 ± 0.02) was not significantly different than that produced by control tissues (pCa -6.27 ± 0.02), (P = 0.10).

The Hill slope (Figure 22 (D)) of the force curve was significantly different when comparing 3µM GF 109203X treated tissues (4.72 ± 0.17) and control tissues (5.30 ± 0.10), (P = 0.04).

3.6.5. **Triton X-100- 10µM GF 109203X**

The minimum (Figures 22 (A)(C)) of the force curve produced by tissues exposed to 10µM GF 109203X (0.002 ± 0.003 F$_2$/F$_1$) was not significantly different than that produced by control tissues (0.007 ± 0.005 F$_2$/F$_1$), (P = 0.50).

The maximum (Figures 22 (A)(C)) of the force curve was significantly different when comparing 10µM GF 109203X-treated tissues (0.51 ± 0.08 F$_2$/F$_1$) and control tissues (0.76 ± 0.05 F$_2$/F$_1$), (P = 0.03).

The LogEC$_{50}$ (Figure 22(B)) produced by tissues exposed to 10µM GF 109203X (pCa -6.21 ± 0.07) was not significantly different than that produced by control tissues pCa -6.25 ± 0.02), (P = 0.59).

The Hill slope (Figure 22 (D)) of the force curve was not significantly different when comparing 10µM GF 109203X treated tissues (6.18 ± 2.29) and control (6.17 ± 1.23), (P = 1.00).
Figure 22. Examples of force tracing (A) LogEC\textsubscript{50} (B1, B2, B3), minimum and maximal contractions (C1, C2, C3) and Hill slope (D1, D2, D3) in 1% Triton X-100 (extensively permeabilized cell in which all membrane functions are eliminated including the loss of receptor and Sarcoplasmic Reticulum (SR) coupling) permeabilized femoral arteries, in the absence of a drug (Control) and presence of GF-109203X (1µM, a potent inhibitor of conventional PKC isotypes; 3µM & 10µM, inhibitor of atypical PKC isotypes). Data in (B1, B2, B3, C1, C2, C3, D1, D2, D3) are averages ± SE. n = 5,3,5- respectively, * = P < 0.05 compared to control values.
3.6.6. Alpha Toxin - 0.1µM Gö 6976

Our protocol requires that a minimum of an \( n = 3 \) are run, in order to perform statistical analysis. However, appears that this antagonist of conventional PKC isotypes does have an inhibitory effect on the \( \alpha \)-toxin permeabilized tissue. This is consistent with the previous data in which 1 and 3µM GF-109203X causes inhibition.

**Figure 23.** Examples of force tracing in 20µg/ml \( \alpha \)-toxin (retains both SR and receptor coupling, forming pores permeable to molecules of up to 3kDa) permeabilized femoral arteries, in the absence of a drug (Control) and presence of 0.1µM Gö-6976 (inhibitor of conventional PKC isotypes). \( n = 1 \).
3.6.7. Alpha Toxin - 10µM PKC 19-31, 20µM PKC 19-31, 10µM PKCζ

Statistics were not run on these compounds. However, it appears that there is variation in the pseudosubstrate PKC inhibitor (PKC 19-31) that affects is reported to inhibit conventional PKC isotypes, based on dosage. The atypical pseudosubstrate peptide inhibitor of PKCζ appeared to have induced calcium sensitization.

**Figure 24.** Examples of force tracing in 20µg/ml α-toxin (retains both SR and receptor coupling, forming pores permeable to molecules of up to 3kDa) permeabilized femoral arteries, in the absence of a drug (Control) and presence of PKC 19-31 (10µM & 20µM, pseudosubstrate PKC conventional isotype inhibitor) and PKCζ inhibitor (atypical pseudosubstrate peptide inhibitor). n = 1, 1, 2, respectively.
3.6.8. **Triton X-100- 20µM PKC 19-31**

The minimum (Figures 25 (A)(C)) of the force curve produced by tissues exposed to 20µM PKC 19-31 (0.003 ± 0.005 F₂/F₁) was not significantly different than that produced by control tissues (0.001 ± 0.002 F₂/F₁), (P = 0.79).

The maximum (Figures 25 (A)(C)) of the force curve was not significantly different when comparing 20µM PKC 19-31-treated tissues (0.64 ± 0.06 F₂/F₁) and control tissues (0.67 ± 0.02 F₂/F₁), (P = 0.65).

The LogEC₅₀ (Figure 25 (B)) produced by tissues exposed to 20µM PKC 19-31 (pCa -6.39 ± 0.03) was significantly different than that produced by control tissues (pCa -6.27 ± 0.03), (P = 0.047).

The Hill slope (Figure 25 (D)) of the force curve was not significantly different when comparing 20µM PKC 19-31-treated tissues (4.54 ± 0.78) and control tissues (3.87 ± 0.74), (P = 0.57).
Figure 25. Examples of force tracing (A) LogEC$_{50}$ (B), minimum and maximal contractions (C) and Hillslope (D) in 1% Triton X-100 (extensively permeabilized cell in which all membrane functions are eliminated including the loss of receptor and Sarcoplasmic Reticulum (SR) coupling) permeabilized femoral arteries, in the absence of a drug (Control) and presence of PKC 19-31 (20µM, pseudosubstrate PKC conventional isotype inhibitor). Data in (B, C, D) are averages ± SE. $n = 3$, * = P < 0.05 compared to control values.
3.6.9. **Triton X-100- 10µM PKCζ**

The minimum (Figures 26 (A)(C)) of the force curve produced by tissues exposed to 10µM pseudosubstrate PKC atypical isotype inhibitor-PKCζ (0.006 ± 0.005 F₂/F₁) was not significantly different than that produced by control tissues (0.001 ± 0.002 F₂/F₁), (P = 0.43).

The maximum (Figures 26 (A)(C)) of the force curve was not significantly different when comparing 10µM pseudosubstrate PKC atypical isotype inhibitor-PKCζ-treated tissues (0.69 ± 0.05 F₂/F₁) and control tissues (0.67 ± 0.02 F₂/F₁), (P = 0.68).

The LogEC₅₀ (Figure 26 (B)) produced by tissues exposed to 10µM pseudosubstrate PKC atypical isotype inhibitor-PKCζ (pCa -6.27 ± 0.03) was not significantly different than that produced by control tissues (pCa -6.33 ± 0.03), (P = 0.14).

The Hill slope (Figure 26 (D)) of the force curve was not significantly different when comparing 10µM pseudosubstrate PKC atypical isotype inhibitor-PKCζ-treated tissue (3.87 ± 0.74) and control tissues (4.17 ± 0.64), (P = 0.77).
Figure 26. Examples of force tracing (A) LogEC<sub>50</sub> (B), minimum and maximal contractions (C) and Hillslope (D) in 1% Triton X-100 (extensively permeabilized cell in which all membrane functions are eliminated including the loss of receptor and Sarcoplasmic Reticulum (SR) coupling) permeabilized femoral arteries, in the absence of a drug (Control) and presence of pseudosubstrate PKC atypical isotype inhibitor-PKCζ (10µM). Data in (B, C, D) are averages ± SE. n = 3.
4.1 Additional Peptide MLCK Inhibitor

We previously established that MLCK was the main kinase responsible for contraction in both the α-toxin and Triton X-100 chemically permeabilized femoral arterial smooth muscles. We also found that the peptide PKC inhibitors were not behaving as expected. To establish a baseline for peptide inhibitors, we added a peptide inhibitor of MLCK, MLCK-18. We did not get the strong inhibition we saw with the wortmannin preparations.

4.1.1. Alpha Toxin - 1µM & 10µM MLCK-18

Our protocol requires that a minimum of an $n = 3$ are run, in order to perform statistical analysis. However, appears that there is variation in the MLCK inhibitor (MLCK-18) based on dosage.
Figure 27. Examples of force tracing in 20µg/ml α-toxin (retains both SR and receptor coupling, forming pores permeable to molecules of up to 3kDa) permeabilized femoral arteries, in the absence of a drug (Control) and presence of MLCK-18 (1µM & 10µM, peptide inhibitor of MLCK). $n = 1$. 
4.1.2. **Triton X-100- 10µM MLCK-18**

The minimum (Figures 28 (A)(C)) of the force curve produced by tissues exposed to 10µM MLCK-18 (0.008 ± 0.008 F₂/F₁) was not significantly different than that produced by control tissues (0.001 ± 0.002 F₂/F₁), (P = 0.49).

The maximum (Figures 28 (A)(C)) of the force curve was significantly different when comparing 10µM MLCK-18-treated tissue (0.53 ± 0.005 F₂/F₁) and control tissues (0.67 ± 0.03 F₂/F₁), (P = 0.003).

The LogEC₅₀ (Figure 28 (B)) produced by tissues exposed to 10µM MLCK-18 (pCa -6.46 ± 0.03) was significantly different than that produced by control tissues (pCa -6.27 ± 0.03), (P = 0.012).

The Hill slope (Figure 28 (D)) of the force curve was not significantly different when comparing 10µM MLCK-18-treated tissues (3.87 ± 0.26) and control tissues (3.87 ± 0.74), (P = 0.57).
Figure 28. Examples of force tracing (A) LogEC$_{50}$ (B), minimum and maximal contractions (C) and Hillslope (D) in 1% Triton X-100 (extensively permeabilized cell in which all membrane functions are eliminated including the loss of receptor and Sarcoplasmic Reticulum (SR) coupling) permeabilized femoral arteries in the absence of a drug (Control) and presence of 10µM MLCK-18, (peptide inhibitor of MLCK). Data in (B, C, D) are averages ± SE. $n = 3$, * = P < 0.05 compared to control values.
KCl-induced contraction has proven to be a valuable tool in the study of smooth muscle regulation (Ratz 2005), as it bypasses GPCR stimulation and activates smooth muscle by changing the $K^+$ equilibrium potential and clamping membrane potential at some value above the resting level (Bolton 1979). The depolarization of the membrane activates voltage activated calcium channels (VOCCS) and allows for an influx of calcium, increasing $[Ca^{2+}]_i$, resulting in smooth muscle cell contraction. It also has been observed that membrane depolarization by KCl can cause $Ca^{2+}$-sensitization (Yanagisawa and Okada 1994; Ratz 2005). The proposal that $Ca^{2+}$-sensitization is caused primarily by inhibition of MLC phosphatase activity has gained widespread support (see (Somlyo and Somlyo 2003) for a review and for review on MLC phosphatase, see (Hartshorne, Ito et al. 1998; Hartshorne, Ito et al. 2004; Ratz 2005) for a review see Ratz 2005). The general model is that GPCR stimulation reduced MLC phosphatase activity by phosphorylation of the MLC phosphatase regulatory subunit MYPT1 and the 17-kDa PKC-activated phosphatase inhibitor CPI-17 (Fujihara, Walker et al. 1997; Hartshorne, Ito et al. 1998; Feng, Ito et al. 1999; Eto 2001; Eto, Kitazawa et al. 2004, for review see (Ratz 2005)). However, whether an increase in $[Ca^{2+}]_i$, due to the activation of VOCCS,
or membrane depolarization itself plays the principle role in KCl-induced Ca\(^{2+}\)-sensitization remains to be determined (Ratz 2005). Several studies using Y-27632, a selective ROK inhibitor, have provided evidence of a decrease in force, without inhibition of KCl-induced increases in [Ca\(^{2+}\)]\(_i\); suggesting that a KCl-induced contraction increases Ca\(^{2+}\)-sensitivity by the activation of ROK (review in (Ratz 2005)). Therefore, the purpose of this study was to test the hypothesis that KCl-induced Ca\(^{2+}\)-sensitization is a result of Ca\(^{2+}\)-induced activation of ROK.

In order to test this hypothesis, we first wanted to determine that ROK, and not PKC, is primarily responsible for the tonic phase of a KCl-induced contraction in intact rabbit femoral artery. This was accomplished by treating tissues with either a ROK or PKC inhibitor before and during a KCl-induced contraction and measuring force over time. We showed (see Figure 8) that neither the conventional PKC isotype inhibitor, 1µM GF-109203X, nor the specific ROK inhibitor, 0.3µM H-1152, significantly reduced the initial peak contraction. However, both inhibitors significantly reduced the earliest portion of the sustained contraction (2 ½ minutes), whereas the ROK inhibitor only (not the PKC inhibitor) significantly reduced the later tonic portion of the sustained contraction (10 minutes). This confirms previous studies in which two other ROK inhibitors, Y-27632 (Mita, Yanagihara et al. 2002; Urban, Berg et al. 2003; Ratz 2005) and HA-1077 (Takizawa, Hori et al. 1993), selectively inhibited tonic KCl-induced contraction (Ratz 2005). These data suggest that during a KCl-induced contraction in the intact rabbit femoral artery, ROK is largely responsible for the sustained contraction,
whereas the conventional PKC isotypes may play a small role, but only during the early phase of the tonic contraction.

We then wanted to determine the degree that the KCl-induced ROK-dependent tonic contraction was contingent on the level of extracellular calcium. To accomplish this, a high potassium calcium contraction-response curve (pCa -4.0 to -2.75) was constructed in intact rabbit femoral artery and compared to tissues treated with a specific ROK inhibitor, 0.3µM H-1152, under the same conditions. As expected, the ROK inhibitor significantly reduced the contraction at all levels of the calcium concentration-response curve (see Figure 9). We also found that the presence of high potassium alone was not sufficient to elicit a contraction in the absence of calcium, in the both the control tissue (the absence of ROK inhibitor) and H-1152 treated tissues. These data suggest that ROK plays a large role in KCl-induced Ca\(^{2+}\)-sensitization, and that calcium is necessary for a KCl-induced contraction.

Additionally, we found that there was no initial peak contraction, and that force developed monotonically to increases in extracellular calcium. Thus, suggesting that the initial peak found in a “standard” KCl-induced contraction (1.6mM Ca\(^{2+}\)) is dependent on a high initial influx of calcium; and supporting the hypothesis that during membrane depolarization, the level of extracellular calcium determines the level of [Ca\(^{2+}\)]\(_i\), and there is a correlation between the amount of extracellular calcium and the strength of the contraction.

For a comparison, the experiment was repeated with a conventional PKC isotype inhibitor, 1µM GF-109203X, and a strong MLCK inhibitor, 1µM wortmannin, to
determine if the high potassium calcium concentration-response curve altered the response seen in the “standard” KCl-induced contraction. The PKC inhibitor did not significantly attenuate force at any calcium concentration, whereas the MLCK inhibitor nearly abolished the contraction at all calcium concentrations. These data provide additional evidence that a KCl-induced contraction, ROK and not PKC is primarily responsible for Ca\(^{2+}\)-sensitization; and that a KCl-induced contraction is highly dependent on MLCK, ruling out the activation of other kinases.

As previously stated, the purpose of this study was to test the hypothesis that KCl-induced Ca\(^{2+}\)-sensitization is a result of Ca\(^{2+}\)-induced activation of ROK. Now that we established that this is possible, we needed to further explore the relationship between \([\text{Ca}^{2+}]_i\) and ROK activation. In order to accomplish this, a methodology was employed permitting precise control of the amount of \([\text{Ca}^{2+}]_i\) in the cell during a contraction. To accomplish this, we “clamped” calcium by chemically permeabilizing isolated rabbit femoral artery rings and exposing them to solutions containing the desired amounts of free calcium as determined using WEBMAXC (Patton, Thompson et al. 2004). We modeled our permeabilization techniques after those described in Kitazawa’s publications (Kitazawa, Kobayashi et al. 1989; Kobayashi, Kitazawa et al. 1989; Kitazawa, Gaylinn et al. 1991; Kitazawa, Masuo et al. 1991; Kitazawa, Takizawa et al. 1999; Kitazawa 2004).

Two different permeabilization detergents were used, \(\alpha\)-toxin, which forms pore sizes allowing molecules smaller than 3kDa to pass, such as ATP and EGTA, but not calmodulin and retains receptor and SR coupling; and Triton X-100, which leads to extensive permeabilization in which all membrane functions are eliminated, including
receptor and SR coupling. A calcium concentration-response curve (pCa -7.5 to -5.5) was constructed in each type of preparation and statistics were run to compare the two. We found that the α-toxin permeabilized tissue responded to lower dose of calcium and that both maximal force and LogEC$_{50}$ were significantly reduced less in the Triton X-100 permeabilized tissues. These data support that of others, showing that there is either membrane bound processes that are being disrupted or there is a loss of several intracellular molecules in the Triton X-100 preparation (Li, Eto et al. 1998; Kitazawa, Takizawa et al. 1999; Adegunloye, Lamarre et al. 2003). We also found that the Hill slope was not significantly different between these two preparations, meaning the cooperativity for calcium is similar.

Before testing the ROK inhibitors, we wanted to first ensure that MLCK was the primary kinase responsible for contraction in the permeabilized artery ring as it is in the intact KCl-induced contraction. To test this, a calcium concentration-response curve (pCa -7.5 to -5.5) was performed in the presence of a strong MLCK inhibitor, 1µM wortmannin, in both permeabilized tissue preparations. As expected, the peak contraction and LogEC$_{50}$ were significantly reduced, but not the Hill Slope, in both preparations. Thus, suggesting that in α-toxin and Triton X-100 permeabilized tissue, MLCK is the primary kinase responsible for force development; and that the cooperativity of calcium is not altered by the MLCK inhibitors.

Armed with this knowledge, we then proceeded to test the relationship between [Ca$^{2+}$]$_i$ and ROK activation by conducting a calcium concentration-response curve (pCa -7.5 to -5.5) in the presence of a specific ROK inhibitor, 0.3µM H-1152, in both the
permeabilized artery preparations. We showed to our surprise that H-1152 did not cause a significant inhibition of contraction in either preparation. We repeated the experiments with higher doses of H-1152 (3µM and 10µM). Again, we did not find any significant difference in peak contraction or LogEC₅₀. However, there was one significant difference in the Hill slope out of all of these H-1152 treated tissues, and that was in the 3µM H-1152 treated α-toxin preparation, but not in the Triton X-100. For a comparison, the experiments were repeated with other known ROK inhibitors, 10µM HA-1077 and 10µM Y-27632, first in the Triton X-100 tissue and then in the α-toxin tissue. These additional ROK antagonists yielded the same results in the Triton X-100 tissue as did the H-1152 compound; there were no significant differences. The first two experiments in the α-toxin preparations, also failed to produce any decline in force. Because these data appeared to simply confirm our findings, we did not pursue additional “n” values. As a result, because of the low “n” values, statistics were not run on the α-toxin preparation treated with HA-1077 and Y-27632. These data suggest that in α-toxin and Triton X-100 permeabilized rabbit artery rings either [Ca²⁺]ᵢ alone does not directly activate ROK or the permeabilization technique itself disrupts the normal ROK signaling system.

As a comparison, we repeated these experiments in the presence of PKC inhibitors, to see if [Ca²⁺]ᵢ activates other Ca²⁺-sensitization pathways in tissues permeabilized by these two methods. We used three different concentrations of GF 109203X, 1µM, 3µM and 10µM, where at 1µM, GF 109203X is a potent inhibitor of conventional PKC isotypes (α, β, γ) and at 3µM & 10µM, GF 109203X is an inhibitor of atypical PKC isotypes (ζ, ι, λ). We also used 0.1µM Gö 6976, a selective inhibitor of the
Ca\(^{2+}\)-dependent PKC\(\alpha\)-isozyme: 10\(\mu\)M and 20\(\mu\)M PKC 19-31, a pseudosubstrate peptide inhibitor of PKC\(\alpha\), and 10\(\mu\)M pseudosubstrate peptide inhibitor of PKC\(\zeta\). We showed that the low dose of GF 109203X (3\(\mu\)M) significantly reduced the maximum contraction in the \(\alpha\)-toxin preparation, but not in the Triton X-100 preparation. These findings were further supported by a single experiment using 0.1\(\mu\)M Gö 6976, a selective inhibitor of PKC\(\alpha\), in the \(\alpha\)-toxin preparation, showing that the maximal contraction was reduced (Gö 6976 was not tested in the Triton X-100 preparation). In the higher doses of GF 109203X, we found that 3\(\mu\)M significantly reduced the Hill slope in both the \(\alpha\)-toxin and Triton X-100 permeabilized tissue preparations, but did not reduce the peak contraction or the LogEC\(_{50}\); at 10\(\mu\)M we found that the peak contraction was significantly reduced in the Triton X-100 preparation (10\(\mu\)M GF 109203X was not tested in the \(\alpha\)-toxin preparation). The conventional pseudosubstrate PKC inhibitor, PKC 19-31, had variable effects in the \(\alpha\)-toxin preparation; at 10\(\mu\)M, the maximum was reduced, but a 20\(\mu\)M the maximum was increased. In the Triton X-100 preparation, 20\(\mu\)M PKC 19-31 increased the LogEC\(_{50}\). The pseudosubstrate peptide inhibitor of PKC\(\zeta\) (10\(\mu\)M ) had no effect in the Triton X-100 preparation.

In summary, the conventional PKC isotype inhibitors, 1\(\mu\)M GF 109203X, 10\(\mu\)M PKC 19-31 and 0.1\(\mu\)M Gö 6976 all appeared to reduce the maximal contraction in the alpha toxin preparation; whereas 1\(\mu\)M GF 109203X and 10\(\mu\)M PKC 19-31 did not reduce the maximal contraction in the Triton X-100 preparation, Gö 6976 was not tested in the Triton X-100 preparation. Our findings support others who have shown that the levels of many molecules such as PKC\(\alpha\) are significantly reduced in smooth muscle tissues.
permeabilized by Triton X-100, but not in tissues permeabilized by α-toxin (Kitazawa, Takizawa et al. 1999). These data suggest that the PKC α isoform is activated in α-toxin permeabilized rabbit femoral artery ring, therefore, either the permeabilization technique or the contracting solution, is activating PKCα-induced Ca\(^{2+}\)-sensitization.

The atypical PKC isotype inhibitors provided various results. In both the α-toxin and Triton X-100 preparations, 3µM of GF 109203X significantly reduced the Hill slope. These data suggests that the cooperativity for calcium is altered. The highest dose of GF 109203X, 10µM, significantly reduced the peak contraction in the Triton X-100 preparation, and although statistics were not performed, it also reduced the maximum contraction in the α-toxin preparation. Finally, the pseudosubstrate peptide inhibitor of PKCζ (10µM ) had no effect in the Triton X-100 preparation. These data present conflicting evidence of the role of PKCζ in permeabilized rabbit femoral artery; presenting the possibility that that the permeabilization technique itself is activating proteases that degrade the peptides before they could exert their effect.

Finally, we wanted to look at another peptide inhibitor to try to determine if the peptide PKC inhibitors were giving variable results because they were peptides. We had already established that MLCK was the primary kinase responsible for contraction the α-toxin and Triton X-100 permeabilized rabbit femoral artery. Therefore, we used a peptide MLCK kinase inhibitor, 10µM MLCK-18. We wanted to establish a control for a peptide inhibitors, PKC 19-31 and the pseudosubstrate peptide inhibitor of PKCζ, since this was the first time we used a peptide inhibitor in permeabilized tissue. We found that at low doses, 1µM, reduced the contraction force, whereas higher doses, 10µM, increased the
contraction in the \( \alpha \)-toxin preparation. In the Triton X-100 preparation, the peak contraction was significantly reduced, but the LogEC\(_{50} \) was significantly increased using 10\( \mu \)M MLCK-18. These data suggest that the permeabilization techniques themselves may be activating proteases that are not normally active in intact rabbit femoral artery.

In conclusion, in both the \( \alpha \)-toxin and Triton X-100 chemically permeabilized rabbit femoral artery rings either \([\text{Ca}^{2+}]\), alone does not directly activate ROK or the permeabilization technique itself disrupts the normal ROK signaling system. Additional investigations are needed to determine which of these scenarios is the most accurate. Proteomics and confocal studies would elucidate the extent of the loss of molecules involved in the ROK pathway as well as the extent of membrane, including caveolar, disruption caused by these two permeabilization techniques.

**CONCLUSIONS**

1. During a KCl-induced contraction in the intact rabbit femoral artery, ROK is largely responsible for the sustained contraction, whereas the conventional PKC isotypes may play a small role, but only during the early phase of the tonic contraction.

2. ROK plays a large role in KCl-induced Ca\(^{2+}\)-sensitization, and that calcium is necessary for a KCl-induced contraction.
3. The initial peak found in a “standard” KCl-induced contraction (1.6mM Ca\(^{2+}\)) is dependent on a high initial influx of calcium.

4. During membrane depolarization, the level of extracellular calcium determines the level of [Ca\(^{2+}\)]\(_i\), and there is a correlation between the amount of extracellular calcium and the strength of the contraction.

5. KCl-induced contraction is highly dependent on MLCK, ruling out the activation of other kinases.

6. The Triton X-100 preparation disrupts more membrane process and/or losses more intracellular molecules compared to the \(\alpha\)-toxin preparation, but the cooperativity of calcium is similar between the two preparations.

7. In both the \(\alpha\)-toxin and Triton X-100 chemically permeabilized rabbit femoral artery, MLCK is the primary kinase responsible for force development; and that the cooperativity of calcium is not altered by the MLCK inhibitors.

8. In both the \(\alpha\)-toxin and Triton X-100 chemically permeabilized rabbit femoral artery rings either [Ca\(^{2+}\)], alone does not directly activate ROK or the permeabilization technique itself disrupts the normal ROK signaling system.
9. In α-toxin chemically permeabilized rabbit femoral artery rings conventional PKC isotypes are activated either by the permeabilization technique or relaxing solution.

10. PKCα is significantly reduced in rabbit femoral artery rings permeabilized by Triton X-100, but not in tissues permeabilized by α-toxin.

11. There is a possibility that in both α-toxin and Triton X-100 chemical permeabilization techniques active proteases not normally activated in intact rabbit femoral artery rings.

FUTURE EXPERIMENTS

1. Use confocal microscopy to look at the different skinned tissue to determine the extent of caveolae and other membrane disruptions. This may account for the inability to induced ROK inhibition in the permeabilized cells.

2. Western Blotting to determine what proteins are being lost during the different skinning preparations. Specifically, look at PKC and ROK as well as their second messengers and compare these values to an intact tissue.

3. Repeat experiments with the addition of GPCR agonists ROK in both the chemically permeabilized tissues preparations to determine if these pathways are intact and can still be activated via GPCRs.
a. If force does not change in the presence of ROK GPRC agonists it would support the hypothesis that the mechanisms involved in ROK activation are disrupted or lost by permeabilization.

b. If force increases in presence of ROK GPRC agonists it would support the hypothesis that $[\text{Ca}^{2+}]_i$ alone does not activate ROK.

4. Repeat experiments with the addition of GPCR agonists PKC in both the chemically permeabilized tissues preparations to determine if these pathways are intact and can still be activated via GPCRs.

5. Repeat the protein inhibitor experiments in the presence of protease inhibitors.

6. Analyze the detergent solutions to determine if the falling tone is due to a reduced effectiveness to permeabilize the cell or if intracellular molecules are contaminating the detergent solutions.

7. Vary the ATP in the relaxing solution/activation solution. That ATP may be causing competitive inhibition.
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VITA

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